

Microorganisms associated with black tooth stain and contemporary strategies for their management

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Abstract

Introduction: Black stain is an extrinsic tooth discoloration, characterized by dark lines or incomplete pigmentation along the gingival margin in the cervical third of the tooth. They can affect both primary and permanent dentitions.

Aim: To investigate the microbial composition of black tooth stains in children and explore potential preventive and therapeutic strategies.

Materials and methods: Supragingival plaque samples were collected from 60 children (30 with and 30 without black stain). Bacterial identification was performed using 16S rRNA gene sequencing. Isolates were classified by morphology and biochemical characteristics. Based on microbial profiles, photodynamic therapy and probiotic supplementation were proposed as adjunctive management strategies. Data were analyzed statistically using the Fisher test.

Results: Children with black stain showed distinct oral microbiota changes compared to controls. *Actinomyces* sp. increased more than three times, while *Streptococcus* sp. significantly decreased three times. *Treponema* sp. and *Eikenella* sp. showed similar relative abundances, whereas *Selenomonas* sp. and *Veillonella* sp. were absent in the group with black stain. *Burkholderia* sp. and *Neisseria* sp. were detected exclusively in black stain samples but were undetected in controls. Morphologically, filamentous rods remained predominant, while the composition of cocci shifted, reflecting a specific microbial profile associated with black stain formation.

Conclusion: Black tooth stain in children is associated with a unique bacterial community structure. Targeted treatments, such as natural antimicrobials, photodynamic therapy and probiotics may help control recurrence and maintain oral microbial balance.

Keywords

16S rRNA gene sequencing, oral microbiota, pediatric dentistry

Introduction

Extrinsic black stain is a form of dental discoloration that is characterized by the presence of dark dots or a continuous pigmented line. These are located predominantly along the cervical third of the tooth and occur on the lin-

gual and buccal surfaces of both primary and permanent dentitions.^[1-3] (Fig. 1).

Epidemiological studies report a variable prevalence (2%–20%) across populations, with most investigations focused on pediatric cohorts and no consistent gender predilection.^[4]



Figure 1. (A) A 4-year-old child presents with black stain deposits on the vestibular surfaces of the maxillary and mandibular anterior teeth. The pigmentation is intense and continuous, forming well-defined linear bands along the gingival margin and middle crown area; (B) An 8-year-old child in mixed dentition showing discrete black dots scattered along the vestibular walls of both anterior and posterior teeth. The pigmentation is mild, discontinuous, and primarily localized near the cervical areas; (C) A 5-year-old child in primary dentition exhibits well-defined black stain deposits of strong intensity along the cervical margins of the mandibular lingual and vestibular surfaces.

The etiology of black stain has been a subject of debate for over a century, and recent research continues to explore the relationship between environmental and microbiological factors in its development.^[1,5]

A systematic review and meta-analysis questioned the protective effect of black stain against dental caries, suggesting that the previously assumed dominance of chromogenic bacteria over cariogenic species may not fully explain this relationship.^[6]

Moreover, recent microbiome analyses indicate that extrinsic black stain may correlate with a lower risk of early childhood caries, as affected children exhibit distinctive oral and gut microbial profiles compared with healthy controls.^[7]

Evidence further suggests that children with a lower risk of dental caries are more prone to developing black stain, as their oral microbiome supports the growth of bacteria involved in its formation.^[6,8]

The characteristic black pigmentation has long been attributed to the reaction between metal ions and bacterially produced hydrogen sulfide, leading to the formation of metal sulfides (e.g., ferric sulfide).^[9,10] These microbial and biochemical distinctions support the concept that black stain represents a specialized biofilm with unique ecological and metabolic properties.^[1,11]

Next-generation sequencing has enabled the identification of black stain-associated microorganisms at a molecular level, revealing specific taxa potentially involved in its formation.^[12] A metagenomic 16S rRNA study demonstrated that black stain exhibits reduced microbial diversity, distinct taxonomic signatures and an enrichment of heme biosynthetic pathways, indicating that heme-dependent iron sequestration is important.^[13]

Recent next-generation sequencing data support these observations. A case control study found that black stains have a distinct microbial profile enriched in *Capnocytophaga*, *Corynebacterium* and *Neisseria*, while caries-free individuals with black stain additionally show increased *Car-*

diobacterium and *Rothia*. These microbial features suggest that black stain represents a specialized plaque phenotype potentially associated with lower caries susceptibility.^[14]

