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Deciphering Black Extrinsic Tooth Stain Composition in Children Using Metaproteomics

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Cite This: *ACS Omega* 2022, 7, 8258−8267 Read Online

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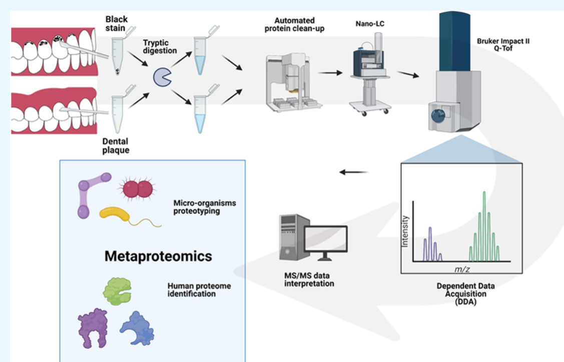
ABSTRACT: The present study focuses on the use of a metaproteomic approach to analyze Black Extrinsic Tooth Stains, a specific type of pigmented extrinsic substance. Metaproteomics is a powerful emerging technology that successfully enabled human protein and bacterial identification of this specific dental biofilm using high-resolution tandem mass spectrometry. A total of 1600 bacterial proteins were identified in black stain (BS) samples and 2058 proteins in dental plaque (DP) samples, whereas 607 and 582 human proteins were identified in BS and DP samples, respectively. A large diversity of bacteria genera (142) in BS and DP was identified, showing a high prevalence of Rothia, Kingella, Neisseria, and Pseudopropionibacterium in black stain samples. In this work, the high diversity of the dental microbiota and its proteome is highlighted, including significant differences between black stain and dental plaque samples.



1. INTRODUCTION

Dental plaque can be briefly described as "the microbial community which develops as a structurally and functionally organized biofilm on the tooth surface, embedded in a matrix of polymers of bacterial as well as host salivary origin".[1] Even if black stain (BS) plaque is characterized by microorganisms embedded in an inter-microbial substance with a tendency to calcify, microbiological studies have advanced contradictory results on differences of oral microbiota between BS and white dental plaque (DP). These findings allow for discussion of the possible etiologic role of microorganisms in BS plaque and therefore some insight as to potentially cariogenic microbiota. Nevertheless, literature is not clear on the topic, as it has been reported that BS was associated with lower carious prevalence[2] in children with BS compared to children without BS.[3] The analyses of oral microbiota may confirm the presence of *Streptococcus mutans*, one of the major carious agents, which represents an opposite to others which do not reveal the presence of a real specific flora "with exception of the presence of oral black-pigmented bacteria".[4] Indeed, there are factors which tend to differ between BS and DP apparition such as (i) fluoride and/or antibacterial agents which minimize the presence of undesired side effects, hence fight against BS formation,[5,6] (ii) fixed prosthetic restorations such as veneers which may lead to the formation of BS,[7] (iii) drinking water with high iron content, water with high pH, having a high salivary pH, and smoking, can all result in BS formation,[8] and (iv) it was also noted that different arrangements of toothbrush bristles including lowered bristles in middle of the brush-field have enhanced the cleaning efficacy compared to planar fields.[9] It has also been described that the high abundance of biosynthetic heme pathways suggests that heme-dependent iron sequestration and subsequent metabolism are directly associated with the formation of BS.[10] From a technological point of view, most early studies were based on the use of PCR to determine and characterize the presence of bacteria involved in BS dental plaque.

Over the last decade, metaproteomics has emerged as a relevant approach to characterize the functioning of a given microbiome through the analysis of its proteins.[11] Based on extensive bottom-up proteomics data, metaproteomics gave the list of the most abundant proteins but also their quantities. Furthermore, the list of peptides identified by high-resolution tandem mass spectrometry allowed identifying the list of the microorganisms present in the sample and even estimating the

Received: August 31, 2021
Accepted: December 20, 2021
Published: February 25, 2022

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ratio of microorganisms in terms of protein biomasses.[12] The recent concept of phylopeptidomics pioneered by Pible et al. is based on the assignation of taxa to the MS/MS spectra and deconvolution of the signals to discriminate the microorganisms present in a given sample. It has not only been applied for the proteotyping of clinical and environmental isolates[13,14] but also for the analysis of microbial communities.[14,15] While a large diversity of pipelines has been developed for interpreting metaproteomics results, efforts at improving the experimental set up and the bioinformatics data treatment are worthwhile. The most recent results from large scale comparative results showed that taxonomic proteotyping by metaproteomics is as valuable as taxonomic characterization by molecular biology approaches.[16]

In this study, we applied metaproteomics to proteotype the microbiota present on the original BSs from a cohort of children and highlighted the differences between BS and DP samples.

