Journal of Dental Sciences 19 (2024) 1369—1379

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Review article

Oral microbial profiles of extrinsic black tooth stain in primary dentition: A literature review

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Received 22 February 2024; Final revision received 28 February 2024
Available online 8 March 2024

KEYWORDS
Bacteria;
Black stain;
Children;
Primary dentition

Abstract The extrinsic black tooth stain (EBS) is commonly found in primary dentition. Patients cannot clean the EBS; this can only be done by professional scaling and debridement. It also has a tendency to reform, which significantly compromises children's aesthetics and even affects their quality of life. However, there is no conclusive evidence on the etiology of the EBS. The associations between the EBS and related oral microbial features is one of the research hot topics. No literature review summarized these research progresses in this area. Therefore, we reviewed the literature on the microbiology of the EBS since 1931 and reported as the following 5 aspects: molecular biotechnology, morphological structure and physiochemical characteristics, microbial etiology hypothesis and core microbial characteristics. The EBS is a special dental plaque mainly composed of Gram-positive bacilli and cocci with scattered calcium deposits that acquired salivary pellicle activates. Early studies showed that the *Actinomyces* was the main pathogenic bacteria. With advances in biological research techniques, the 'core microbiome' was proposed. The potential pathogenic genera were *Actinomyces*, *Prevotella nigrescens*, *Pseudotropinibacterium*, *Leptotrichia*, *Neisseria* and *Rothia*. However, the pathogenic species of the above genera were still unclear. Currently, it is believed that the EBS consists of iron compounds or black substances that oral bacterial metabolism produces or that the bacterial metabolites formed after chemical reactions in the micro-ecological environment.

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https://doi.org/10.1016/j.jds.2024.02.028
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Introduction

The extrinsic black tooth stain (EBS) is commonly found in primary dentition (Fig. 1). The main clinical characteristics are the discontinuous black dots or dark lines deposited on the crown, which can cover most of the tooth surface or even affect the pits and grooves in severe cases. The prevalence of the EBS ranges from 2.4% to 26% worldwide.[1–12] These black stains adhere tightly to the tooth surface. Patients cannot remove the EBS with daily oral health self-care methods, such as using a toothbrush and toothpaste. Currently, the EBS is mainly removed through professional scaling and debridement.[13] However, the EBS often reform quickly, lacking a fundamental solution.[14,15] The EBS significantly compromises children's appearance and confidence and causes long-term troubles for the patients and their families. Therefore, studying the causes and formation mechanisms of the EBS is of crucial significance for providing fundamental prevention and treatment measures in clinical practice.

Currently, there is no consensus on the etiology of the EBS. The association between the EBS and related oral microbial features is a research direction. In 1931, researchers found that black-stain samples mainly comprised various bacteria under the microscope.[16] Between the black-stain samples and the tooth surface, researchers observed a structure similar to the acquired pellicle. The development of molecular biotechnology has enhanced the understanding of the EBS microecological environment. Many speculate that changes in certain oral microorganisms may lead to the EBS formation. However, there is no literature review summarized these research progresses in this area. Therefore, we reviewed the literature on the microbiology of the EBS since 1931 and reported as the following 5 aspects: molecular biotechnology, morphological structure and physiochemical characteristics, microbial etiology hypothesis and potential core microbial characteristics.

Molecular bio-techniques

Next-generation sequencing (NGS) technology and proteomics have empowered researchers to explore the etiology of the EBS formation (Fig. 2).

Next-generation sequencing technology

Gene sequencing technology originated from the Sanger sequencing technology. With the development of high-throughput sequencing technology, Roch 454, Illumina HiSeq/MiSeq and SOLiD are the main sequencing platforms that emerged in the mid-1990s. NGS is a DNA sequencing technology based on PCR and gene chips, which can simultaneously determine the sequence of hundreds of thousands to millions of DNA molecules.[17] Due to the high throughput and short read length of NGS, the amplicon sequencing uses the variable regions of microorganisms, such as 16S, 18S and ITS to obtain the microbial community structure, evolutionary relationship and the correlation between microbiomes and their ecological environment. Unlike Sanger sequencing, the NGS technology does not rely on cultivable microorganisms. Some dead and uncultivable microorganisms can also be detected. It has significant advantages in processing large-scale samples, improving sequencing speed, increasing sequencing throughput and reducing the cost and time of microbial identification.[18,19] NGS is the core sequencing technology up to now.

