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Review

Anaerobic culture to detect periodontal and caries pathogens ☆

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ABSTRACT

Background: Anaerobic culture has been critical in our understanding of the oral microbiotas.
Highlight: Studies in advanced periodontitis in the 1970s revealed microbial complexes that associated with different clinical presentations. Taxonomy studies identified species newly-observed in periodontitis as *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans*, *Campylobacter* (*Wolinella*) *rectus* and other *Campylobacter* species, and *Tannerella* (*Bacteroides*) *forsythia.* Anaerobic culture of initial periodontitis showed overlap in the microbiota with gingivitis, and added *Selenomonas noxia* and *Filifactor alocis* as putative periodontal pathogens. *Porphyromonas gingivalis* and *T. forsythia* were found to be associated with initial periodontitis in adults. The dominant microbiota of dental caries differs from that of periodontitis. The major cariogenic species are acidogenic and acid tolerant species particularly *Streptococcus mutans*, and *Lactobacillus* and *Bifidobacterium* species. Anaerobic culture of severe early childhood caries revealed a widely diverse microbiota, comparable to that observed using cloning and sequencing. The PCR-based cloning approach, however, underestimated Actinobacteria compared with culture. Only a subset of the caries-associated microbiota was acid tolerant, with different segments of the microbiota cultured on blood agar compared to a low pH acid agar. While the major caries-associated species was *S. mutans*, a new species, *Scardovia wiggsiae*, was significantly associated with early childhood caries. Higher counts of *S. wiggsiae* were also observed in initial white spot carious lesions in adolescents.
Conclusion: In periodontitis and dental caries, anaerobic culture studies of advanced disease provided a comprehensive analysis of the microbiota of these infections. Anaerobic culture highlighted the limitation of PCR with standard primers that underestimate detection of Actinobacteria.

Contents

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1. Introduction anaerobic culture of plaque biofilm samples

Dental plaque samples were first examined microscopically by van Leeuwenhoek, then by culture as methods and bacterial growth media were developed. The cultivability of dental plaque bacteria improved over time with the use of complex, blood-containing media, and with application of anaerobic methods to process and culture bacteria. Studies in the 1960s were critical to demonstrate the value of anaerobic methods, which were successfully applied to both periodontal and caries samples in the 1970s. Major investigators in anaerobic culture of plaque samples in the 1970s and 1980s were those at the Forsyth institute in the Socransky and co-workers laboratories, at the laboratories of Holdeman and Moore (Virginia Polytechnic Institute, USA), Walter Loesche (Michigan, USA), William Wade (UK), and Etsuro Hoshino (Niigata, Japan) among others.

Anaerobic cultural methods continue to expand our understanding of the oral microbiota of periodontitis and dental caries although approaches for strain identification have changed from biochemical tests to 16S rRNA sequence based identifications. This review will focus on selected anaerobic culture studies that have provided the basis of our understanding of the oral microbiota. Non-cultural molecular analyses of plaque samples, mainly based on analysis of the 16S rRNA gene, have highlighted both strengths and limitations of culture to describe the complete oral microbiota. Nevertheless, when bacteria are detected by molecular methods, the focus then becomes to devise methods to cultivate them [1] which frequently involves use of anaerobic methods.

2. Periodontitis

2.1. Advanced periodontitis

Clinically, periodontitis affects the tooth supporting structures and if left unchecked leads to tooth loosening and loss. Periodontitis is recognized by increased depth in the gingival sulcus leading to periodontal pockets, and by loss of periodontal attachment and the surrounding alveolar bone. Periodontitis is frequently associated with gingival inflammation, gingivitis, which may include spontaneous bleeding of gingival tissues.

Based on the observation that periodontal loss increased with age, in the 1960s it had been assumed that this was a slowly progressing chronic infection that was difficult to arrest. One form of periodontitis variously recognized as periodontosis, juvenile periodontitis, and currently aggressive periodontitis, however, progressed very rapidly. Permanent teeth, principally central incisors and 1st permanent molars that erupted in childhood could be lost in adolescence. Anaerobic culture of bacteria from periodontal pockets associated with aggressive periodontitis in adolescents in Socransky's laboratory in the 1970s revealed a microbiota containing bacteria not previously recognized from periodontal samples of other patients [2,3]. Further many isolates were difficult to maintain in culture. This form of rapidly destructive periodontitis in adolescents is now recognized as characterized by a microbiota frequently dominated by *Aggregatibacter actinomycetemcomitans*.

Advanced adult periodontitis sites were also found to have progressing disease as demonstrated by increasing alveolar bone loss observed from sequential radiographs. This contrasted from periodontal pockets that were not progressing and represented disease remission or repair. A second study of advanced periodontitis was undertaken in the 1970s in Socransky's laboratory. In this study adult periodontitis was defined as advanced disease with sites that either had a record of increasing alveolar bone loss on radiographs within the previous two years or young adults with very advanced periodontitis. Healthy, control sites in the same subjects (when present) had no attachment loss, and minimal if any gingival inflammation [4].