Metaproteomics is a powerful emerging technology that successfully enabled human protein and bacterial identification of this specific dental biofilm using high-resolution tandem mass spectrometry. Recent metaproteomic analyses of black stain and conventional dental plaque identified hundreds of microbial and human proteins and revealed a high diversity of bacterial genera.^[15]

The microbial shifts observed highlight the potential influence of interspecies metabolic exchanges, particularly between facultative and obligate anaerobes, in sustaining the chromogenic biofilm and distinguishing it from conventional dental plaque.^[1,11]

Growing interest in minimally invasive approaches has led to the exploration of new therapeutic strategies for managing extrinsic black stains. Among these, probiotic and photodynamic therapies have gained attention for their potential to influence the oral microbial ecosystem and improve clinical outcomes. Probiotics such as *Lactobacillus reuteri* and *Streptococcus salivarius* M18 have demonstrated beneficial effects in reducing plaque accumulation, gingival inflammation, and modulating dysbiotic taxa through the production of bacteriocins and metabolites like reuterin.^[16,17] These strains may also affect the occurrence or severity of extrinsic staining, though evidence remains limited and depends on strain specificity, dosage and host factors. A recent case study demonstrated that direct application of probiotic powder over black-stained tooth surfaces facilitated stain removal with routine brushing and flossing. Microbiome analysis revealed a reduction in *Corynebacterium*.^[18]

Complementarily, photodynamic therapy represents an emerging adjunctive method for black stain management. This method uses a photosensitizing agent activated by targeted light to generate reactive oxygen species, selectively disrupting pigmented and anaerobic microorganisms in

the biofilm. By achieving antimicrobial effects without mechanical abrasion, it limits bacterial recolonization while minimizing damage to dental surfaces.^[19]

These innovative approaches highlight a shift from purely mechanical stain removal toward biologically oriented strategies aimed at restoring microbial balance and maintaining oral health.

Aim

To investigate and compare the microbial composition, morphology and metabolic characteristics of dental plaque associated with extrinsic black stains in children and plaque from stain-free individuals using 16S rRNA gene sequencing (Fig. 2).

Materials and methods

Subject selection

A total number of 60 children aged 5-12 years participated in the study. The first group included 30 children without black stain (control group), while the second group comprised 30 children with at least six black-stained teeth (experimental group). No significant differences in age or sex were observed between the groups. None of the participants had systemic or infectious diseases, nor had they used antibiotics two weeks prior to sample collection. The study was conducted in accordance with the Helsinki Declaration and was approved by the Committee for Scientific Research

Ethics at the Medical University of Plovdiv, Bulgaria (Approval No. 6/05.10.2023). The research was carried out at the Department of Pediatric Dentistry in Plovdiv, Bulgaria, between January and June 2024. Written informed consent was obtained from the parents of all children enrolled in the study.

Sample collection and analysis

Samples from both groups were collected at the Department of Pediatric Dentistry between 8:00 and 12:00 a.m. Participants were instructed not to brush their teeth the evening before sample collection and to refrain from eating breakfast on the day of the visit. These instructions were given to standardize oral conditions before sample collection and to minimize the influence of recent oral hygiene or food intake on the results. Subjects were asked to rinse their mouths with clean water before sample collection. Dental plaque and black stain samples were collected by gently scraping tooth surfaces with individual sterile metal cures, taking care not to remove enamel unnecessarily.^[11] Approximately 1-2 mg of each sample was transferred onto sterile endodontic paper point^[20] and stored in sterile 1.5 ml Eppendorf tubes at -22°C until further processing. All samples were placed on ice before being transported to the laboratory. The collection, handling, and storage procedures are presented in Fig. 3.

DNA extraction

Genomic DNA was extracted using a commercial bacterial DNA kit (Genaxxon Biosciences, Germany) according to a protocol optimized for bacterial samples. Samples were

