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Original Research Article

Biochemical and microbial profile of saliva in children with black extrinsic tooth stains

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Abstract

Background: Black stain (BS) is a type of extrinsic discoloration seen in children, often along the cervical third of teeth. Though BS is linked to chromogenic bacteria and has been associated with lower caries prevalence, its aetiology is unclear. This study aimed to evaluate salivary mineral composition and bacterial profile of children with BS.

Methodology: Oral examination was conducted on 240 children aged between 3–14 years. Salivary levels of copper, iron, sodium, calcium, and phosphorus were analyzed in children with and without BS. Bacterial profiling involved culture techniques and identification via VITEK® 2 and 16S rRNA sequencing.

Results: Children with BS showed significantly higher salivary copper and lower iron and sodium levels. Actinomyces species, including A. israelii and S. odontolytica, predominated the bacterial profile. Cariogenic microorganisms were less prevalent.

Conclusion: High copper and Actinomyces dominance may contribute to the lower caries risk in children with BS, suggesting a protective role.

Keywords: Black stain, Children, Dental caries, Saliva.

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1. Introduction

Black stain (BS) is a characteristic form of extrinsic dental discoloration, predominantly observed in children, and typically presents as dark lines or dots along the cervical third of the tooth surface, closely following the gingival margin.[1] While its global prevalence ranges from 2.4% to 26%,[2] it is most common in childhood and tends to decrease with age.[3] The etiology of BS is not fully understood, but it is believed to result from the formation of ferric sulfide—an iron compound formed by the reaction between hydrogen sulfide, a bacterial byproduct, and iron found in saliva or gingival crevicular fluid.[4] Microbiological analyses have consistently shown that BS is associated with a distinct bacterial profile, particularly dominated by chromogenic bacteria such as *Actinomyces* spp., *Prevotella*, *Porphyromonas*, and *Neisseria*. Notably, several studies have also reported a lower incidence of dental caries in children with BS, though the mechanisms underlying this apparent protective effect remain unclear.[5] There is limited data on the chemical composition of saliva in children with BS. Spatial chemical analysis using wavelength dispersive spectrometry has supported the involvement of metal ions—such as iron and copper—in the pigmentation process.[6] Therefore, this study aims to compare and assess salivary levels of copper, iron, sodium, calcium, and phosphorus in children with and without black stain, and to evaluate the microbial content of black stain.

2. Methodology

Oral examinations were done in children of age group 3-14 years for black stain and were divided into two groups.

Group 1- 120 children with black stain

Group 2- 120 children without black stain,

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Selection criteria included children without any systemic diseases and without any medication including chlorhexidine, iron preparate and antibiotics for atleast 1 month. Prior parental consent was taken for their inclusion in the study.

5ml of unstimulated saliva sample was collected two hours after the last meal. The subjects were asked to rinse thoroughly with distilled water before the collection of salivary samples. Five minutes after the oral rinse, subjects were asked to swallow any residual saliva that may be present in their mouth. Un-stimulated whole saliva was collected by making the patient to sit in upright position at rest and bow their head. The saliva samples were transported to the laboratory for mineral estimation within 24 hrs using standard gel coolant pack in order to maintain the temperature between 2 ºC to 4ºC.

Estimation of iron was done by Ferrozine method,[7] Copper by Di-Br-PAESA method,[8] sodium by Phosphanazo Method,[9] inorganic salivary calcium by OCPC method[10] and salivary phosphorous by Molybdate U.V. method.[11]

For microbiological analysis, the collected saliva samples were serially diluted (10^{-2} to 10^{-6}) with sterile distilled water before inoculation onto selective media such as Tryptone Yeast Extract Agar (TYA) and Actinomycetes Isolation Agar (AIA). Plates were incubated at 28°C for 1–3 weeks to allow colony formation. Distinct colonies were selected, subcultured for purity, and characterized based on morphology, Gram staining, and standard biochemical tests (e.g., catalase, oxidase, starch, gelatin, lipid hydrolysis, nitrate reduction, and citrate utilization).[12,13] Isolates were stored at 4°C on slants and at −20°C in glycerol stocks. Pure colonies were further identified using the VITEK® 2 Compact system, which provides rapid biochemical profiling for species-level identification.[14] For Actinomycetes, genomic DNA was extracted using the Hi-Media DNA extraction kit, and the 16S rRNA gene was amplified via PCR using universal primers (27F/1492R). PCR products were sequenced (Sanger method) and taxonomically identified by comparison with databases such as NCBI BLAST.[15]

Comparison of mean salivary copper, iron, calcium, sodium and phosphorous levels between children with & without black stain was done using Independent Student t Test. A significance level of $p \leq 0.05$ was used.