Subgingival samples were collected and processed using anaerobic methods, with prolonged incubation of samples since pilot sampling indicated that only a portion of the microbiota formed colonies with incubation times less than 10–14 days. Microbiological methods included use of prereduced anaerobically sterilized (PRAS) solutions in Hungate or roll tubes for sample processing and biochemical tests. Strain characterization relied on detection of acid end products of metabolism to define genera, and an array of fermentation and other tests for isolate speciation.

The major finding of clinical importance was that the microbiotas differed between subjects, and from that of the adolescent aggressive periodontitis. Furthermore, microbiotas of adult periodontitis could be grouped into distinct disease types. Hindsight allows renaming these disease groups from “young adult” to generalized aggressive periodontitis, “minimal inflammation” to post-antibiotics refractory periodontitis, and “moderate inflammation” to chronic periodontitis (Fig. 1). Differentiation of clinical and microbial subgroups within periodontitis led to a profound change in the description of periodontitis with the recognition of distinct and different “periodontal diseases”.

Healthy sulci microbes were dominated by gram positive species, mainly *Streptococcus* and *Actinomyces* species, although over 10% could not be identified. Gram negative anaerobic rods dominated the advanced periodontitis microbiotas (Fig. 1). The “young adult”, aggressive periodontitis group was characterized with a species tentatively identified as *A. actinomycetemcomitans* with *Bacteroides asaccharolyticus* (now *Porphyromonas gingivalis*). The “minimal inflammation”, refractory periodontitis group harbored had higher proportions of *Bacteroides melaninogenicus* subsp. *intermedius* (now *Prevotella intermedia*), *Eikenella corrodens* and unidentified isolates grouped as “fusiform *Bacteroides*” (now *Tannerella forsythia*). The third “moderate inflammation”, chronic periodontitis group was dominated by a complex of *P. gingivalis*, *F. nucleatum* and “fusiform *Bacteroides*”. As in the previous Newman studies of periodontosis [3], many isolates did not fit species recognized from the earlier studies from periodontal pockets [5]. These unidentified isolates were grouped as *A. actinomycetemcomitans*-like, “vibrio-corroders” and “fusiform *Bacteroides*”.

2.2. Species new to periodontal pockets

Characterization of the *A. actinomycetemcomitans*-like isolates included determining guanine plus cytosine (G+C) content of cells and DNA–DNA hybridizations in addition to biochemical tests. Findings confirmed that some isolates were indeed *A. actinomycetemcomitans* [6]. The *A. actinomycetemcomitans* isolates included strain Y4 from adolescent aggressive periodontitis and this isolate has become a widely used reference strain. Isolates from advancing

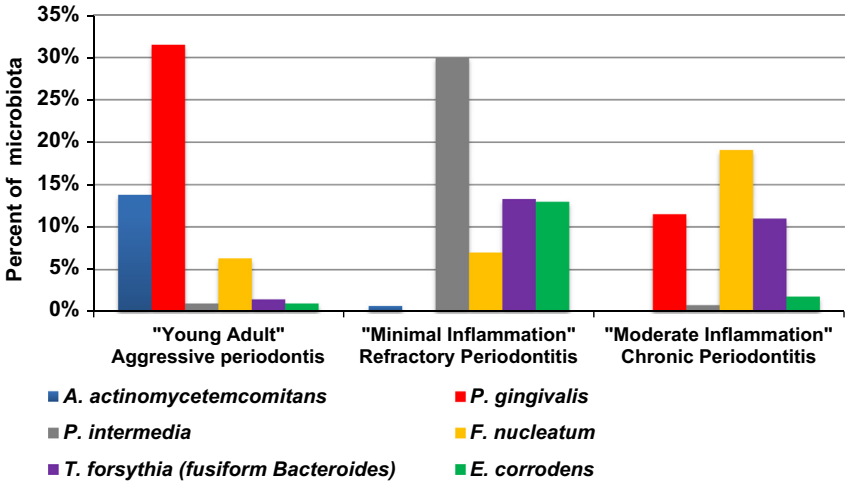

Fig. 1. Gram negative species associated with progressing advanced periodontitis. Samples from deep periodontal pockets were cultured anaerobically and species identified using biochemical tests. Three microbial complexes were observed in different clinical categories. Samples from "Young Adults" or aggressive periodontitis were dominated by *A. actinomycetemcomitans* and *P. gingivalis;* from "minimal inflammation" or refractory periodontitis by *P. intermedia*, *T. forsythia* and *E. corrodens*; and from "Moderate inflammation", chronic periodontitis by *P. gingivalis*, *F. nucleatum* and *T. forsythia*. Data modified from Tanner et al. [4].

periodontitis included strains of the closely related *Haemophilus aphrophilus* [6].

