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Metagenomic analysis of black-stained plaques in permanent dentition

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A R T I C L E I N F O

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A B S T R A C T

Objectives: We aimed to determine the aetiologic agent responsible for black staining of permanent dentition using next-generation sequencing and determine the relationship between caries and black stains.
Materials and methods: A total of 52 systemically healthy patients with black-stained and caries-free (n = 13), black-stained and carious (n = 13), black stain-free and caries-free (n = 13), and black stain-free and carious (n = 13) teeth were enrolled in the study. The International Caries Detection and Assessment System (ICDAS II) was used for caries classification. Between 08:00 and 10:00, supragingival plaque samples were collected after a minimum of 8–12 h of accumulation and DNA samples were isolated. The samples were processed using the ZymoBIOMICS™ Service. Bioinformatics analysis was performed using mothur at usegalaxy.org. Data were analysed statistically using the Pearson chi-square and Fisher tests.
Results: The number of caries-free teeth (ICDAS 0, 1, and 2) was significantly higher in patients with black stains ($p = 0.007$).*Capnocytophaga* (4.8 %), *Corynebacterium* (3.9 %), and *Neisseria* (5.4 %) species were the most abundant among all black-stained plaques (carious and caries-free) ($p < 0.05$). *Capnocytophaga* (10.8 %), *Cardiobacterium* (3.6 %), and *Rothia* (1.72 %) species were detected in the black-stained plaques of caries-free patients ($p < 0.05$).
Conclusion: This study is one of the first studies examining the microbial composition of dental plaques with black staining in carious and caries-free adult patients using next generation sequencing technology. In the presence of black staining, plaques have an ultimate complex microbial structure. A lower caries burden was noted in the presence of black staining.

1. Introduction

Dental discolouration is a common aesthetic and clinical problem for which people seek dental care and treatment. When classified according to the location of the stain, tooth discolouration falls into three categories—extrinsic, intrinsic, and internalised (Hattab, Qudeimat, & al-Rimawi, 1999). Black staining of the teeth is a type of extrinsic discolouration that is most frequently located parallel to the gingival margin, appearing as firmly attached dark black circular formations (Heinrich-Weltzien, Bartsch, & Eick, 2014). Black stains have been suggested to be a special type of dental plaque containing highly insoluble iron salts, copper, calcium, and phosphate (Reid & Beeley, 1976). It consists of ferric sulphite from insoluble iron salts that are formed by the reaction of salivary iron with bacterial hydrogen sulphide (Reid, Beeley, & MacDonald, 1977; Żyła, Kawala, Antoszewska-Smith, & Kawala, 2015). Iron supplements and iron-rich diets increase black staining by increasing the amount of iron in the saliva (Adcock & Hogan, 2008). Black staining can be seen in both deciduous and permanent dentition, and its prevalence is not significantly different between sexes (Akyuz, Garan, & Kaya, 2015; Chen et al., 2014; Garcia Martin et al., 2013), ranging from 1% to 20 % depending on the population (Li et al., 2015). It has great importance in terms of public health significance and implications for research, clinical, and public health practice.

In 1923, the concept of 'caries-resistant individual' was introduced

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as patients with black staining showed lower susceptibility and higher resistance to dental caries; the primary cause of this condition was shown to be the specific type of dental plaque (Slots, 1974). Many epidemiological studies (Gasparetto et al., 2003; Heinrich-Weltzien, Monse, & van Palenstein Helderman, 2009, 2014; Koch et al., 2001; Li et al., 2015; Żyła et al., 2015) have found that patients with black stains have a lower prevalence of caries. Further studies have revealed that chromogen bacteria that produce black pigment are responsible for the formation of black stains, and it has been argued that further large carefully designed microbiome studies should be performed for a more complete understanding of the black stain phenomenon (Costa, Dorta, Ribeiro-Dias, & Pimenta, 2012; Heinrich-Weltzien et al., 2014; Slots, 1974). Molecular methods are highly advantageous for the diagnosis of oral bacteria that are difficult to cultivate (Li et al., 2015). Polymerase chain reaction (PCR) is one of the currently utilised molecular methods. The PCR method, also known as Sanger sequencing, only identifies bacteria that are predicted to be found, and it is not suitable to fully identify the microbial flora, unlike next-generation sequencing (NGS), which allows sequencing of the genome without replication in any host cell (Ustek, Abacı, Sırma, & Cakiris, 2011). NGS can be performed with high accuracy and in a short time. It is possible to determine the microbiological diversity of the environment due to the random sequencing of all microbiological genomes (microbiomes) present in the sample examined using the NGS method (Mardis, 2008). This study aimed to identify the bacteria responsible for black stain formation in permanent dentition at the genus level using NGS.