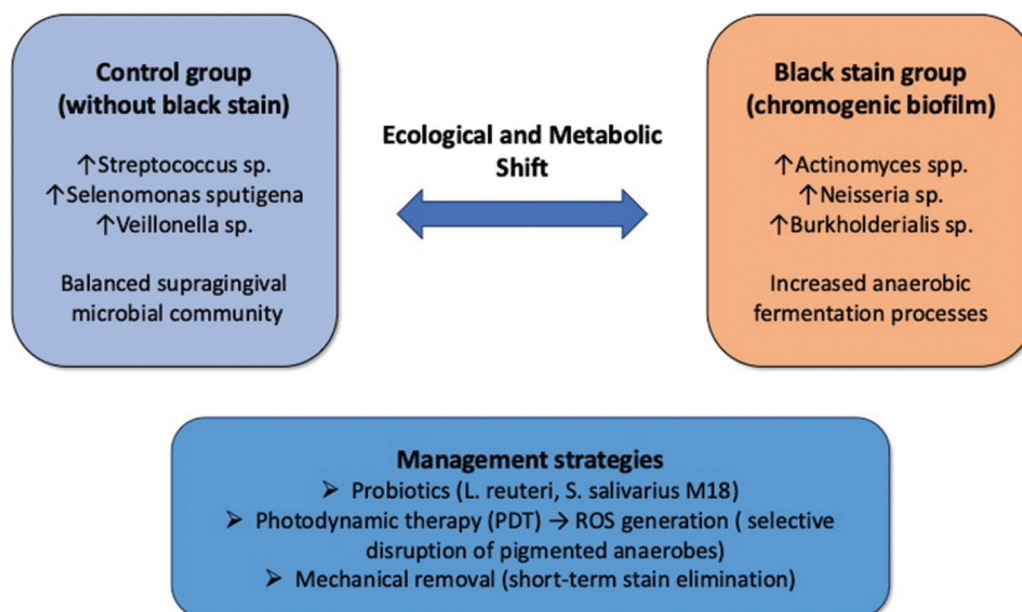


Figure 2. Graphical abstract summarizing the microbial differences between children with and without black stain and proposed management strategies.