3. Results

Figure 1 illustrates descriptive analysis of the mean mineral concentration in children with and without stains Results show that the mean sodium (Na) concentration of children with stain was 0.810787±0.239136 mg and in children without stains was 1.151257±0.187889mg. The mean Iron (Fe) concentration of children with stain was 63.30833±28.13606 mg and in children without stain was 101.4903±17.09075 mg. The mean calcium (Ca) concentration of children with stain was 5.64319±2.678556 mg and in children without stain was 5.347939±3.250228mg. The mean Phosphorus (P) concentration of children with stain was 62.7732±30.90006mg and in children without stain was 58.89521±28.57526mg. The differences between the children with stain and without stain for calcium and phosphorus was not statistically significant. The mean Copper (Cu) concentration of children with stain was 174.5512±37.78641 mg and in children without stain was 97.56393±21.35257 mg. The differences between the children with stain and without stain for sodium, iron, copper was statistically significant (P value <0.001).

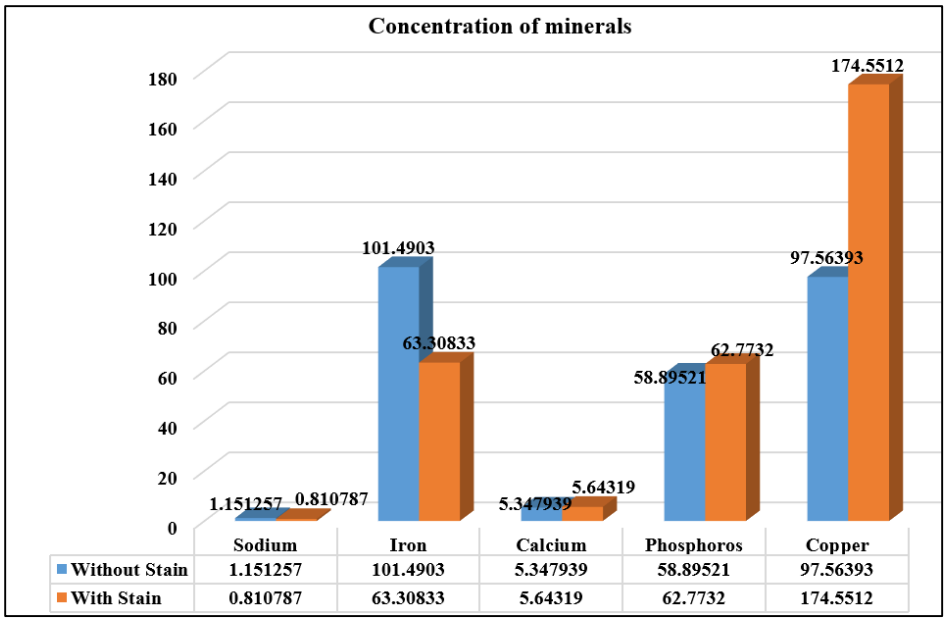

Figure 1: Mineral concentration in children with and without stains

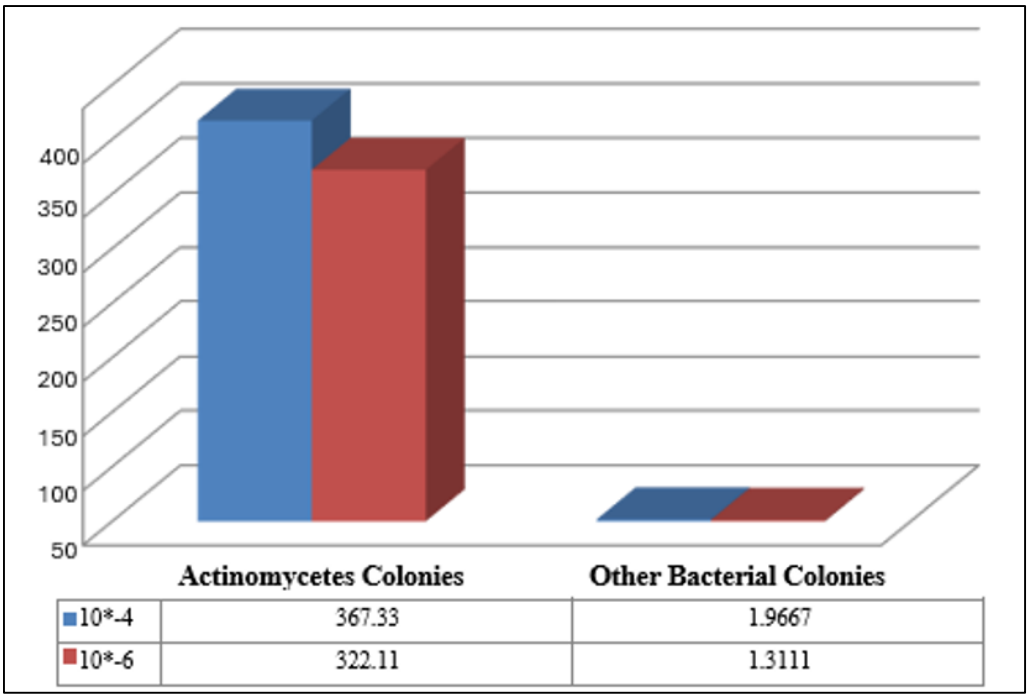

Figure 2: Distribution of bacterial colonies and Actinomycetes colonies with Lobene stain

Figure 2 illustrates a significantly higher mean count of Actinomycetes colonies compared to other bacterial colonies in subjects with black stain across both 10^{-4} and 10^{-6} dilutions. At 10^{-4} dilution, Actinomycetes colonies averaged 367.33 ± 10.18, decreasing slightly to 322.11 ± 0.50 at 10^{-6} dilution, while other bacterial colonies showed much lower counts of 1.97 ± 1.61 at 10^{-4} and 1.31 ± 1.91 at 10^{-6} dilution.

The Actinomycetes species identified in black-stained plaque included *Actinomyces israelii*, *Schaalia odontolytica*, *Schaalia meyeri*, *Actinomyces oris*, and *Schaalia funkei*, highlighting the predominance of Actinomycetes compared to other bacterial populations. Additional bacterial species detected were *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Neisseria meningitidis*, *Prevotella* spp., *Treponema denticola*, *Bacteroides melaninogenicus*, *Leptotrichia buccalis*, *Lactobacillus acidophilus*, *Streptococcus mutans*, *Bacillus* spp., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Klebsiella pneumoniae* ssp. *ozaenae*.

4. Disscussion

Tooth discoloration in children refers to changes in the natural colour of their teeth. Black stain is a form of extrinsic staining affecting the buccal and palatal surfaces of primary and permanent teeth.[16] It presents as an incomplete line of dark dots formed at the cervical third of the tooth, typically following the gingival margin and not extending to the proximal areas. Black stain is aesthetically unwelcome to patients, and clinicians frequently attempt its removal.[17] Dental black stain has a prevalence rate of 2.4%–26% affecting both genders equally and may appear throughout the lifetime. Despite be high prevalence, the aetiology is unclear.[3] This study aimed to evaluate salivary mineral composition and bacterial profile of children with BS.