2. Materials & methods

Fifty-two systemically healthy adults aged 18–50 years who had not received any antibiotic treatment within the last 6 weeks and whose oral hygiene practices were similar (cleaning teeth at least once a day and use of fluoridated toothpaste) were included after ethical approval was obtained from the Marmara University Faculty of Dentistry Clinical Researches Ethics Committee (Date: 06.06.2017, Decision no: 2017-100). Caries classification was performed based on the ICDAS II criteria.

The concept of ICDAS is to provide a standard caries detection and evaluation method to allow evidence-based detection of early and advanced caries and their severity (Pitts, 2004).

Participants were divided into groups as follows: 1) Group 1 (with black staining and caries free): Patients with all teeth with ICDAS 0–2 and at least six teeth with black staining (Fig. 1a) 2) Group 2 (with black staining and caries): Patients with black staining in at least six teeth with ICDAS 3–6 (Fig. 1b) 3) Group 3 (black stain free and caries free): Patients with all teeth with ICDAS 0–2 and no black staining (Fig. 1c) 4) Group 4 (black stain free with caries): Patients with black stain-free teeth and at least six teeth with ICDAS 3–6 (Fig. 1d)

All patients signed an informed consent form; before sample collection, the time of the previous day of sampling participants were instructed to avoid any dietary or oral hygiene practices after 20:00. Plaque samples (10–12 h) were collected from the buccal and lingual regions of teeth with and without black staining using a sharp periodontal curette at 08:00–10:00 and transferred to DNA/RNA shield solution, which is a special storage medium for DNA and RNA transportation. The DNA/RNA shield preserves the genetic integrity and expression profiles of samples at ambient temperatures (no refrigeration or freezing needed). DNA isolation was performed in 13 steps according to the manufacturer's instructions provided with the isolation kit. Samples were processed and analysed using ZymoBIOMICS™ Service-Targeted Metagenomic Sequencing (Zymo Research, Irvine, CA, USA). 16S library preparation: Bacterial 16S ribosomal RNA gene-targeted sequencing was performed using the Quick-16S™ Next Generation Sequencing Library Preparation Kit (Zymo Research, Irvine, CA, USA). Bacterial 16S primers expanded the V3–V4 region of the 16S rRNA gene, which contains the maximum nucleotide heterogeneity and allows for sensitive discrimination between bacterial genera. The sequencing library was prepared using an innovative library preparation process in which real PCR reactions were performed using real-time PCR machines to control cycles and, thus, prevent PCR chimaera formation. The final PCR products were collected based on equal molarity, as measured using qPCR. Finally, the common library was filtered using Select-a-Size DNA Clean & Concentrator™ (Zymo Research, Irvine, CA), and then measured using TapeStation® (Agilent Technologies) and Qubit^(^(Life Technologies™).

The final library was sequenced using a V3 reagent kit (600 cycles) on an Illumina® MiSeq™. Sequencing was performed using a >10 %