For the EBS microbial studies, the V3–V4 hypervariable region of the 16S rRNA gene is the most common research part. Among them, Roche 454 pyrosequencing and Illumina HiSeq/MiSeq sequencing platforms were used to study the EBS microbial communities.[20–26] However, due to the limitation of NGS, which can read up to 250–800bp, the full length of the bacterial 16S rRNA gene sequence (1540bp) could not be completed.[19] Therefore, the associated microbiomes only can be identified in the genus level, not exactly to the species. Regarding the relationship between

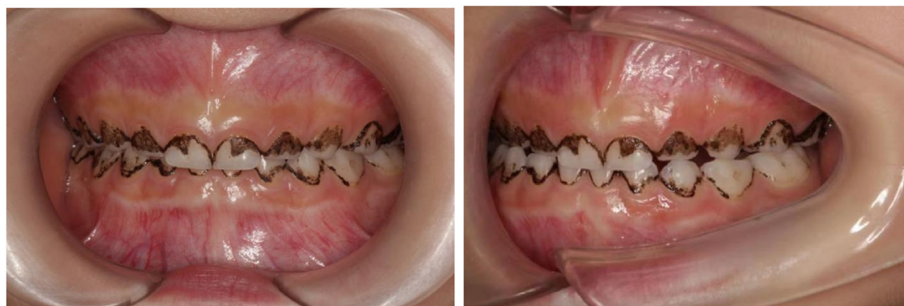

Figure 1 Clinical pictures of the EBS in primary dentition.

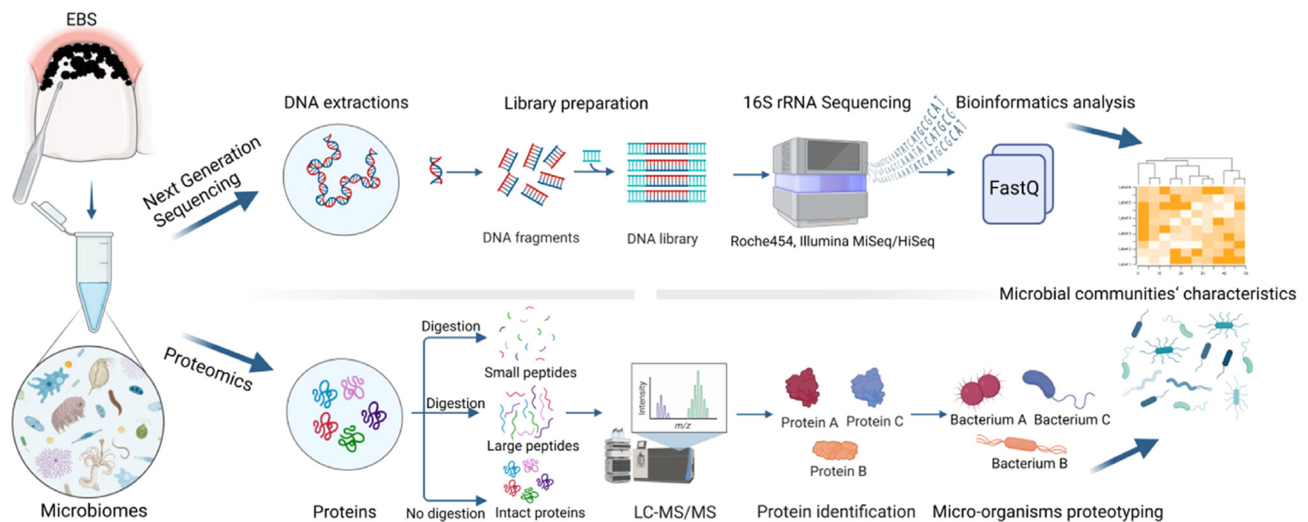

Figure 2 Workflows of next-generation sequencing (NGS) and proteomics.

microorganisms and diseases, it is more valuable to define the species or even the strain. Thus, adopting the third-generation sequencing technology to obtain longer reading sequences will help to obtain a more concise microbial information and classification for further pathogenesis mechanism studies.

Proteomics

Proteomes are all the proteins a cell or tissue produce under a certain time or life span. Proteomics is a research method used to separate the complex mixed proteins into single proteins by 2-dimensional gel electrophoresis or multidimensional liquid chromatography.[27] Trypsin digests the single proteins into peptides. These peptides are compared with the standard microbial proteomic fingerprint mass spectrometry database for microbial identification and classification. Meanwhile, the dynamic protein composition, expression level, modification state and protein function can also be revealed.[27,28] Compared to sequencing technologies, proteins have more stability than genes do, the extraction process affects them less and they can better reflect the dynamic biological functions expressed in situ. Therefore, proteomics is a promising research method in future.

Morphological features

Under confocal microscope, the matured supragingival plaque biofilm mainly comprised Gram-positive aerobic or facultative anaerobic bacillus and cocci, which aggregate into a 'hedgehog' structure. The framework contains a base comprised of *Corynebacterium* filaments, an annulus with bacillus and filamentous bacteria and a periphery of corncob structures consisting of cocci.[29] Bibby et al. reported that the main bacterial components of the EBS also included Gram-positive aerobic or facultative anaerobic bacillus and cocci.[10,16,30–32] There was a cell-less and unstructured basement membrane similar to acquired membranes on which rod-shaped bacteria vertically aggregated and adhered tightly to each other, lacking an intercellular matrix and forming a dense 'network' structure.[10,32] In areas with less attachment of bacillus, amorphous calcium deposits were covered by a large amount of coccus, which were considered as a special type of dental plaque.[10,32]