The "vibrio-corroder group" presented a new challenge by showing minimal to no growth in conventional glucose broth, and when growth occurred, did not ferment carbohydrates so most routine biochemical tests were not useful for strain characterization. As a group, colonies were transparent and many pitted or corroded agar surfaces. Some isolates resembled *Eikenella corrodens* that uses amino acids as an energy source and nitrate as electron acceptor. Other isolates with identical colony morphologies were highly motile and resembled "*Vibrio succinogens*", a cow rumen species that uses formate or hydrogen gas as energy sources, and fumarate (or other organic acids) as electron acceptors. A taxonomy study was performed and these strains were classified and identified as several new species, *Wolinella recta* (now *Campylobacter rectus*), *Campylobacter concisus*, and *Bacteroides gracilis* (now *Campylobacter gracilis*) [7]. Characterization of the strains used DNA hybridizations, as in the *A. actinomycetemcomitans* study [6], and sensitivity to a range of antibiotics, dyes and indicators. Companion reports classified the strains serologically [8] and described the cell ultrastructure of species [9].

The third group of unidentified isolates grew slowly as tiny colonies, composed of bacterial cells with tapered ends (fusiform) and formed succinate as an acid end product of metabolism, a characteristic of *Bacteroides* species, giving the group name of "fusiform *Bacteroides*". Multiple additives were tested to enhance growth, without much success, although growth agar was enhanced by co-culturing with *Fusobacterium nucleatum*. To characterize the "fusiform *Bacteroides*" strains, SDS-PAGE (polyacrylamide gel electrophoresis for whole cell protein profiles) combined with inhibitor tests and DNA hybridizations were used. The strains did not resemble any reference named *Bacteroides* species and a new species *Bacteroides forsythus* was proposed [10]. Subsequently, 16S rRNA sequence data demonstrated that this species did not fit in *Bacteroides*, *Prevotella*, or *Porphyromonas* [11]. The species was reclassified to *Tannerella forsythensis* [12] with the final name of *Tannerella forsythia* proposed shortly afterwards [13].

Parallel microbiology studies on periodontal and gingival diseases using anaerobic methods were performed by Holdeman (Moore) and Moore at the Virginia polytechnic institute in Blacksburg, Virginia, USA. Their studies [14] led to description of several additional new species that are recognized as significant bacteria in the microbiology of periodontal infections. These new species included *Selenomonas noxia*, *Selenomonas flueggei* [15], *Actinomyces georgiae*, *Actinomyces gerencseriae* [16], *Lactobacillus* (now *Olsenella*) *uli*, *Lactobacillus rimae* [17], *Oribaculum catoniae*, *Dialister pneumosintes* [18], *Prevotella tannerae*, *Prevotella enoeca* [19]. *Fusobacterium* (now *Filifactor*) *alocis* and *Fusobacterium* (now *Eubacterium*) *sulci* [20]. Taxonomy studies from Wade's laboratory further expands our understanding of the taxonomy of cultured oral species including for *Shuttleworthia satelles* [21], *Dialister invisus* [22], *Prevotella marshii* and *Prevotella baroniae* [23], and *Propionibacterium acidifaciens* [24]. Studies from Hishino's group have further clarified the oral microbiology and taxonomy of oral anaerobes including for *Mogibacterium diversum* and *Mogibacterium neglectum* [25], *Eubacterium saphenum* [26], *Eubacterium* (*Atopobium*) *minutum* [27] and *Eubacterium exiguum* (now *Slackia exigua*) [28].

2.3. Initial periodontitis in adults

The early stages of adult periodontitis can be difficult to differentiate from gingivitis. Bacteria from periodontal pockets can spread around and infect gingivally healthy sites of the same individual [29], making healthy sites from subjects with gingivitis or periodontitis inappropriate as non-diseased control sites. Anaerobic cultural analysis of the microbiota of initial periodontitis in adults compared the microbiota of healthy sites from healthy subjects, a gingivitis site in subjects with gingivitis (but no evidence of recent periodontal attachment loss) and an active initial periodontitis site that showed attachment loss in the last few months [30]. Of particular interest was whether the species of advanced periodontitis would also be detected in initial periodontitis.

Microbiologically, differences were detected between health, gingivitis and active (progressing) periodontitis. Active periodontitis was characterized by *T. forsythia*, *Selenomonas noxia*, then a new *Selenomonas* species [15], *C. rectus*, *Fusobacterium* (now *Filifactor*) *alocis* [20] *Eubacterium brachy* (Table 1). Most of these species were also detected in gingivitis. *Campylobacter gracilis* and *P. gingivalis* were associated with gingivitis, whereas *P. intermedia*, *P. nigrescens* and *E. corrodens* were elevated in both initial periodontitis and gingivitis. Sites with progressing attachment loss were detected at buccal sites, typical of gingival recession. In contrast to the interproximal active sites, these sites harbored a microbiota similar to that of gingival health particularly *Actinomyces naeslundii*, *Actinomyces israelii* and *Rothia dentocariosa* (Table 1).

Table 1
Microbiota of initial active periodontitis compared with health, gingivitis and recession (mean % species ± SD)*.