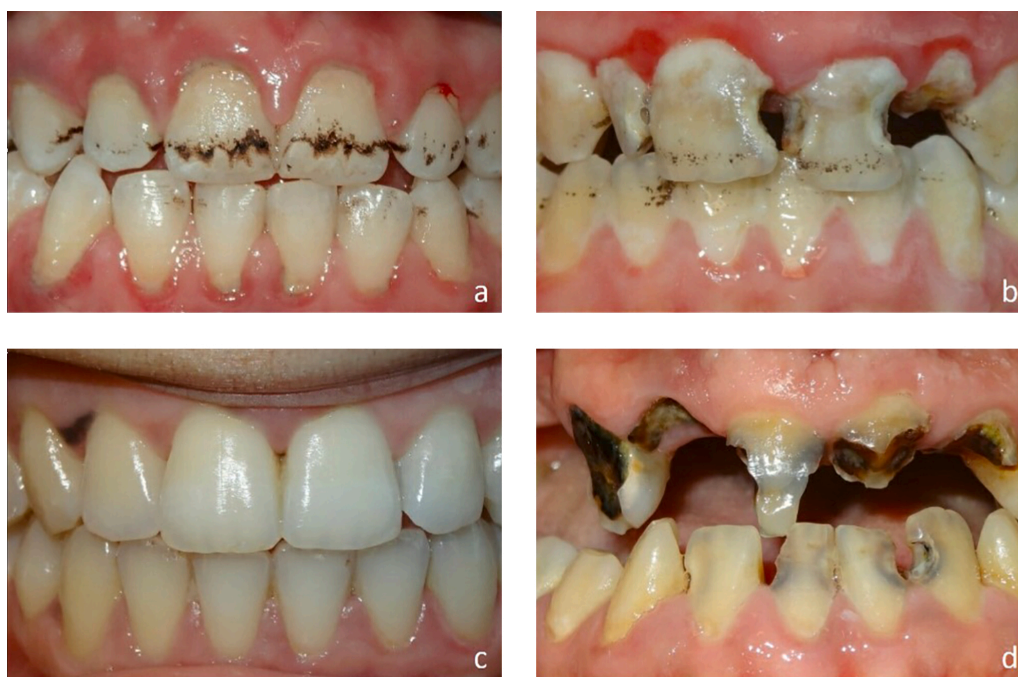

Fig. 1. Intraoral presentation of patients from Group 1 (a), Group 2 (b), Group 3 (c), Group 4 (d).

PhiX. Bioinformatics analysis was performed using mothur software at the usegalaxy.org open-source address. The booklet published with Galaxy software was used to ensure that the mothur program could be used by researchers without making errors. The analyses were performed according to the sample booklet 'Microbial analysis of 16S rRNA' in mothur. The raw data were bioinformatically analysed following the standard operating procedure provided by the Schloss laboratory at Galaxy (Schloss, 2018) by Drs Aris Cakiris, Zeynep Ceren Çelik, and Halil Celik.

Data were analysed descriptively using Stata v. 15.1 (StataCorp, College Station, TX, USA), with the level of significance set at $p < 0.05$, excluding Bonferroni correction. The Pearson chi-square and Fisher tests were used to evaluate the relationships between categorical variables. The Kruskal–Wallis test was used for the evaluation of groups, and the Mann–Whitney *U* test was used for inter-group post hoc analyses.

3. Results

Among the 52 individuals included in our study, 33 were females and 19 were males. The ages of the patients ranged from 18 to 50 years, with a mean age of 26.4 ± 8.4 years. The possibility of being caries free (ICDAS 0–2) was found to be significantly higher in black-stained teeth compared to black stain-free teeth. ($p = 0.007$). The caries rate (ICDAS 3–6) in black-stained and black stain-free posterior teeth was 18.2 % (n = 164) and 43.2 % (n = 201), respectively ($p = 0.0011$).

A total of 825,823 bacteria and 165 different types of bacteria were detected in the samples of the 13 patients included in Group 1. The percentage median values of common bacteria are displayed as a pie chart. Visualised bioinformatics was applied to all groups. According to this bioinformatic analysis, Group 2, Group 3, and Group 4 patients had a total of 851,137, 718,834, and 644,683 bacteria, with 168, 142, and 161 different types of bacteria, respectively.

Table 1 shows the microbial composition of the biofilms in Groups 1 and 3. The bacteria that are responsible for black staining in caries-free teeth are as follows: *Bergeriella*, *Brachymonas*, *Capnocytophaga*, *Cardiobacterium*, *Mannheimia*, *Propionibacterium*, *Rothia*, *Corynebacterium*, and *Neisseria*. Table 2 shows the microbial composition of the biofilms in Groups 2 and 4. NGS revealed that *Actinomyces*, *Brachymonas*, *Capnocytophaga*, *Cardiobacterium*, *Corynebacterium*, *Neisseria*, and *Rothia* species were the most prevalent species responsible for black staining in carious teeth.