Physiochemical characteristics

Past researchers have proposed that the EBS was a type of special dental plaque containing organisms and insoluble inorganic compounds, such as ferric, calcium and phosphorus plasma (Fig. 3A).[16,31,33–35] Several studies found that iron and microbiota were closely related with the EBS. In 1977, Reid et al. observed the absorption peaks of iron, ferrous ions, iron sulphide and ferrous sulphide using α,α'-bipyridine spectrophotometry.[31] These results confirmed the presence of iron in the EBS. However, iron is an essential element for all microorganisms' growth. Its discovery in the EBS was not surprising. However, in 2017, Zhang et al. analysed the EBS samples by inductively coupled plasma-mass spectrometry. The results showed that the concentration of iron in the EBS groups was 76.12—116.88 $\mu g\ g^{-1}$, which was significantly higher than that in normal plaque ($P < 0.001$), suggesting that iron might be related to the EBS.[35] In 2022, Zhang et al. used phylogenetic investigation of communities by reconstructing unobserved states (PICRUSt) software to determine the microbial function profiles.[25] They found that the iron complex outer membrane receptor protein, the iron complex transport system and the iron (III) transport system (KO2010, KO2011, KO2012) were enriched in the EBS plaque samples. These systems' activities were significantly enhanced. In fact, bacteria could not produce iron but secreted the siderophore. Siderophore chelated the iron ions in the environment, and these ions were bound to the outer membrane iron receptor proteins in the bacteria due to enzymes (Fig. 3B).[36] Subsequently, iron was imported to the cell and began further biological utilisation. Therefore, the enrichment and enhanced activity of iron-complex outer membrane receptor proteins and iron-related

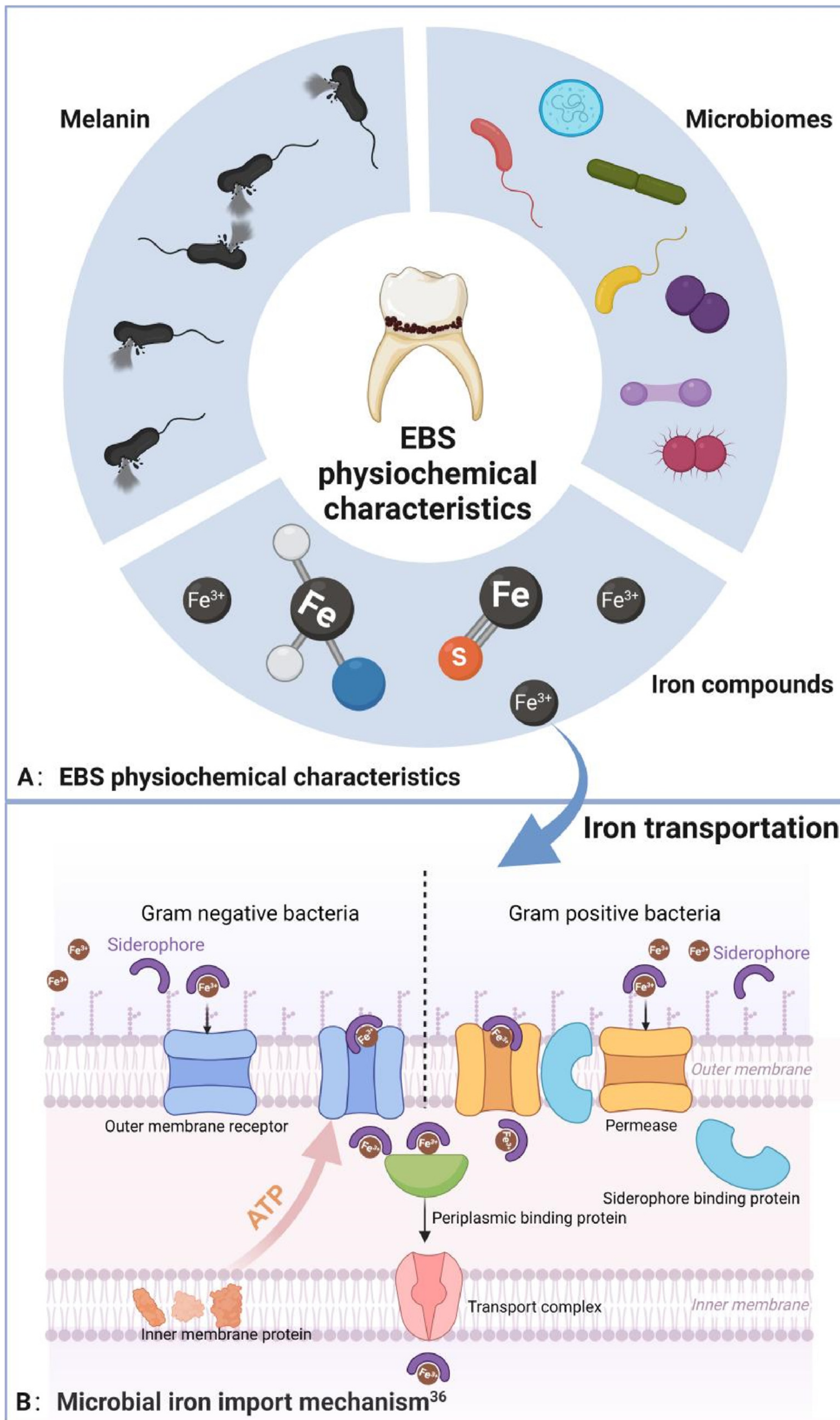

Figure 3 (A) EBS physiochemical characteristics. (B) Microbial iron import mechanism.