Species	Healthy	Gingivitis	Active	Recession
N subjects (sites sampled)	10 (14)	10 (15)	9 (10)	5 (5)
Tannerella forsythia	0.1 ± 0.1^{ga}	5.3 ± 2.9^{hr}	9.0 ± 3.1^{hr}	0
Selenomonas noxia [15]	0.9 ± 0.5^{a}	0.8 ± 0.8^{a}	5.9 ± 2.2^{hgr}	0^{a}
Campylobacter rectus	0.2 ± 0.2^{a}	1.2 ± 0.7	4.9 ± 2.2^{hr}	0^{a}
Selenomonas flueggeii [15]	0	0.2 ± 0.2	1.6 ± 1.0	0
Eubacterium lentum	0	0	1.3 ± 1.3	0
F. nucleatum ss. *fusiforme*	0.5 ± 0.3	0.1 ± 0.1	1.3 ± 0.7	0.4 ± 0.0
Filifactor alocis [20]	0^{a}	0.8 ± 0.6	1.6 ± 0.6^{h}	0
Eubacterium brachy	0.2 ± 0.2^{ga}	2.3 ± 0.7^{hr}	3.4 ± 1.8^{h}	0^{g}
Prevotella intermedia	0	2.9 ± 1.7	2.7 ± 2.4	0.4 ± 0.4
Prevotella nigrescens	2.2 ± 2.0	2.2 ± 1.3	2.2 ± 1.8	0.8 ± 0.8
Streptococcus anginosus	2.0 ± 1.4	1.2 ± 1.2	2.0 ± 1.8	0.4 ± 0.4
Campylobacter gracilis	1.9 ± 0.8	4.5 ± 1.9^{r}	1.9 ± 0.5^{r}	0^{ga}
Porphyromonas gingivalis	0	2.8 ± 2.2	0.7 ± 0.7	0
Capnocytophaga sputigena	0.1 ± 0.1	1.8 ± 1.1	0.9 ± 0.5	0
Capnocytophaga gingivalis	0.9 ± 0.5	1.6 ± 0.8	0.2 ± 0.2	0
Selenomonas sputigena	0.7 ± 0.6	1.5 ± 0.6	0.6 ± 0.4	0
Olsenella uli [17]	0	1.1 ± 1.1	0	0
Eikenella corrodens	0.2 ± 0.2	1.0 ± 0.8	1.3 ± 0.8	0.8 ± 0.5
Streptococcus oralis	14.2 ± 7.1^{ga}	0.8 ± 0.3^{hr}	1.3 ± 0.9^{h}	8.0 ± 3.7^{g}
Actinomyces naeslundii	13.6 ± 6.0	7.6 ± 3.3^{r}	3.7 ± 1.4^{r}	23.6 ± 7.9^{ga}
Actinomyces gerensceriae [16]	10.8 ± 5.9	2.6 ± 1.5	0.9 ± 0.6	2.0 ± 2.0
Parvimonas micra	4.9 ± 2.3	3.4 ± 1.6	3.2 ± 1.0	0.8 ± 0.5
Streptococcus intermedius	4.1 ± 2.6	3.3 ± 1.3	3.8 ± 2.4	0.4 ± 0.4
Veillonella parvula	2.5 ± 1.1	0.8 ± 0.5	2.7 ± 2.2	0
Streptococcus mutans	2.4 ± 1.8	0.1 ± 0.1	1.8 ± 1.2	0
Eubacterium nodatum	1.4 ± 1.4	0.4 ± 0.3	0	0
Rothia dentocariousa	1.3 ± 1.3	0.3 ± 0.2	0^{F}	14.4 ± 12.0^{a}
Gemella morbillorum	1.5 ± 1.0	0.3 ± 0.2	0.2 ± 0.2	0
Streptococcus sanguinis	0.7 ± 0.6	0.4 ± 0.4	0	8.4 ± 7.9
Actinomyces israelii	0.2 ± 0.2	2.3 ± 1.6^{a}	0.0 ± 0.0^{g}	10.0 ± 10.0
Actinomyces odontolyticus	0.5 ± 0.3	1.1 ± 0.6	0.9 ± 0.4	2.4 ± 1.9
Haemophilus aphropilus	0.8 ± 0.6	0.5 ± 0.4	0.7 ± 0.3	1.2 ± 1.2
Mean pocket depth (mm)	2.5 ± 0.5^{ar}	3.0 ± 0.6^{ar}	4.0 ± 1.0^{hgr}	1.5 ± 0.4^{hga}
Mean attachment level (mm)	1.3 ± 0.4^{ar}	1.9 ± 0.8^{ar}	3.7 ± 1.1^{hg}	3.3 ± 0.5^{hg}
Mean % of site with:				
Plaque	10 ± 32^{g}	70 ± 42^{hr}	78 ± 44^{r}	0^{ga}
Redness	10 ± 32^{g}	80 ± 42^{hr}	33 ± 50^{g}	0^{ga}
Bleeding on probing	0	17 ± 33	22 ± 44	0

Superscripts values that differed between groups < 0.05 Kriskal–Wallis rank test. h=health, g=gingivitis, a=interproximal active, r=buccal active/recession. 32 additional species were detected at less than 1% of the cultivable microbiota.

* Data modified from Tanner et al. [30], with reference links to species newly described when paper published.