The oral cavity is a dynamic ecosystem, and the balance between different bacterial species, including Actinomycetes and S. mutans, can shift under different conditions. While S. mutans is a major contributor to dental caries, studies suggest that Actinomycetes may play a role in maintaining a stable oral environment and may even be involved in the initiation and progression of oral biofilm formation and dental plaque disease.[18] Actinomycetes are among the initial bacteria to colonize teeth, forming a base layer of plaque. *S. mutans* may attach to the existing Actinomycetes biofilm. However, studies have shown that S. mutans in dual-species biofilms with Actinomycetes produce less lactic acid compared to single-species S. mutans biofilms.[19] Actinomycetes don't completely prevent S. mutans from colonizing teeth, but they can influence the composition and activity of the dental biofilm, potentially limiting S. mutans growth.[20]

The present findings highlight a significantly higher prevalence of *Actinomyces* colonies compared to other bacterial species in samples of black stain, at both 10^{-4} and 10^{-6} dilutions. Other Identified bacterial species included *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Neisseria meningitidis*, *Prevotella* spp., *Treponema denticola*, *Bacteroides melaninogenicus*, *Leptotrichia buccalis*, *Lactobacillus acidophilus*, *Streptococcus mutans*, *Bacillus* spp., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Klebsiella pneumoniae ssp. ozaenae*. The Actinomycetes species identified were *Actinomyces israelii*, *Schaalia odontolytica*, *S. meyeri*, *A. oris*, and *S. funkei*, underscoring the predominance of Actinomycetes in black stain compared to other bacterial populations. This

observation agrees with earlier studies that have consistently associated *Actinomyces* spp., with black stain. Heinrich-Weltzien et al.,[21] Zyla et al.,[3] Zhang F et al,[6] Gayathri A et al,[23] Mutsaddi S et al,[22] Chen L et al,[27] Veses V et al,[24] Çelik ZC et al.,[25] Luoyuan Zheng et al.[26]

Actinomyces spp. has shown to exhibit lower acidogenic potential and thereby may play a protective role against cariogenic bacteria such as *Streptococcus mutans*. This supports clinical observations that children with black stain often show a lower incidence of dental caries. Persistence of high Actinomycetes counts even at lower concentrations underscores their robust presence in the biofilm matrix.[28]

Saliva has a significant role in remineralization of dental enamel. It not only has a buffering capacity to neutralize the oral cavity's low pH generated after acidic encounters, but also acts as a carrier of essential ions, such as fluoride, calcium and phosphate, which have a positive role in enamel's remineralization[29] Few studies have explored the association of the mineral content of saliva with black stains and its impact on dental caries. Surdacka et al have reported a higher content of copper, iron, total calcium, inorganic phosphates, and sodium in the saliva of children with black stains.