Moreover, regardless of the caries status, *Aggregatibacter*, *Bergeriella*, *Brachymonas*, *Capnocytophaga*, *Corynebacterium*, *Neisseria*, and *Rothia* species may play a role in the aetiology of black staining (Table 3). In addition, the relative abundance of *Streptococcus* bacteria was found to be significantly higher ($p = 0.0061$) in black stain-free patients (5.6 %) than in patients with black staining (2.05 %; Table 1).

Table 1
Microbial composition of biofilms in Group 1(black stain and caries-free) and Group 3 (black stain-free and caries-free).

Name of bacteria	Group 1 n = 13 (p50 - %)	Group 3 n = 13 (p50 - %)	*p*
Bergeriella	0.0015	0	0.0351
Brachymonas	0.32	0.004	0.0360
Capnocytophaga	10.58	6.38	0.0014
Cardiobacterium	3.6	1.2	0.0006
Corynebacterium	4.08	3.62	0.0422
Granulicatella	0.02	0.13	0.0032
Haemophilus	0.0094	0.23	0.0001
Kingella	0.4	0.74	0.0061
Mannheimia	0.00124	0	0.0093
Mogibacterium	0	0.002	0.0126
Neisseria	2.2	1.97	0.0731
Propionibacterium	0.0094	0.0037	0.0111
Rothia	1.72	0.64	0.0293
Streptococcus	2.05	5.6	0.0061
Veillonella	0.94	8.4	0.0000

Table 2
Microbial composition of biofilms in Group 2 (black stain with caries) and Group 4 (black stain-free with caries.

Name of bacteria	Group 2 n = 13 (p50 - %)	Group 4 n = 13 (p50 - %)	*p*
Actinomyces	3.24	1.56	0.0378
Bifidobacterium	0	0.023	0.0374
Brachymonas	0.022	0	0.0095
Capnocytophaga	9.38	5.12	0.0032
Cardiobacterium	1.8	0.24	0.0004
Corynebacterium	7.08	3.12	0.0378
Dialister	0.156	0.166	0.0378
Fusobacterium	4.8	7.7	0.0096
Lactobacillus	0.00188	0.036	0.0077
Neisseria	9.5	2.07	0.0111
Phocaeicola	0	0.004	0.0004
Rothia	1.106	0.258	0.0149

Table 3
Microbial composition of total black stain (Group 1 + Group 2) and black stain-free (Group 3 + Group 4) plaques.

Name of bacteria	Black stain n = 26 (p50 - %)	Black stain-free n = 26 (p50 - %)	*p*
Aggregatibacter	0.0011	0	0.0163
Bergeriella	0.0006	0	0.0035
Brachymonas	0.0219	0.0009	0.0003
Capnocytophaga	4.8406	2.5946	0.0000
Corynebacterium	3.8696	1.9915	0.0064
Neisseria	5.4184	1.2459	0.0015
Rothia	0.6314	0.1953	0.0019
Streptococcus	1.1235	1.7400	>0.05

4. Discussion

In this study, we aimed to determine the bacteria responsible for black stain formation on permanent dentition using NGS technology.

Many clinical and epidemiological studies (Bhat, 2010; França-Pinto et al., 2012; Gasparetto et al., 2003; Heinrich-Weltzien et al., 2009, 2014; Koch et al., 2001; Li et al., 2015; Saba et al., 2006; Shourie, 1947; Żyła et al., 2015) have shown that children with black stains have a significantly lower caries burden. Saba et al. (2006) found that 10.3 % and 31.3 % children with black stains and without black stains, respectively, had a DMFT (total number of decayed, missing, and filled teeth) >3 status.

Shmuly, Zini, Yitschaky, and Yitschaky (2014)) reported that young adults with black staining had lower DMFT scores than adults without black staining in the clinical setting. Pehlivan, Yanikoglu, Tagtekin, and Hayran (2017)) found a positive correlation between having black stains and being caries free in a Turkish adult population. In this study, the number of teeth with ICDAS 0, ICDAS 1, and ICDAS 2 was found to be significantly higher in people with black staining than in people without black staining ($p = 0.0127$). In addition, when only the posterior teeth were evaluated based on their carious status, it the number of black-stained teeth with ICDAS 3–6 was significantly lower compared with ICDAS 0−2. ($p = 0.0011$; $p < 0.05$).