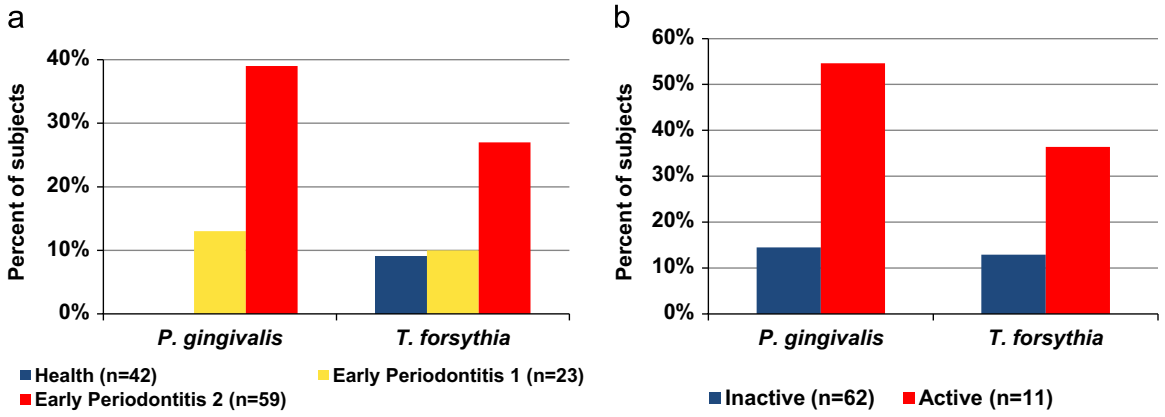

Fig. 2. *P. gingivalis* and *T. forsythia* in (a) early periodontitis and (b) active initial periodontitis in adults. Samples from periodontitis and healthy control sites were analyzed by PCR. (a) In the cross sectional analysis of early periodontitis, increased detection frequencies of *P. gingivalis* ($p < 0.001$) and *T. forsythia* ($p < 0.03$) were observed in subjects with over 1 site with at least 2 mm periodontal attachment loss. (b) In longitudinal analyses, detection frequency of *P. gingivalis* ($p < 0.01$) was associated with progressing/active periodontitis whereas the association with *T. forsythia* did not reach significance ($p = 0.075$). Data modified from Tanner et al. [35,36].

Anaerobic culture of progressing initial periodontitis thus added *Selenomonas noxia* and *Filifactor alocis* as candidate periodontal pathogens, to those species associated advanced periodontitis. Other studies on the same patient population examined subgingival temperature and microbiota in initial periodontitis [31], *Campylobacter* species in health, gingivitis, and periodontitis [32], and Serum IgG reactivity to subgingival bacteria in initial periodontitis, gingivitis and healthy subjects [33].

While the strength of anaerobic culture is the ability to detect all species present cultured in a sample, culture is time consuming and labor intensive which limits the number of subjects that can be analyzed. Furthermore, culture can underestimate the presence of fastidious species. For example, we observed that more subjects were *P. gingivalis* positive when subgingival samples were assayed using an immunofluorescence assay compared with anaerobic culture [34], suggesting that culture was underestimating the *P. gingivalis*. Thus to test our culture findings of initial periodontitis, we designed a study with more subjects and assayed samples using molecular assays, 16S rRNA [35] and whole genomic probes [36], in reverse capture and conventional checkerboard assays respectively. To specifically target *P. gingivalis* and *T. forsythia* we used a multiplex PCR assay [37]. In this larger population of 190 subjects, *P. gingivalis* and *T. forsythia* were significantly associated with early periodontal loss in comparison with healthy and gingivitis sites, whereas *P. gingivalis* was detected more frequently in progressing compared with inactive sites, while the association of *T. forsythia* with active periodontitis did not reach significance (Fig. 2). The gingival crevice fluid host in this population was also studied and a strong association between IL-1β and early periodontitis was observed [38].

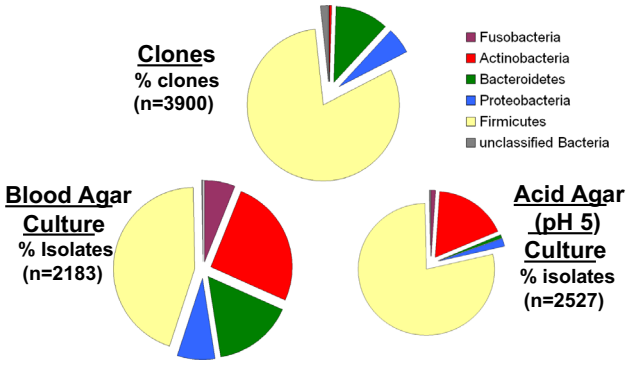

Fig. 3. Bacterial phyla from clonal and cultural analyses of early childhood caries and caries-free children. Samples from 80 children were processed by cloning and sequencing [58] and by anaerobic culture [57]. All clones and isolates were identified from 16S rRNA sequences in the Human Oral Microbiome Database (HOMD) [61]. Higher proportions of Actinobacteria were observed in cultural analysis on blood or acid agars compared with the cloning/sequencing analysis. Total bacterial counts on blood agar were 10 times higher than acid agar, and the greatest microbial diversity was observed from anaerobic blood agar isolation. Sequence based analyses by Natalia I Chalmers.