transport systems suggested increased iron bioavailability in the EBS. Iron is an important component of many enzymes; it play roles in regulating microbial surface movement and stabilising the polysaccharide matrix. Under the iron-deficient condition, the surface hydrophobicity of microbiota decreases, resulting in limited biofilm formation. Meanwhile, iron also contributes to the respiratory chain process as well as the catalytic reactions of DNA deoxyribozymes and several DNA polymerases.[37] Therefore, iron might affect biofilm formation, bacterial metabolism and the growth and reproduction of the EBS. Zhang et al. also proposed that the EBS may be the iron compounds that microbial metabolism produced.[35]

Reid et al. detected sulphide using N, N-dimethyl-p-phenylenediamine. They observed blue regions in the EBS samples under a handheld magnifying glass, suggesting that these regions were sulphide. Combined with the results of spectrophotometric studies, they speculated that the sulphide might be ferric sulphide.[31] Zhang et al. employed the PICRUSt to predict the functional characteristics of the EBS microbiota.[25] The results indicated that the metabolic pathways of methionine and cysteine were highly abundant in the EBS group. Both of these amino acids contained sulphur. Methionine could be converted into cysteine. Methionine and cysteine could produce H_2S through fermentation.[38] It was presumed that H_2S bacteria produced reacted with iron in saliva or gingival crevicular fluid and formed ferric sulphide. Therefore, the EBS formation might be the product of the interaction between microbial metabolites and the ecological environment.

In addition, researchers separated *Bacteroides melaninogenicus* from the EBS samples. *B. melaninogenicus* is a type of chromogenic bacteria.[39] It can produce black substances, including extracellular colloidal ferrous sulphide, extracellular melanin, intracellular haemoglobin and intracellular derivatives of haemoglobin, suggesting that bacteria might also directly produce the EBS. Moreover, Zhang et al. performed functional analysis and found that tyrosine metabolism was active.[35] Tyrosine metabolism can produce melanin, but whether melanin was the main component of the EBS remains unclear.

Microbial etiology hypothesis

Before the 20th century, using electron microscopy and in vitro culture technology, scholars proposed that filamentous bacteria or *Actinomyces* were associated with the EBS formation.[16,30] With the emergence of NGS technology, a special micro-ecological environment was discovered in the EBS, where other suspicious pathogenic bacteria were also detected. The authors categorized these findings into a specific plaque hypothesis and a core microbiome hypothesis, respectively.

Specific plaque hypothesis

In 1931, Bibby et al. cultured the EBS samples in aerobically and anaerobically environments.[16] They cultured 7 filamentous bacteria, of which 4 bacterial were anaerobic and the rest were aerobic. Some microorganisms' adherent grew on the tube wall in liquid media, indicating the possibility of plaque formation on smooth surfaces. Meanwhile, these strains exhibited protein degradation characteristics, suggesting that proteins in saliva could be utilized. In total, these 7 filamentous bacteria were considered the pathogenic bacteria for the EBS. However, these microbiota were not identified due to the technical limitations at the time.[16]

In 1974, Slots et al. cultured bacteria in the EBS samples. Biochemical testing, colony morphology and cell morphology observations were also conducted. They found that Gram-positive bacteria accounted for about 90% of the cultivable microorganisms in the EBS, of which 82% were *Actinomyces*. This indicated that *Actinomyces* was the main microbial community in the EBS.[30] In 2016, Tripodi et al. used multiple microbial cultivation media for black sedimentation, and the results showed that *Actinomyces* spp. accounted for 63% of the total cultivable microorganisms. *Streptococcus* spp. was 31%. *Capnocytophaga* spp. and *Fusobacterium* were 2%, respectively and *Actinomyces actinomycetemcomitans* and *Neisseria* spp. accounted for 1%, respectively. They believed that the high presence of *Actinomyces* might have been related to the EBS formation.[10] In all, these results suggested that *Actinomyces* might be a specific microbial community in the EBS formation. The strains of this microbial flora might be the main pathogenic bacteria of the EBS, which provided a targeted goal for the EBS prevention and treatment.

'Core microbiome' hypothesis

After 2000, with the development of biofilm theory and advances in the culture technology and molecular biological methods, more researchers believed that the EBS formation was not only due to a single or specific bacteria but also to the 'core microbiome'. The term 'core microbiome' refers to a set of microbial taxa or the genomic that are the characteristics in a defined environment. Usually, core microbiomes are identified as the microbiomes shared among 2 or more samples. These microbial taxa have a high detection rate and can be the predominant microbiomes, or it not affected by their abundance, have direct or synergistic influence on the microbial community function.[20—26,40]