Moreover, *A. naeslundii* and *S. mutans* adhesion have been shown to be associated with low and high rates of caries formation, respectively (Stenudd et al., 2001). A significant increase in the number of *A. naeslundii* and a low number of *S. mutans* was associated with a low caries burden in children with black staining (Heinrich-Weltzien et al., 2014). Another study suggested that a lower number of caries occurred with *Actinomyces* colonisation due to an increase in the level of *Actinomyces* antibody (Levine, Owen, & Avery, 2005). A comprehensive

literature search revealed that most authors endorse the effect of the composition of bacterial microbiota on modulating sensitivity and resistance, especially when dominated by *Actinomyces* species in dental caries. (Heinrich-Weltzien et al., 2014; Levine et al., 2005; Stenudd et al., 2001) Similarly, in our study, the number of *Actinomyces* was significantly different between the caries-free black stain (1.56 %) and carious black stain (3.24 %) groups (p = 0.0378) (Table 2).

When different stages of colonisation are compared, *Fusobacterium nucleatum*, which acts as a bridge between the early and late colonisation stages of dental plaques, was found to be more common in black stain-free plaques (Kolenbrander & London, 1993). *Fusobacterium* bacteria belong to the Bacteroidaceae family and are non-spore forming, gram-negative, anaerobic bacilli. Similarly, in our study, the *Fusobacterium* genera was present in Group 2 (4.8 %) and Group 4 (7.7 %; Table 2). The prevalence rate was higher in Group 4 individuals than in Group 2 individuals (p = 0.0096).

In another study, *A. actinomycetemcomitans*, which is a gram-negative, black pigment-producing bacterium that acts as a periodontal pathogen, is thought to play a role in the formation of black stains, with a prevalence of 70 % in black stain samples and 20 % in black stain-free plaque samples (Saba et al., 2006). In another study conducted using real-time PCR, no significant differences were found between the prevalence of the periodontopathogenic bacteria *A. actinomycetemcomitans* and *Prevotella intermedia* in teeths with and without black stains (Heinrich-Weltzien et al., 2014). In the present study, *Aggregatibacter* bacteria showed significancy in paired comparisons and in the analyses comparing the black stain and black stain-free groups collectively (Table 3). When all black stain and black stain-free mouths were compared, the number of *Aggregatibacter* was significantly higher in black-stained plaques (p = 0.0163); the burden of *A. actinomycetemcomitans*, which is considered the main pathological aetiologic agent in aggressive periodontitis, was found to be significantly abundant in patients with black staining, suggesting that they may be susceptible to aggressive periodontitis (Heinrich-Weltzien et al., 2014).

Li et al. (2015) conducted a study using 16S rRNA gene sequencing to evaluate the oral microbiota in children with and without black staining. In their study with two groups, caries-free with black stains and caries- and black stain-free, Li et al. found that the microbial diversity of the saliva and plaques of children who were caries free and had black staining was significantly less than that in children without black staining. Microbiological NGS in the same study showed that *Actinomyces*, *Cardiobacterium*, *Haemophilus*, *Corynebacterium*, *Tannerella*, and *Treponema* were more common in plaque samples collected from children with black staining. According to the results of our study, the following bacteria can be considered to form black stains without causing caries: *Capnocytophaga*, *Cardiobacterium*, and *Rothia*. Similarly, Li et al. (2015) found that relative abundance of *Cardiobacterium* and *Corynebacterium* were significantly higher in black-stained plaques (p = 0.0064; p = 0.0000).

Capnocytophaga bacteria are gram-negative rods found in the healthy oral microbiota. When classified based on its taxonomic ranks from kingdom to genus, it follows the following order: Bacteria, Bacteroidetes, Flavobacteria, Flavobacteriales, Flavobacteriaceae, and *Capnocytophaga*. 'Capno' in the bacterium's name means carbon dioxide, referring to its need for an atmosphere containing 5%–10 % carbon dioxide for growth, making it capnophilic (Pudakalkatti, Baheti, Hattarki, Kambali, & Naik, 2016). *Capnocytophaga* in subgingival plaque produces bacteriocin, which in turn inhibits the proliferation of *S. sanguis*, *S. mitis*, *S. mutans*, and *P. acnes* fibroblasts (Yalcin Cakir, Gurgan, & Attar, 2010). We believe that *Capnocytophaga* may decrease the cariogenic potential of carious bacteria and contribute to the lower incidence of caries in the presence of black stains due to bacteriocin production.