2.4. Periodontitis by anaerobic culture

Findings from these anaerobic culture studies on advanced and initial adult periodontitis provided the species for DNA probes for checkerboard of Socransky and co-workers [39]. There is a vast literature in periodontal microbiology based on these species. Other studies using anaerobic microbiology to characterize periodontitis that improve our understanding of the cultivable microbiota, include those of Slots [40–42], Zambon [43], and most notably those of Drs. LVH and WEC Moore [14]. Culture of pericoronitis [44], dentoalveolar abscesses [45] and endodontic infections [46] Wade's group expand periodontal species and their role in oral infections. Additionally having cultured isolates provides strains for antimicrobial and periodontal pathogenicity testing.

In defining pathogens for human periodontitis, the associations of *P. gingivalis* and *T. forsythia* with advanced and initial periodontitis in adults, suggests a continuum of disease progression involving these species. They are not, however, the only periodontal pathogens, which likely vary between patients. Further, additional unnamed species that are better detected using molecular methods [47] need considering in the pathogenesis of periodontitis.

3. Dental caries

Dental caries begins as decalcified enamel or white spot lesions which progresses into cavities in enamel and finally into dentin. If left untreated, bacteria in dentin invade the pulp then root canals. Dental caries is recognized to have three major risk factors; cariogenic bacteria, a susceptible host or compromised teeth and frequent ingestion of dietary carbohydrates which are used as

Table 2
Major phylotypes and species detected more frequently from either blood or acid (pH 5) agars from childhood caries and caries-free children.

Detected more frequently blood agar		Detected more frequently acid agar	
Firmicutes	*Streptococcus sanguinis* ($p < 0.0001$)	Firmicutes	*Streptococcus thermophius* ($p < 0.0001$)
	Gemella morbillorum ($p < 0.0001$)		*Streptococcus mutans* ($p < 0.0001$)
	Abiotrophia defectiva ($p < 0.0001$)		*Streptococcus anginosus* ($p < 0.0001$)
Actinobacteria	*Actinomyces naeslundii* ($p < 0.0001$)		*Streptococcus salivarius* ($p = 0.001$)
	Actinomyces sp. HOT 171 ($p < 0.0001$)		*Lactobacillus gasseri* (acid only)
	Actinomyces massiliensis ($p < 0.0001$)	Actinobacteria	
	A. gerensceriae ($p < 0.0001$)		*Scardovia* HOT195 (*S. wiggsiae*) ($p < 0.003$)
	Actinomyces sp. HOT 178 ($p < 0.0001$)		*Parascardovia denticolens* ($p = 0.034$)
	Actinomyces sp. HOT 169 ($p < 0.0001$)		*Bifidobacterium dentium* (acid only) ($p = 0.05$)
Veillonellaceae	*Selenomonas noxia* ($p < 0.0001$)	Veillonellaceae	
	Selenomonas infelix ($p < 0.0001$)		*Veillonella atypical* ($p < 0.0001$)
	Selenomonas artemidis ($p < 0.0001$)		*Veillonella dispar* ($p = 0.003$)
	Selenomonas sputigena ($p < 0.0001$)		
Bacteroidetes	*Capnocytophaga granulosa* ($p < 0.0001$)	Bacteroidetes	None
	Capnocytophaga gingivalis ($p < 0.0001$)		
	Capnocytophaga sp. HOT 326 ($p < 0.0001$)		
	Capnocytophaga sp. HOT 336 ($p < 0.0001$)		
	Prevotella melaninogenica ($p < 0.0001$)		
	Prevotella nigrescens ($p < 0.0001$)		
Fusobacteria	*F. nucleatum ss polymorphum* ($p < 0.0001$)	Fusobacteria	None
	Leptotrichia bucallis ($p < 0.0001$)		
Proteobacteria	*Campylobacter concisus* ($p < 0.0001$)	Proteobacteria	
	Campylobacter showae ($p < 0.0001$)		None
	Eikenella corrodens ($p < 0.0001$)		

Data from Tanner et al. [57].

substrates by bacteria to produce acid that will demineralize tooth surfaces. The rate of caries progression varies based on the levels of these risk factors. In young children dental caries can progress very rapidly and spread throughout the child's dentition.

The major species most frequently associated with dental caries are the acid-producing and acid-tolerant *Streptococcus mutans* and *Lactobacillus* species [48]. Several other bacterial groups have been associated with carious lesions including non-mutans streptococci, *Actinomyces* and *Bifidobacterium* species [49–51]. A sequence of colonization of bacterial groups in the progression of dental caries was presented in the seminal paper of Takahashi and Nyvad [52]. Under this hypothesis an initial stage of stability, albeit associated with mild and infrequent acidification, was associated with non-mutans streptococci and *Actinomyces*. This could lead to second acidogenic stage characterized by low pH species. The last aciduric stage was characterized by species that were highly tolerant to acid, including *Streptococcus mutans*, *Lactobacillus* and *Bifidobacterium* species.

Many other species, however, have been detected in dental caries, particularly in studies performed in the last decade using 16S rRNA identifications of clones [53–55] or isolates [56]. The identification of a wide diversity of species, including uncultivated species, suggested that our knowledge of the cultural microbiota of early childhood caries is incomplete.