In recent years, many studies adopted NGS to investigate the EBS microbial diversity by using Roche 454 or the Illumina MiSeq/HiSeq platform, targeting at the V1—V3 or V3—V4 hypervariable regions of microbial 16S rRNA. However, multiple studies have shown different potential pathogens in the EBS samples (Table 1). The dominant microbial flora reported included *Actinomyces*, *Cardiobacterium*, *Haemophilus*, *Corynebacterium*, *Tannerella*, *Treponema*, *Leptotrichia*, *Fusobacterium*, *Streptococcus*, *Neisseria*, *Capnocytophaga*, *Pseudopropionibacterium*, *Rothia*, *Pseudomonas fluorescens*, *Aggregatibacter Prevotella*, *Bacteroides*, *Kingella* and *Lautropia*. Some studies found that *Actinomyces* was significantly abundant.[20,22,24,25] Chen and Zheng et al. revealed that *Pseudopropionibacterium* was strongly

Table 1 Oral microbial profiles of the EBS in primary dentition.

Year	Country	Participants, n	Samples	Detection methods	Oral microbial profiles
2015[20]	China	10 EBS; 15 HG	saliva plaque EBS	NGS (V3–V4 regions, 16SrRNA)	Relative abundance: • Plaque-EG vs. HG: *Actinomyces*, *Cardiobacterium*, *Haemophilus*, *Corynebacterium*, *Treponema* and *Tannerella* ($P < 0.05$, EG↑); *Campylobacter* ($P < 0.05$, HG↑) • Saliva-No significant difference
2016[21]	China	40 EBS; 26 HG	saliva plaque EBS	454 pyrosequencing (V1–V3 regions,16SrRNA)	Relative abundance: • Plaque-EG vs. HG: *Leptotrichia* and *Fusobacterium* ($P < 0.05$ –0.001, EG↑) • Saliva-No significant difference
2019[22]	China	10 EBS; 10 HG	plaque EBS	Illumina MiSeq sequencing (V3–V4 regions,16SrRNA)	Relative abundance: • Plaque-EG vs. HG: *Actinomyces naeslundii* ($P < 0.05$, EG↑); *Candidate_division_TM7* ($P < 0.05$, HG↑)
2021[24]	China	17 EBS; 26 HG	saliva EBS	Illumina MiSeq sequencing (V3–V4 regions,16S rRNA)	Relative abundance: • Saliva-EG vs. HG: *Neisseria* ($P < 0.05$, EG↑); *Catonella*, *Granulicatella*, *Prevotella*, *Actinomyces*, *Acinetobacter*, *Corynebacterium*, *Streptomyces*, *Kingella*, *Butyrivibrio*, *Atopobium*, *Oribacterium*, *Cardiobacterium*, *Rhodococcus*, *Bulleidia*, *Campylobacter*, *Neisseria*, *Rothia*, *Abiotrophia*, *Bacillus*, *Tannerella* and *Peptococcus* ($P < 0.05$, HG↑) Biomarkers [LDA score (log10) >2]: • EG: Neisseria • HG: *Stenotrophomonas*, *Actinomyces*, *Corynebacterium*, *Rothia*, *Porphyromonas*, *Prevotella*, *Capnocytophaga*, *Sphingobacterium*, *Granulicatella*, *Oribacterium*, *Peptococcus* and *Campylobacter*
2021[23]	China	15 EBS; 18 HG	plaque EBS	Illumina MiSeq sequencing (V3–V4 regions,16S rRNA)	Relative abundance: • Plaque-EG vs. HG: *Selenomonas_3* and *Capnocytophaga* ($p < 0.05$, EG↑); *Lautropia* and *Abiotrophia* ($P < 0.05$, HG↑) Biomarkers [LDA score (log10) >4]: • Plaque-EG: *Actinomyces*, *Rothia*, *Pseudopropionibacterium*, *Capnocytophaga* and an uncultured *Lentimicrobiaceae* genus • HG: *Lautropia*
2022[25]	China	21 EBS; 48 HG	saliva plaque EBS	Illumina MiSeq sequencing (V3–V4 regions,16S rRNA)	Relative abundance: • EG vs. HG: an unclassified *Pseudomonas* sp., *Lautropia mirabilis*, *Pseudomonas fluorescens* and *Rothia aeria* ($P < 0.05$ –0.001, EG↑); an unclassified *Streptococcus* sp., *Corynebacterium matruchotii*, *Actinomyces naeslundii*, *s__hongkongensis*, *s__catoniae* and *Leptotrichia* sp.*_HMT_392* ($P < 0.01$–0.001, HG↑) • Saliva-EG vs. HG: an unclassified *Neisseria* sp., *Lautropia mirabilis* and an unclassified *Haemophilus* sp., ($P < 0.05$ –0.001, EG↑); *Prevotella melaninogenica* and an unclassified *Prevotella* sp. ($P < 0.05$–0.01, HG↑) Biomarkers [LDA score (log10) >3]: • EBS-EG: an unclassified *Pseudomona* sp., *Pseudomonas fluorescens*, *Leptotrichia* sp.*_HMT_212*, *Actinomyces* sp.*_HMT_169*, *Aggregatibacter* sp.*_HMT_898*, an unclassified *Prevotella* sp., an unclassified *Bacteroides* sp. and an unclassified *Selenomonadaceae(family) sp.* • Plaque-EG: *Lautropia mirabilis*, *Rothia aeria*, *Corynebacterium durum*, *Kingella oralis* and an unclassified *Fusobacterium* sp. • Plaque-HG: *Corynebacterium matruchotii*, *Actinomyces naeslundii*, an unclassified *Streptococcus* sp., *Prevotella shahii*, *s__hongkongensis*, *Leptotrichia* sp.*_HMT_392*,