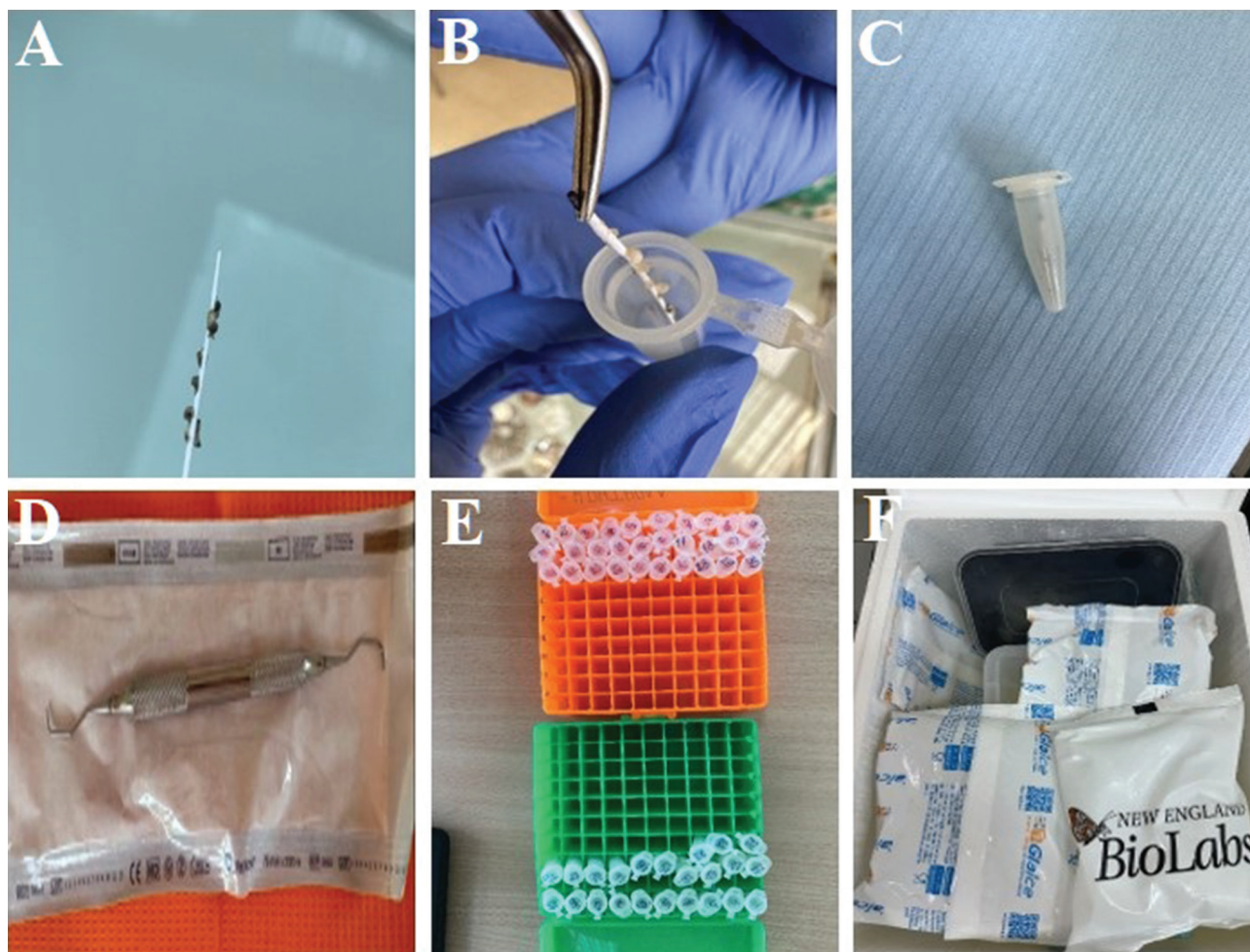


Figure 3. (A) Dental black stain collected on an endodontic paper point; (B) A sterile endodontic paper point with collected dental plaque placed into a sterile Eppendorf tube; (C) Eppendorf tube containing biological material; (D) Periodontal curette used for sample collection; (E) Storage containers for sample preservation; (F) Supragingival plaque samples placed in a transport box containing ice packs for preservation during transport to the laboratory.

incubated with 200 μ L Tris-EDTA buffer (pH 7.9) supplemented with 40 μ L lysozyme (100 mg/mL) at 37°C for 2 hours. After enzymatic lysis, 300 μ L lysis buffer and 17 μ L proteinase K (20 mg/mL) were added, followed by incubation at 55°C for 10 minutes. DNA was purified using silica spin columns, washed, and eluted in 25 μ L elution buffer.

PCR amplification

Primers targeting the V1–V3 and V4–V6 regions of the 16S rRNA gene were selected based on Church et al. (2020).^[21]

V1–V3:

Forward 5'–AAGAGTTTGATCATGGCTCA–3'

Reverse 5'–TTACCGCGGCTGCTGGCAC–3'

V4–V6:

Forward 5'–GTGCCAGCAGCCGCGGTAA–3'

Reverse 5'–GAGCTGACGACAGCCATGC–3'

Each 25 μ L PCR reaction contained 12.5 μ L 2 \times Master Mix, 1 μ L of each primer (10 μ M), 5 μ L template DNA, and 5.5 μ L deionized water. PCR cycling conditions:

V1–V3: 95°C/10 min; 30 cycles of 94°C/30 s, 57°C/30 s,

72°C/30 s; final extension 72°C/10 min.

V4–V6: 95°C/10 min; 30 cycles of 94°C/30 s, 53°C/30 s, 72°C/30 s; final extension 72°C/10 min.

PCR products (~500 bp) were verified on 2% agarose gels with a 50 bp DNA ladder.

PCR product purification and sequencing

Specific PCR products were enzymatically purified using Exonuclease I and Shrimp Alkaline Phosphatase (37°C/15 min, enzyme inactivation 85°C/15 min). Sequencing reactions were performed with the forward primer using the BigDye Terminator v. 3.1 kit, followed by capillary electrophoresis on an ABI PRISM 310 Genetic Analyzer.

Sequence analysis

Chromatograms were analyzed using Sequencing Analysis v. 5.2. Bacterial species were identified by BLAST against the NCBI GenBank database. Representative sequences were recorded for reference.

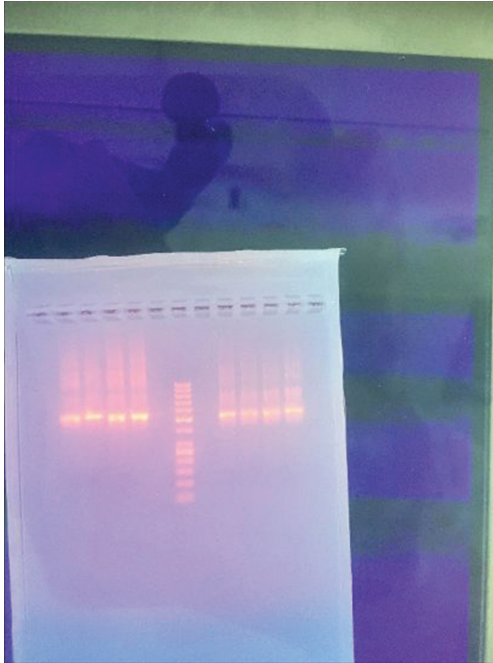
Results

Overview of the molecular analysis

For each of the 60 analyzed samples, two specific PCR products were successfully obtained, corresponding to the