3.1. Early childhood caries

Study of dental caries using anaerobic culture examined a patient population of children with advanced, severe early childhood caries that were scheduled for treatment under general anesthesia for the extensive restorations and extractions needed [57]. This allowed clinical measurements and sampling procedures to be performed under anesthesia avoiding any behavioral or compliance issues that could interrupt clinical procedures in very young, and frequently frightened, children. A dietary and dental history survey was also obtained from the child's parent or caregiver.

Samples for culture were taken and put in PRAS Ringers salt solution, processed and incubated anaerobically as for the previous studies in advanced and initial periodontitis. Samples from caries and caries-free control children were cultured on a complex blood agar to facilitate growth of fastidious anaerobes, including species without culture representatives, but that had been observed from cloning and sequencing analysis. Samples were also cultured on a pH 5 acidic agar medium to select for acid tolerant species, to enrich for novel caries pathogens [57]. Bacterial samples were co-processed by sequencing and cloning analysis [58] and by 16S rRNA (HOMIM) microarray [59].

There were several advantages to using an anaerobic culture method to study early childhood caries as has previously been observed for childhood caries [48] and carious dentin [60]. The cultural approach [57] detected a wider diversity of taxa than the parallel molecular cloning and sequencing [58] mainly because culture improved detection of Actinobacteria, including *Actinomyces* and *Bifidobacterium*/*Scardovia* species (Fig. 3). Improved detection of Actinobacteria from dental caries by culture compared to cloning/sequencing analysis had been observed in samples from deep dentine [56] and was attributed to limitations in the 16S rRNA primers used to amplify the DNA in samples before cloning. Anaerobic culture on blood agar allowed cultivation of several unnamed and previously uncultured species, particularly from blood agar isolation and prolonged primary incubation of about 10 days. Newly cultured species were mainly in *Streptococcus* and Actinobacteria and these isolates are currently undergoing further characterization under the Human Oral Microbiome Database (HOMD) project [1,61].

Several species were preferentially isolated on either blood (24 species) or the acidic (10 species) agar with marked differences within individual genera (Table 2) [57]. Species suppressed by the acidic agar included *Streptococcus sanguinis*, *Actinomyces*, *Selenomonas*, *Capnocytophaga*, *Prevotella*, *Fusobacterium and Campylobacter* species. This would suggest these species would not fit into species of the advanced aciduric phase of dental caries from the Takahashi/Nyvad model [52]. In contrast *S. mutans*, *Streptococcus anginosus*, *Streptococcus salivarius*, *Lactobacillus gasseri*, *Scardovia wiggsiae*, *Parascardovia denticolens* and *Bifidobacterium dentium* were detected more frequently from the acid agar and could be candidates for the advanced aciduric stage of caries progression.

Comparing the microbiota of severe early childhood caries with caries free children, the major caries-associated species from both blood and acid agar isolation were *S. mutans* and a new *Bifidobacterium*-group species, *Scardovia wiggsiae* [62] (Fig. 4). Not

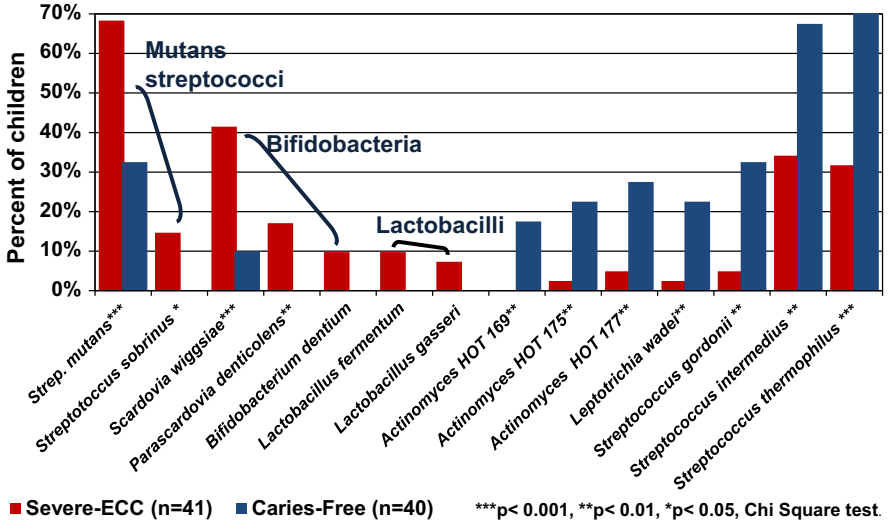

Fig. 4. Major acid-tolerant species cultured from early childhood caries and caries-free children. Samples were processed by anaerobic culture and isolates were identified from 16S rRNA sequences in HOMD. The detection frequencies of the major acid tolerant species associated with childhood caries were *S. mutans*, *S. wiggsiae*, *Parascardovia denticolens* and *Streptococcus sobrinus*. Other acid resistance species were detected more frequently from caries-free children including several non-mutans streptococci, and unnamed *Actinomyces* species as denoted by the Human Oral Taxon (HOT) number. Data modified from Tanner et al. [57].

all species isolated on the acid agar, however, were caries-associated including some unnamed *Actinomyces* species, *Streptococcus gordonii*, *Streptococcus intermedius* and *Streptococcus thermophilus*.