Table 1 (*continued*)

Year	Country	Participants, n	Samples	Detection methods	Oral microbial profiles
					*s__cationiae, s__sp__HMT_286, Leptotrichia shahii, s__TM7_G-1_bacterium_HMT_348, s_massiliensis, s__sp__HMT_326,*an unclassified *Selenomonas* sp., *s__morbillorum, Prevotella melaninogenica, Lachnoanaerobaculum umeaense, s__intermedius, s__sp__HMT_481* and *Selenomonas artemidis* • Saliva-EG: an unclassified *Neisseria* sp., *Lautropia mirabilis*, an unclassified *Haemophilus* sp., an unclassified *Aggregatibacter* sp., *Neisseria oralis, Aggregatibacter* sp.*_HMT_898* and *s__propionicum* • Saliva-HG: *Prevotella melaninogenica,* an unclassified *Prevotella* sp., *Streptococcus parasanguinis clade 411*, an unclassified *Lactobacillales(order) sp.*
2022[40]	France	47 EBS; 47 HG	plaque EBS	MS	Significantly modulated: • Plapue-EG vs. HG: *Alcanivorax, Conchiformibius, Methylococcus, Vibrio, Kingella, Rothia, Methylocaldum, Pseudopropionibacterium, Tessaracoccus* and *Neisseria* ($P < 0.003$—0.001, EG↑); *Clostridium, Lachnoclostridium, Leptotrichia, Centipeda, Selenomonas, Alloprevotella, Enterococcus, Treponema, Catenibacterium* and *Prevotella* ($P < 0.003$—0.001, HG↑)
2023[26]	China	10 EBS; 10 HG	plaque EBS	Illumina MiSeq sequencing (V3—V4 regions,16S rRNA)	Relative abundance: • Plaque-EG vs. HG: *Lautropia* and *Pseudopropionibacterium* ($P < 0.05$, EG↑) *Veillonella, Lachnoanaerobaculum, Kingella* and *Dialister* ($P < 0.05$, HG↑)

Abbreviations: EG, EBS group; HG, healthy group.

enriched.[23,25] *Rothia* was enriched in the study conducted by Chen and Zhang et al.[23,25] In another study that Han and Zhang et al. conducted,[24,25] *Neisseria* also had a higher abundance compared with the control group of *Leptotrichia* and *Fusobacterium*, which had a high account in Li and Zhang's studies.[21,25] Note that *Actinomyces*, *Pseudopropionibacterium* and *Corynebacterium* belonged to Actinobacteria. However, Li et al. indicated that *Actinomyces* might not be the only bacteria involved in the EBS formation, and the changes of abundance of other species might affect the formation process.[20] Hirtz et al. used proteomic methods for the first time to study the EBS microbiomes.[40] The results were analyzed using the Swiss—Prot protein sequence database. They found that *Rothia*, *Kingella*, *Neisseria* and *Pseudopropionibacterium* were highly abundant at the genus level in the EBS group.

Therefore, *Actinomyces*, *Pseudopropionibacterium*, *Neisseria*, *Rothia*, *Leptotrichia* and others in the EBS had high abundance in many studies. However, no single oral microorganism was shared within all the included studies (Fig. 4). No experiments had further verified the functions of these potential EBS microbial biomarkers until now. Furthermore, the impact of the medium- and low-abundance microbiota to the EBS ecological environment remains unknown. This indicated that more research using omics data and experimental validation should be conducted to confirm the pathogenic composition and structure of the EBS 'core microbiome'.

Characteristics of core microbiome

Based on current speculations about the physiochemical components of the EBS, we presented the characteristics of the core microbiota that were reported at least in 2 included studies (Table 2).

Actinomyces

Actinomyces is a Gram-positive facultative anaerobic bacillus. It is a part of the normal oral microbial flora and it plays an important role in forming oral biofilm.[41] Thus far, 33 species have been identified in this genus. For years, *Actinomyces* was considered an important microbial community affecting the EBS formation. One of the reasons was that certain *A.* spp. could produce H_2S, especially *Actinomyces naeslundii*, which might lead to the formation of ferric sulphide.[42] The other reason was that *Actinomyces* could produce various siderophores with high affinity for Fe^{3+}. Hydroxamic acid siderophores and catechol siderophores were the most common.[43—45]

Prevotella nigrescens

Prevotella nigrescens is a Gram-negative specialised anaerobic bacillus that forms a black colony on blood agar medium and can produce pigments ranging from dark brown