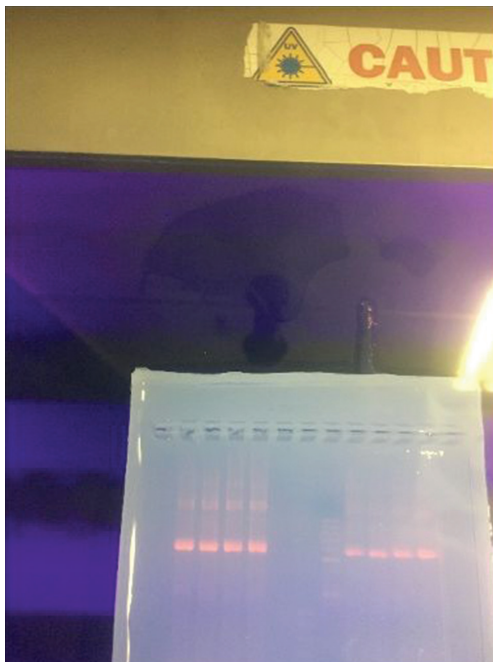
V1–V3 and V4–V6 variable regions of the 16S rRNA gene. Representative amplicons (~500 bp) are shown in **Figs 4 and 5**.

All tested samples yielded specific PCR products of approximately 500 bp, corresponding to the expected size for the V1–V3 variable region of the 16S rRNA gene, confirming successful DNA amplification and suitability for subse-



Lane 1 - empty
 Lane 2 - sample 1C (V1/V3 region)
 Lane 3 - sample 2C (V1/V3 region)
 Lane 4 - sample 3C (V1/V3 region)
 Lane 5 - sample 4C (V1/V3 region)
 Lane 6 - empty
 Lane 7 - 50 bp molecular marker
 Lane 8 - empty
 Lane 9 - sample 1BS (V1/V3 region)
 Lane 10 - sample 2BS (V1/V3 region)
 Lane 11 - sample 3BS (V1/V3 region)
 Lane 12 - sample 4BS (V1/V3 region)
 Lane 13 - empty

Figure 4. 2% agarose gel electrophoresis showing PCR products (~500 bp) specific for the 16S rRNA V1–V3 region obtained from control (lanes 2–5) and black stain (lanes 9–12) plaque samples.



Lane 1 - empty
 Lane 2 - sample 5C (V4/V6 region)
 Lane 3 - sample 6C (V4/V6 region)
 Lane 4 - sample 7C (V4/V6 region)
 Lane 5 - sample 8C (V4/V6 region)
 Lane 6 - empty
 Lane 7 - empty
 Lane 8 - 50 bp molecular marker
 Lane 9 - sample 5BS (V4/V6 region)
 Lane 10 - sample 6BS (V4/V6 region)
 Lane 11 - sample 7BS (V4/V6 region)
 Lane 12 - sample 8BS (V4/V6 region)
 Lane 13 - empty

Figure 5. 2% agarose gel electrophoresis showing PCR products (~500 bp) specific for the 16S rRNA V4–V6 region obtained from control (lanes 2–5) and black stain (lanes 9–12) plaque samples.

quent sequencing. Clear and distinct single bands observed in both control (lanes 2-5) and black stain (lanes 9-12) samples indicate successful and specific amplification of the target region, confirming that the extracted DNA was of good quality and suitable for further sequencing analysis. Similar amplification patterns were obtained for the V4-V6 region, confirming consistent DNA quality and amplification efficiency across both variable regions.

Taxonomic composition and comparison between groups

The predominant bacterial species identified in both groups are summarized in Fig. 6, while the presence of selected bacterial genera and the results of Fisher’s exact test are presented in Table 1. Distinct microbial profiles were observed between children with and without black stain. Notably, black stain samples exhibited a significant increase in *Actinomyces* sp. and the presence of newly detected

Gram-negative taxa (*Burkholderia* and *Neisseria*), whereas the control group was dominated by *Streptococcus* and *Veillonella* species. Comparative analysis revealed a shift in the Gram-positive/Gram-negative balance. *Actinomyces* sp. showed a strong association with black stain, increasing from 3 to 10 occurrences, while *Streptococcus* sp. decreased (from 10 to 3). *Treponema* sp. exhibited a slight increase, potentially reflecting its contribution to subgingival biofilm activity. In contrast, *Selenomonas* sp. and *Veillonella* sp. were absent in the experimental group with black stain. Newly detected taxa, *Burkholderia* and *Neisseria*, appeared exclusively in black stain samples, indicating a possible adaptation to the rich in iron and low oxygen environment of the chromogenic biofilm.