In the current study, children with black stains exhibited significantly elevated salivary copper levels. This finding is supported by Klimuzko et al., who reported that copper ions can reduce enamel dissolution.[30] They proposed that a protective copper phosphate layer forms on the tooth surface, which helps stabilize the enamel's crystal lattice and inhibits demineralization.[31] Moreover, copper demonstrates cariostatic properties through several mechanisms:[32,33]

1. Copper ions alter the redox potential within bacterial cytoplasm, triggering nitrative stress that leads to cell death.
2. Copper suppresses the expression of key biofilm-related genes in *Streptococcus mutans*, including glucosyltransferase genes (*gtfB, gtfC, gtfD*) and glucan-binding protein genes (*gbpB, gbpC*).
3. Copper irreversibly inhibits F-ATPase activity in *S. mutans*, disrupting glycolysis under acidic conditions and resulting in bacterial cell death.

Khan et al., in a systematic review, found that copper reduces acid production in dental plaque biofilm. Their study showed a significant decrease in acid formation following the use of a copper sulfate mouth rinse.[34] These results were further validated by randomized controlled trials conducted by Afseth et al.[35] Additionally, cross-sectional studies by Duggal et al. and Bhandary et al. revealed lower salivary copper levels in individuals with high caries activity [36]. Similarly, Hussein et al. reported reduced salivary copper concentrations in children with early childhood caries (ECC) compared to their caries-free counterparts.[37] These findings are consistent with the current study, which observed higher salivary copper levels in children with black stains, accompanied by lower caries prevalence.

Regarding iron, the current study found significantly lower salivary iron levels in children with chromogenic stains. Ferritin, an acidic iron-binding protein, may lower salivary pH and reduce its buffering capacity, thereby increasing the risk of caries.[39] A decrease in salivary pH has been associated with a shift toward acidogenic and aciduric bacterial communities, creating conditions conducive to caries development. Salivary sodium levels were also significantly lower in children with black stains.[38] According to Siddharth Chandel et al., bacterial acids attack tooth mineral content, causing sodium to leach from the apatite crystals.39 Consequently, children without black stains, who may have more active caries, show elevated salivary sodium levels. This suggests that increased sodium is likely a by product of the carious process rather than a contributing factor.

Furthermore, the study found elevated salivary calcium and phosphorus levels in children with black stains. Surdacka et al. noted that higher concentrations of these minerals are typically observed in individuals with lower caries susceptibility.[10] Calcium and phosphorus are involved in microbial aggregation and the stabilization of salivary micelles, which help maintain oral homeostasis. Salivary micelles consist of various immune proteins, including low molecular weight mucin, secretory IgA, lactoferrin, amylase, glycosylated proline-rich proteins, and lysozyme.[40] The increased levels of calcium and phosphorus observed in this study may promote the formation of more micelles, which act as reservoirs for remineralization.

5. Conclusion

This study provides comprehensive evidence linking the presence of black stain (BS) in children to both distinctive microbial and biochemical profiles that may contribute to a lower incidence of dental caries. Among the various salivary minerals assessed, copper levels were found to be significantly higher in children with black stain. The elevated copper may play a protective role by forming a stable copper phosphate phase on the tooth surface, reducing enamel demineralization. Additionally, higher salivary calcium and phosphorus levels in this group are likely to enhance the buffering capacity of saliva and promote remineralization.

Microbiologically, the study demonstrated a consistent predominance of *Actinomyces* species—including *Actinomyces israelii*, *Schaalia odontolytica*, *Schaalia meyeri*, *Actinomyces oris*, and *Schaalia funkei*—across serial dilutions of salivary and plaque samples. These bacteria were present in significantly higher concentrations compared to other species such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Neisseria meningitidis*, and *Streptococcus mutans*.

Taken together, these findings suggest that the lower caries prevalence observed in children with black stain may be attributed to both the protective biochemical environment and a microbiota less conducive to acid production.

6. Source of Funding

None.

7. Conflict of Interest

None.

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