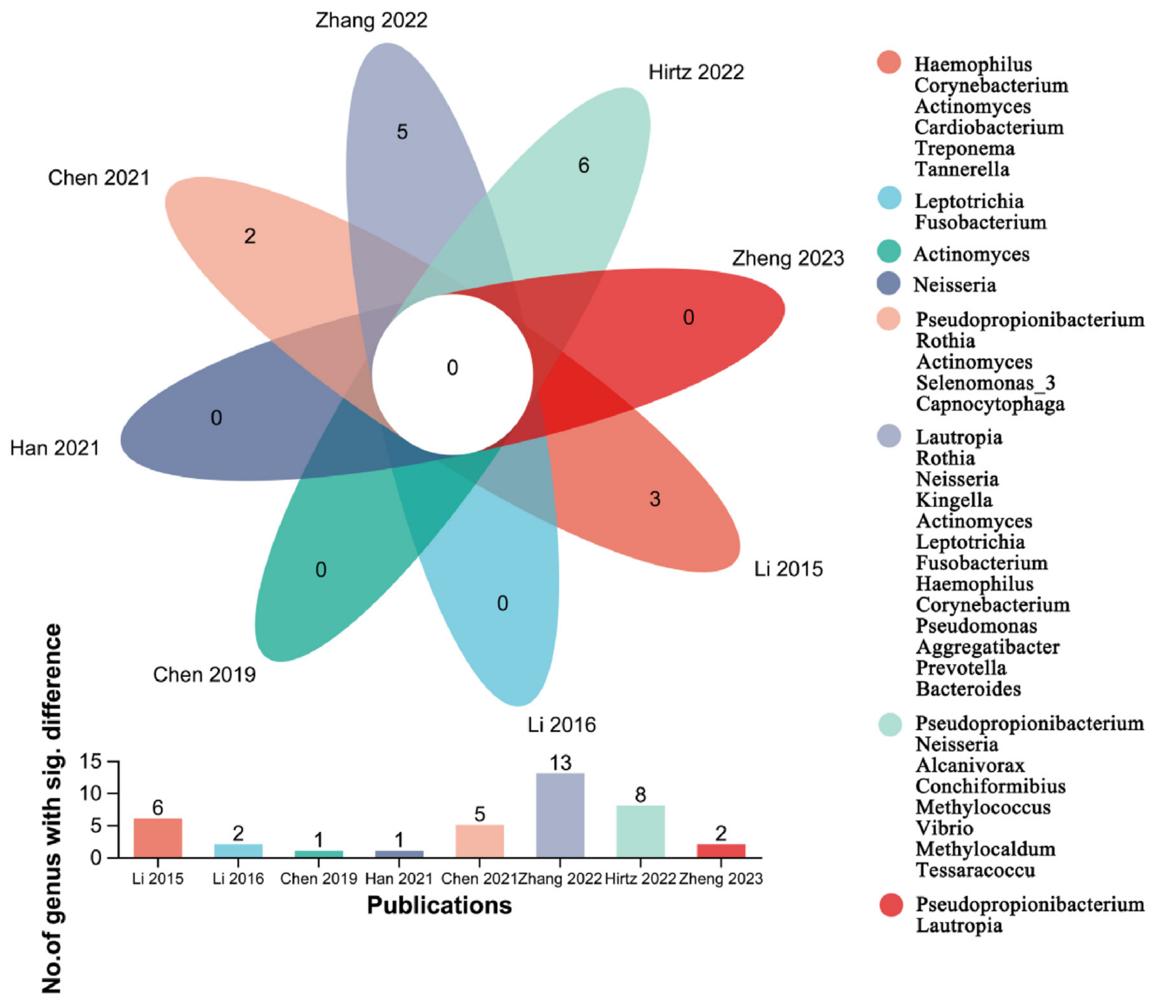

Figure 4 Venn diagram of the core microbiome at the genus level.

to black.[46] *Prevotella nigrescens* relied on haemoglobin as the iron source required for growth.[47] It could produce H_2S, which might be involved in the EBS formation.[48] However, several studies reported no significant differences were found in the relative bacterial abundances between the EBS samples and normal plaques.[49,50] Although *Prevotella nigrescens* can produce a black substance, it might not be the pathogenic bacterium for the EBS.

Pseudopropionibacterium

Pseudopropionibacterium is a Gram-positive facultative anaerobic bacillus that belongs to Actinobacteria, a member of the human oral microbial community. In 2016, it was reclassified as a new genus of bacteria from *Propionibacterium*.[51] Archaeological findings depicted that this genus was present in ancient humans' dental calculus, and it may be related to infectious endocarditis and other diseases.[52] Moreover, in 2021, through PICRUSt functional prediction, Chen et al. detected high levels of carbohydrates and amino acid metabolism processes in this genus of bacteria.[23] Amino acid metabolism helped neutralise acid in dental plaque, suggesting that it might relate to caries inhibition in the EBS patients. At present, there are 3 known bacterial species under the *Pseudopropionibacterium*, namely *Pseudopropionibacterium propionicum*, *Pseudopropionibacterium rubrum* and *Pseudopropionibacterium massiliense*. Research has shown that H_2S production could also be detected in the metabolites of *P. propionicum*.[53,54] Interestingly, these 3 bacterial strains were of dark orange, red, or brown colonies in specific media, which relate to the EBS formation.[54–56]

Neisseria

Neisseria is a Gram-negative obligate aerobic diplococcus, which is a member of the host's normal microbiota. This genus contains 31 bacterial species, among which *Neisseria meningitidis* and *Neisseria gonorrhoeae* can synthesise cysteine.[57] H_2S can be produced through cysteine metabolism, which indicates that *Neisseria* might be associated with the EBS formation.