Presence of selected bacterial genera was evaluated in the control group versus the black stain (BS) group using Fisher’s exact test. None of the genera yielded a p-value below the conventional significance threshold of 0.05, therefore no statistically significant associations were demonstrated

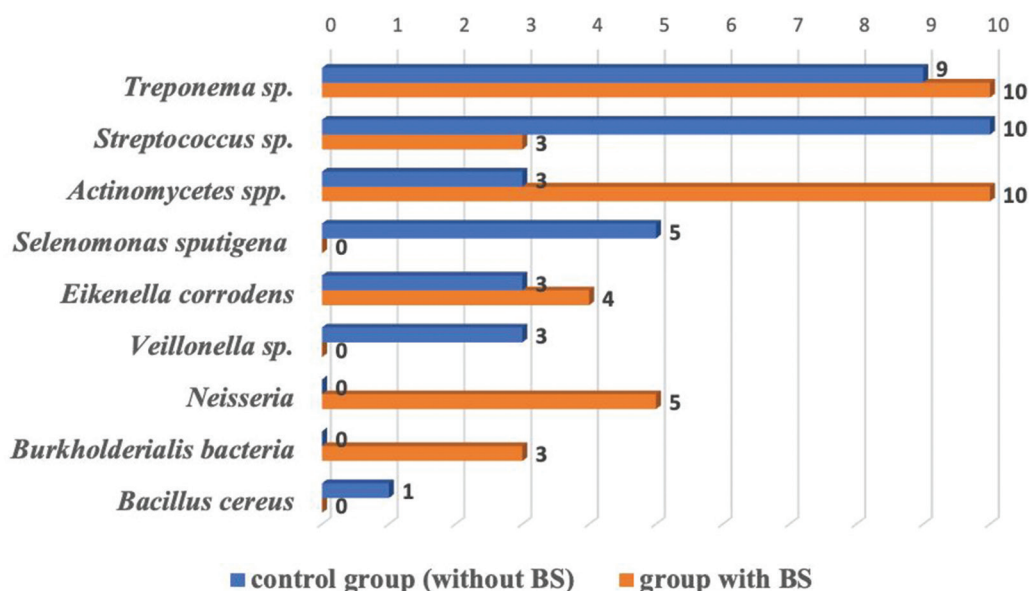


Figure 6. Predominant microbial species (quantitative comparison).

Table 1. Presence of selected bacterial genera in control vs. black stain group with Fisher’s exact test

Bacterial genus	Control (n=30) present n (%)	BS (n=30) present n (%)	Fisher’s exact pvalue
<i>Treponema</i> sp.	9 (30.0%)	10 (33.3%)	1.000
<i>Streptococcus</i>	10 (33.3%)	3 (10.0%)	0.057
<i>Actinomyces</i> sp.	3 (10.0%)	10 (33.3%)	0.057
<i>Selenomonas</i>	5 (16.7%)	0 (0.0%)	0.052
<i>Eikenella</i>	3 (10.0%)	4 (13.3%)	1.000
<i>Veillonella</i>	3 (10.0%)	0 (0.0%)	0.237
<i>Neisseria</i>	0 (0.0%)	5 (16.7%)	0.052
<i>Burkholderiales</i> bacteria	0 (0.0%)	3 (10.0%)	0.237
<i>Bacillus cereus</i>	1 (3.3%)	0 (0.0%)	1.000

in this dataset. *Treponema* sp. and *Eikenella* each produced $p=1.000$, clearly indicating non-significance. *Streptococcus* and *Actinomyces* sp. both returned $p=0.057$, which is close to but still above 0.05. *Selenomonas* and *Neisseria* each returned $p=0.052$, slightly above the threshold. *Veillonella*, *Burkholderiales* bacteria and *Bacillus cereus* yielded $p=0.237$ or 1.000 , also non-significant. Although the raw counts show descriptive differences for some genera, such as a higher frequency of actinomyces in the BS group and a higher frequency of *Streptococcus* in the control group, these differences did not attain formal statistical significance with the current sample size and observed distribution of data. Consequently, the findings remain descriptive and confirmation of any associations would require larger samples or additional analytical approaches.

Taxonomic categorization, as presented in **Table 2**, highlights broader ecological patterns in the microbial communities. In black stain samples, the microbial community shifts toward filamentous and rod-shaped forms, while Gram-negative taxa increase in diversity. Cocci such as *Veillonella* are replaced by *Neisseria*, and *Actinomyces* remains dominant among filamentous bacteria, consistent with the biofilm's structural stability.

The identification of these taxa supports the hypothesis that black extrinsic stain in children represents a complex, metabolically active biofilm rather than a simple pigment accumulation. The coexistence of *Actinomyces* with anaerobic genera such as *Eikenella* and *Treponema* reflects a structured ecological consortium potentially involved in biofilm maturation, iron accumulation and pigment biosynthesis.