The association of *S. mutans* with severe early childhood (nursing) caries from cultural analysis was also observed in previous culture studies with anaerobic incubation of samples [63,64]. One study from David Beighton's laboratory was a comprehensive analysis that incorporated selection of acid-tolerant bacteria from broth isolation. In all the anaerobic cultural analysis using non-selective blood agar for strain isolation, the dominant species detected were *Streptococcus* and *Actinomyces* species [57,63,64]. Cultural detection of several species in Bifidobacteriaceae was performed using a modified selective agar, and while *S. wiggsiae* was detected, the major species isolated childhood caries was *Bifidobacterium dentium* [65]. Bifidobacteriaceae including *Bifidobacterium* and *Scardovia* species, however, are major caries-associated species being highly associated with root caries and other lesions in older adults [49,50] in addition to childhood caries [53,66,67]. A separate culture study of infected pulps in children identified high proportions of *S. wiggsiae* (as *Bifidobacterium* spp. 2) [68] suggesting that this species is a significant part of the microbial complex involved in the progression of deep dentinal caries.

Few *Lactobacillus* species were detected in the anaerobic cultivation of severe early childhood caries suggesting that they colonize at low levels of the microbiota in very young children [57]. Several *Lactobacillus* species were detected in the Beighton laboratory study of similar childhood caries infection, nursing caries, but only after use of selective isolation [64] which facilitates detection of species at low levels in samples. The major species detected, *Lactobacillus fermentum* was similar in both culture studies.

3.2. White spot initial carious lesions

The associations of *S. mutans* and *S. wiggsiae* with severe early childhood caries were observed from assay by species specific PCR [59] in addition to anaerobic culture. To evaluate whether *S. mutans* and *S. wiggsiae* were also involved in initial carious lesions, the PCR assay was modified to be quantitative (q-PCR) to examine the microbiota of initial white spot carious lesions [69]. The microbiota of white spot lesions in adolescents with fixed orthodontic bands was selected to study. White spots or zones parallel to the gingival margin representing enamel decalcification may develop within 6–12 months after orthodontic bands are placed, particularly when oral hygiene is poor [70]. White spot lesions are generally considered a cosmetic problem, although if enamel decalcification progresses to cavities, bands are generally removed to prevent further caries progression.

Several studies have examined the development of gingivitis associated with fixed orthodontic appliances [71,72]. The microbiology of incipient white spot carious lesions was studied using anaerobic culture by Bowden and co-workers [73]. The culture study focused on *Streptococcus*, *Lactobacillus* and *Actinomyces* species associated with the development of initial enamel decalcification below orthodontic bands and reported detection of *S. mutans* in only a few children.

Using qPCR, *S. mutans* and *S. wiggsiae* were associated with white spot lesions [69]. Both species were also associated with gingival inflammation, but, more importantly the caries associations remained significant after adjusting for presence of gingivitis. The caries-associations for *S. mutans* and *S. wiggsiae* were not significant using 16S rRNA probes in the HOMIM microarray that was performed in parallel with the qPCR assay [69]. Using the same HOMIM microarray caries-associations for *S. mutans* and *S. wiggsiae* were positive but were significant only for *S. mutans* [74]. Other white spot lesion-associated species varied between studies using the HOMIM microarray, possibly reflecting the longitudinal [74] compared to cross-sectional [69] study designs.

The detection of *S. mutans* and *S. wiggsiae* in initial caries, based on previous culture of these species using anaerobic methods of samples from advanced caries suggests that similar acidogenic and acid tolerant species can be involved in early and later stages of this disease, and could be good makers to detect aggressive disease.

4. Conclusions

Anaerobic culture of bacteria associated with advanced periodontitis and dental caries, compared to healthy, non-diseased, sites has proved extremely valuable in expanding our knowledge of the bacteria associated with these major clinical conditions of the oral cavity. By using rich non-selective media and anaerobic incubation long enough for the dominant microbiota to grow, a wide diversity of bacteria has been detected with improved detection of Actinobacteria compared to PCR-based methods using standard primers. Anaerobic culture studies have provided species for rapid detection using molecular methods which can be used on studies of larger populations of subjects. Anaerobic culture, however, is limited in the number of samples that can be processed as it remains a laborious and time consuming approach. Furthermore, species in lower proportions of the overall microbiota, and species for which the nutritional requirements are as yet unknown remain undetected by anaerobic culture [1].

Of clinical importance, pathogens to advanced periodontal and carious lesions were detected in initial disease. This suggests that pathogens from advanced disease are candidates for disease risk assessment. Since dental pathogens may also colonize healthy sites assessment of periodontal and caries risk will require addition of other risk markers for example host factors in periodontitis [75] and diet in dental caries [76].

Ethical approval

This review cites papers that have already been published and each had own ethical approval and thus was documented in the original publications.

Conflict of interest

None.

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