Leptotrichia

Leptotrichia is a Gram-negative anaerobic bacillus containing 6 species. This bacterium is commonly found in dental plaque and associated with dental caries,

Table 2 Characteristics of the EBS potential core microbiome in the reviewed studies.

Core microbiomes	Significance in oral cavity	Characteristics
Actinomyces	Associated with maturation of dental plaque[64] Related to root caries[65]	Produce melanin[66] Produce H_2S[42] Adjust the pH of the oral environment[67] Able to bind to acidic proline-rich protein[68] Produce siderophores with high affinity for iron[45]
Prevotella nigrescens	Related to periodontitis[69]	Produce melanin[46] Produce H_2S[48] Rely on haem in haemoglobin as the iron source required for growth[47]
Pseudopropionibacterium	Inhibits dental caries[23] Related to acute and chronic pulp infections[70]	Produce H_2S[54] Carbohydrate metabolism and amino acid metabolism are abundant[23]
Neisseria	Normal oral microbial community[71]	Synthetic cysteine[57]
Leptotrichia	Related to periodontal disease and oral abscess[58—60]	Abundant in carbohydrate metabolism[72] Produce H_2S[61] Promote oral colonization of *Fusobacterium*[62,63]
Rothia	Related to dental caries and oral leukoplakia[73]	Activate macrophages and immune regulatory systems in the human gums Contain catechol-siderophore like biosynthetic genes that may be associated with the EBS[40]

periodontal disease and oral abscess.[58—60] The functional feature analysis Chen et al. conducted showed that *Leptotrichia* had a high carbohydrate metabolism, which could produce more organic acids and extracellular polysaccharides than the control could.[23] However, the role of this function in the EBS is unclear. In addition, studies showed that *Leptotrichia* could produce H_2S, which is a definite odour-causing bacterium.[61] In 2016, Li et al. found that *Leptotrichia* promoted the *Fusobacterium* colonized in the oral cavity, which could contribute to the EBS formation in primary dentition.[21] *Fusobacterium* could co-aggregate with chromogenic bacteria, such as *Porphyromonas gingivalis* and *Prevotella melaninogenica*, leading to further infection.[62,63] However, the association between this microbiota and the EBS still needs further investigation.

Rothia

Rothia is a Gram-positive irregular bacillus belonging to Actinobacteria, which has many shared characteristics with *Actinomyces*. This genus has 11 bacterial species. Uranga et al. conducted a genome-mining technique targeting the biosynthetic gene clusters in *Rothia*.[40] The results found a catechol-siderophore-like biosynthetic gene possibly associated with the EBS in *Rothia mucilaginosa*, *Rothia dentocariosa* and *Rothia aeria*.

Conclusions

In conclusion, the EBS formation was considered related to the 'core microbiome', such as *Actinomyces* (especially *A. naeslundii*), *Prevotella nigrescens*, *Pseudopropionibacterium*, *Leptotrichia*, *Neisseria* and *Rothia*. However, controversy remains over the EBS core microbiota due to the differences in research methods and experimental designs. The pathogenesis of the EBS is still unclear because the current research methods only identify the microbial flora at the genus level. In addition, the lack of experimental validation at the species level and the complex composition of the EBS components also slowed the progress in pathogenic mechanisms.

In this regard, we proposed the following recommendations: 1) to obtain more specific information about the microbial community information, the third-generation sequencing with a longer reading length is recommended for 16S rRNA amplification sequencing. Furthermore, comprehensive studies from multi-omics, for example, macrogene sequencing, macroproteome, metabolome and transcriptome, should be conducted for more consist findings of the EBS core microbiome. 2) It is necessary to culture and preserve the EBS-related microorganisms or to establish an the EBS oral microbial bank for future experiment. 3) The EBS pathogenic mechanism should be solved by multiple disciplines. The help of chemists is crucial to discover the EBS material components as well as the possible chemical reaction between and within the micro-ecological environment. Based on these fundamental findings, microbiologists should identify relevant pathogenic microorganisms, and they should establish the relationship between chemical constituents and microorganisms. Combined with valuable epidemiological findings and dentists' clinical experience, a comprehensive and multidimensional analysis of the EBS pathogenesis can profoundly promote prevention and treatment.

Declaration of competing interest

The authors declare no potential conflict of interest with respect to the authorship and/or publication of this article.

Acknowledgments

The authors gratefully acknowledge the financial support from the Yunnan Provincial Science and Technology Department and Kunming Medical University Conjoint Fund, China (No. 202201AY070001-172), Yunnan Provincial High-level Science and Technology Talents Project for Youth and Middle-aged Scholars and Leaders (No.202405AC350008), and National Key Clinical Specialty Development Project of Pediatric Dentistry Division, China.

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