Corynebacterium bacteria are gram-positive, filamentous, facultative anaerobic rods (Yalcin Cakir et al., 2010). They are known to form black-pigmented colonies on blood agar (Shukla, Harney, Jhaveri, Andrews, & Reed, 2003). *Rothia* bacteria are gram-positive, filamentous, facultative anaerobic cocci. *Rothia* belong to the family Actinomycetaceae. The species of the bacteria found in oral flora is typically *R. denticariosa* (Yalcin Cakir et al., 2010). *Actinomyces* bacteria are very similar to Rothia in most of its features. In addition, *Actinomyces* form charcoal black colonies, similar to those of *Corynebacterium* bacteria, in vitro (Daneshvar et al., 2004).

Some studies (Heinrich-Weltzien et al., 2014; Li et al., 2015; Saba et al., 2006) investigating the formation of black stains have shown that *Actinomyces* species are significantly more abundant in children with black staining. Our study confirms the findings of these previous studies and significantly contributes to the existing literature, especially for an age group that has not been well studied. *Actinomycetes* species were found to be more abundant in patients with black staining and caries in Group 2 (3.24 %) than in black stain-free patients with caries in Group 4 (1.56 %; p = 0.0378). In addition, the fact that *Actinomycetes* species were found to be more abundant in Group 3 (black stain-free and caries-free) patients (3.45 %) suggests that *Actinomycetes* species may be related to caries-free status. Therefore, it can be concluded that microflora dominated by *Actinomycetes* may be associated with black stain formation and may have lower cariogenic potential than dental plaques without black stains.

In a microbiological study, Slots (1974) suggested that the low incidence of caries in some patients was due to the low number of streptococci. According to Heinrich-Weltzien et al. (2014), the abundance of *S mutans* and *S. sobrinus* was not significantly different in children with or without caries and with or without black stains, but *Lactobacillus* species were significantly more abundant in black-stained plaque samples. In our study, *Lactobacillus* were found to be more abundance in black-stained plaques (Table 2) (p = 0.0077).

In a recent study, plaque samples of children with black staining were found to have a greater abundance of *Actinomyces*, *Cardiobacterium*, *Haemophilus*, *Corynebacterium*, *Tannerella*, and *Treponema* species; the abundance of *Streptococcus* species did not differ significantly between black-stained and black stain-free plaques (Li et al., 2015). Similarly, in our study, the abundance of *Streptococcus* did not differ in the samples of patients with black stains (p > 0.05). The study by Li et al. (2015) was the first study to use NGS to determine the microflora of black stains in children. However, no previous NGS studies have been conducted among adult patients (Albelda-Bernardo, Mar Jovani-Sancho, & Veses, 2018).

A limitation of this study is that the microbial analysis was performed at the genus level. Further studies can be conducted at the species level with a larger sample size. In addition to the small sample size, the similar nutritional habits of the individuals selected for standardisation can be considered another limitation. However, the major strengths of our study are the inclusion of adult participants and the use of a comprehensive metagenomic method such as NGS.

To our knowledge, many previous studies have been conducted among the paediatric population and have used classical microbiological and molecular methods, such as PCR and real-time PCR, to identify the microorganisms present in black stains. This is the first study to use NGS techniques to assess the microflora of black stains in the adult population. This study contributes to the literature by evaluating the bacterial composition of black stains in great detail using a novel approach that has the power to produce many sequences per analysis in a cost- and time-efficient manner, showing that there may be additional bacterial species that may be responsible for the formation of black stains and have not been mentioned in previous studies.

Both black-stained and black stain-free plaques contain a wide variety of bacteria. Alterations in the oral microflora may occur with the formation of a black stain. Detecting the presence of different bacteria in black-stained plaques suggests that there are many different microorganisms in addition to *Actinomyces* that play a role in the formation of black stains. The microflora composition of black stains differs between

carious and caries-free states. Consistent with most epidemiological studies worldwide, we found that adults with black-stained teeth had a lower prevalence or history of caries. Utilisation of NGS has enabled the accurate analysis of oral microflora in a short time.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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