Discussion

Actinomyces sp.

Actinomyces sp. are Gram-positive, facultatively anaerobic or microaerophilic rods. They are among the earliest colonizers of the dental biofilm and participate in the initial stages of plaque development through strong adhesion to the acquired pellicle and coaggregation with other oral microorganisms.^[22] Our findings demonstrate a significant increase in *Actinomyces* sp. in patients presenting with black stain, rising from three in the control group

to ten in the black stain group. This observation corroborates earlier studies reporting the frequent detection of *Actinomyces* sp. in black stain plaque.^[3,5,11,23-25] Members of the *Actinomyces* are common residents of supragingival plaque. Some strains produce hydrogen sulfide, which can react with iron derived from saliva or gingival fluid to form ferric sulfide.^[9] *Actinomyces* sp. are considered important members of the oral biofilm community, where their metabolic activity may contribute to maintaining ecological balance and influencing plaque characteristics associated with black stain formation.

Treponema sp.

Treponema species showed no significant difference in abundance between children with and without black stain. These motile, Gram-negative spirochetes are associated with periodontal disease due to their proteolytic activity, tissue invasiveness and ability to evade the host immune response.^[26] Consistent with our findings, *Treponema* sp. was also detected in the studies conducted by Li et al.^[11] *Treponema denticola* produces volatile sulfur compounds (VSCs), such as hydrogen sulfide and methyl mercaptan^[26], which not only contribute to halitosis but may participate in the formation of iron sulfide pigments characteristic of black stains. Interactions between *Treponema* and other biofilm members may facilitate nutrient exchange and influence the redox balance within the dental plaque.

Streptococcus sp.

Streptococcus sp., which are dominant early colonizers involved in biofilm initiation and carbohydrate metabolism, showed decreased relative abundance in black stain samples compared to controls, suggesting an ecological shift toward less acidogenic and more iron- or sulfur-metabolizing taxa. Our results are consistent with those of Slots et al.^[5], who reported lower levels of *Streptococcus* sp. in children with black stain, whereas Heinrich-Weltzien et al.^[27], and Li et al.^[11] found no significant difference between groups. Reid and Beeley proposed that the reduced caries incidence in individuals with black extrinsic stains may be related to the higher calcium and phosphate content of the stain biofilm.^[9] However, the hypothesis that the microbial composition of black stain alone accounts for the lower caries experience in affected children remains uncertain.

Table 2. Taxonomic/functional categorization

Category	Control group	Black stain group
Gram-positive	<i>Streptococcus</i> , <i>Actinomyces</i> , <i>Bacillus</i>	<i>Streptococcus</i> , <i>Actinomyces</i>
Gram-negative	<i>Treponema</i> , <i>Selenomonas</i> , <i>Eikenella</i> , <i>Veillonella</i>	<i>Treponema</i> , <i>Eikenella</i> , <i>Burkholderia</i> , <i>Neisseria</i>
Cocci	<i>Streptococcus</i> , <i>Veillonella</i>	<i>Streptococcus</i> , <i>Neisseria</i>
Filamentous/rod-shaped bacteria	<i>Actinomyces</i> , <i>Bacillus</i>	<i>Actinomyces</i> , <i>Burkholderia</i>

***Neisseria* sp.**

Li Yue et al. reported that *Streptococcus*, *Neisseria*, and *Capnocytophaga* were among the dominant genera in black stain samples.^[11] Similarly, in our study, *Neisseria* were also identified as predominant taxa. Hwang et al. and Zhang et al. also detected *Neisseria* sp. in children with black stain, whereas these bacteria were absent or less prevalent in children without discoloration.^[23,28] *Neisseria* sp. are Gram-negative, aerobic diplococci that act as early colonizers of the oral cavity. Moreover, hydrogen sulfide (H₂S) can be produced through cysteine metabolism, suggesting that *Neisseria* sp. may also be involved in the pigmentation processes associated with black stain formation.^[29]

***Burkholderia* sp.**

Members of the order *Burkholderiales* have not been previously reported in studies of black stain. *Burkholderia* species are Gram-negative, aerobic rods recognized for their metabolic adaptability, biofilm formation and resistance to oxidative stress. *Burkholderia* sp. can produce siderophores that can mobilize and stabilize ferric ions.^[30]

***Selenomonas* sp.**

In agreement with our findings, Hwang et al. reported that *Selenomonas* sp. were more abundant in children without black stain, suggesting that these bacteria may be associated with a non-pigmented, health-related oral biofilm.^[31] *Selenomonas* is a Gram-negative, anaerobic, motile bacterium commonly found in the subgingival of healthy individuals. It is generally regarded as a commensal microorganism frequently detected in mature supragingival biofilms, particularly near the gingival margin and interproximal sites. When present in moderate abundance, it is typically associated with biofilm maturation and ecological balance rather than pathology.^[32] Overall, the distribution of *Selenomonas* sp. appears consistent with their presence as commensal members of a stable oral biofilm community.

Eikenella corrodens and *Veillonella* sp. were detected in our study, with *Eikenella* showing no significant difference between children with and without black stain, while *Veillonella* was observed exclusively in the control group. In contrast to our findings, Hwang et al.^[28] reported the presence of *Veillonella dispar* and *V. rodentium* as positive candidates for black stain formation, whereas in our study, *Veillonella* sp. were predominant only in the control group. *Eikenella corrodens* is a microaerophilic, Gram-negative rod normally present in the oral cavity. Although usually part of mixed infections, it can occasionally cause serious infections independently, and its slow growth and CO₂ requirement may lead to under-recognition.^[33] *Veillonella* sp. are Gram-negative, obligate anaerobic cocci that metabolize lactate into weaker acids, contributing to pH balance and microbial diversity in oral biofilms.^[31] Their exclusive

presence in the control samples may indicate reduced buffering capacity and a shift toward a less diverse microbiota in the black stain group.

Bacillus cereus

Bacillus cereus was identified as the predominant species in one control sample. It is a Gram-positive, spore-forming rod widely distributed in the environment and frequently isolated from various foods. This opportunistic bacterium is best known for causing foodborne illness through the production of emetic and diarrheal toxins. Beyond its well-established pathogenic function, recent studies emphasize the diversity of its virulence factors, genetic determinants of toxin synthesis, and growing antimicrobial resistance.^[34]

Limitations

This study has several limitations that should be taken into account when interpreting the results. First, the sample size was relatively small, which may have reduced the statistical power to detect significant differences in bacterial distribution between the groups. Second, the microbiological assessment relied on presence/absence data rather than quantitative measurements of relative abundance, limiting the ability to evaluate proportional microbial shifts within the biofilm. Third, although participants were matched by age, factors such as individual oral hygiene practices, dietary habits and fluoride exposure were not fully controlled and may have acted as confounding variables influencing microbial composition.

In addition, only publications written in English were considered when reviewing the existing literature, which may have excluded relevant evidence reported in other languages. The limited number of available studies on black stain further restricted the ability to explore additional environmental, behavioral or host-related factors that could influence the etiology and microbial profile of this condition. Future research incorporating larger cohorts, quantitative sequencing approaches, and broader multilingual literature coverage is warranted to strengthen and expand upon these findings.

Conclusion

This study provides evidence that black extrinsic dental stains in children are associated with distinct shifts in the oral microbial community compared to controls without stains. The results demonstrate a marked increase in *Actinomyces* sp., accompanied by the appearance of newly detected Gram-negative species such as *Burkholderia* and *Neisseria*, and a concurrent reduction in *Streptococcus* and *Veillonella*. These microbial transitions suggest that black stain represents a specialized ecological biofilm charac-

terized by filamentous species. Collectively, these findings support the hypothesis that black dental stains are not a superficial discoloration but rather reflect a unique, metabolically active microbial consortium. Understanding this microbial balance provides a basis for developing targeted preventive and therapeutic strategies aimed at modulating the oral microbiota and reducing black stain formation in pediatric populations.

Ethical approval

The present study was conducted in compliance with the Helsinki Declaration and received approval from the Ethics Committee at the Medical University of Plovdiv, Bulgaria (Approval No. 6/05.10.2023).

Conflict of interest

The authors declare no conflict of interest.

Ethical statements

- The authors declared that no clinical trials were used in the present study.
- The authors declared that no experiments on humans or human tissues were performed for the present study.
- The authors declared that written informed consent was obtained from the parents of all children enrolled in the study.
- The authors declared that no experiments on animals were performed for the present study.
- The authors declared that no commercially available immortalized human and animal cell lines were used in the present study.

Use of AI

In order to improve the structure of two sections of the article and check the text's grammar, the authors employed an AI tool during the preparation of this work. The authors then accepted full responsibility for the publication's content and reviewed and edited it as necessary.

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Author contributions

KT conducted the study, performed the microbiological analyses, interpreted the data and wrote the manuscript. MS supervised the study, contributed to the design and interpretation of the results and critically revised the manuscript. Both authors read and approved the final version of the manuscript.

Data availability

All data used are referenced or included in the article.

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