2. RESULTS

2.1. Sample Characteristics. In our clinical study, two groups of plaque samples (47 BS vs 47 DP) were obtained from young patients followed at the Department of Pediatric Dentistry, CSERD Dental Hospital, CHRU Montpellier (France). Patients included were paired by age ±2 years (DP mean age 6.7 ± 1.6; BS mean age 6.6 ± 2.2) and diagnosed using clinical examination including extrinsic tooth discoloration and gender. The patients' information is shown in Table 1. Children were 44.7% male in the DP group and 66.0% in the BS group. Participant gender, age, and plaque index were not significantly different between DP and BS samples. The majority of children (61.7% of BS and 51.1% of DP) exhibited good oral hygiene habits with daily brushing and interdental hygiene. Based on Gasparetto et al. criteria, children with BS were categorized as following: 51.1% score 1, 31.9% score 2, and 17.0% score 3 (Table 1).[3] Score 1° corresponds to the occurrence of pigmented dots or lines with incomplete coalescence parallel to the gingival margin. Score 2° corresponds to the presence of continuous pigmented lines, and score 3° corresponds to the presence of pigmented stains covering beyond half of the cervical third of the tooth surface. Multiple logistic regression used on clinical data (Table 1) between BS and DP showed that DMFT in the BS group (4.8 ± 4.6) is significantly lower than that in the DP group (6.6 ± 3.9) ($p < 0.05$). The presence of BSs in our population seemed not to be directly linked to gender or plaque indices. Clinical and metaproteomics sample group characteristics are statistically similar (Table S1).

Table 1. General Patients' Information Selected for the Clinical Study

	patient status	
	BS	DP
population	47	47
age, average	6.60 ± 2.21	6.74 ± 1.63
Sex		
male	31 (66.0%)	21 (44.7%)
female	16 (34.0%)	26 (55.3%)
Oral Hygiene Habits		
daily	29 (61.7%)	24 (51.1%)
4 to 6 times a week	10 (21.3%)	20 (42.5%)
1 to 3 times a week	7 (14.9%)	2 (4.3%)
never	1 (2.1%)	1 (2.1%)
BS Score		
score 1	24 (51.1%)	
score 2	15 (31.9%)	
score 3	8 (17.0%)	
Dental Plaque Index		
0	7 (14.9%)	3 (6.4%)
1	27 (57.5%)	18 (38.3%)
2	8 (17.0%)	19 (40.4%)
3	5 (10.6%)	7 (14.9%)
DMFT index	4.83 ± 4.64	6.64 ± 3.87

2.2. Plaque Protein Distribution/Diversity. Plaque samples from DP and BS groups were analyzed using LC−MS/MS, and all spectra were searched against the Nextprot database to identify proteins from Homo Sapiens and against the SwissProt database to identify bacteria diversity with "Bacteria" as Taxonomy. Peptide FDR was controlled below 1%. 607 human proteins (4042 peptides) were identified in the BS group and 582 human proteins (3450 peptides) in the DP group. We observed (Figure S1A) a distribution of identified proteins with 318 common identification "pairs", 289 uniquely identified proteins for the BS group and 264 for the DP group, showing an important heterogeneity. Interestingly, on bacterial proteins, 1600 proteins (5287 peptides) were identified in the BS group and 2058 proteins (6979 peptides) in the DP group, as shown in Figure S1B, representing an increase for bacterial protein identification (by a factor 2.6 to 3.5), respectively. The distribution of relevant identified proteins (human as well as bacterial) regarding the patients can be shown in Figure 1A,B. The number of identified proteins among bacteria was higher than identified human proteins, whatever the two groups. If the proportion of human proteins identified in BS and DP was equivalent (51 vs 49%), we identified 28.5% more bacterial proteins in DP than in BS.

2.3. Taxonomical Comparative Analysis. In our metaproteomics study, a spectral counting label-free method was adapted to relative comparison of Taxon-to-Spectrum Matches (TSMs).[17] The metaproteomics-based proteotyping of DP and BS performed at the genus level allowed the assignation of 142 bacterial genera based on 2 specifics to validate a genus using validated Taxon-to-Spectrum Matches (TSMs) at a Mascot p-value of 0.05. These 142 genera correspond to 10 phyla, 22 classes, 47 orders, and 79 families.

For the taxonomical comparative analysis, there were significant differences between DP and BS groups for 17 bacterial genera based on their LC−MS/MS abundances (means of their TSMs) and normalized by the total number of bacterial TSMs per sample (Figure 2). These genera were *Alcanivorax*, *Clostridium*, *Lachnoclostridium*, *Leptotrichia*, *Vibrio*, *Centipeda*, *Selenomonas*, *Kingella*, *Rothia*, *Alloprevotella*, *Enterococcus*, *Treponema*, *Pseudopropionibacterium*, *Catenibacterium*, *Prevotella*, *Tessaracoccus*, and *Neisseria*.

Furthermore, the species, genus, family, order, class, phylum, and super kingdom taxonomy which are linked to number of taxon to spectrum matches "TSMs" are presented as in Table 2. The 17 bacterial genera that were found significantly modulated between BS and DP groups (more abundant in children with BS) are listed in Table 3 including the relevant characteristics for each genus.

2.4. Pathway Analysis on Human Proteins. After ingenuity pathway analysis, a relevant overlapping of canonical pathways was observed for DP and BS groups in Figures S2 and S3, respectively. Identifying the pathways (within DP vs BS) association partners and positioning them (separately in each

https://doi.org/10.1021/acsomega.1c04770
ACS Omega 2022, 7, 8258−8267

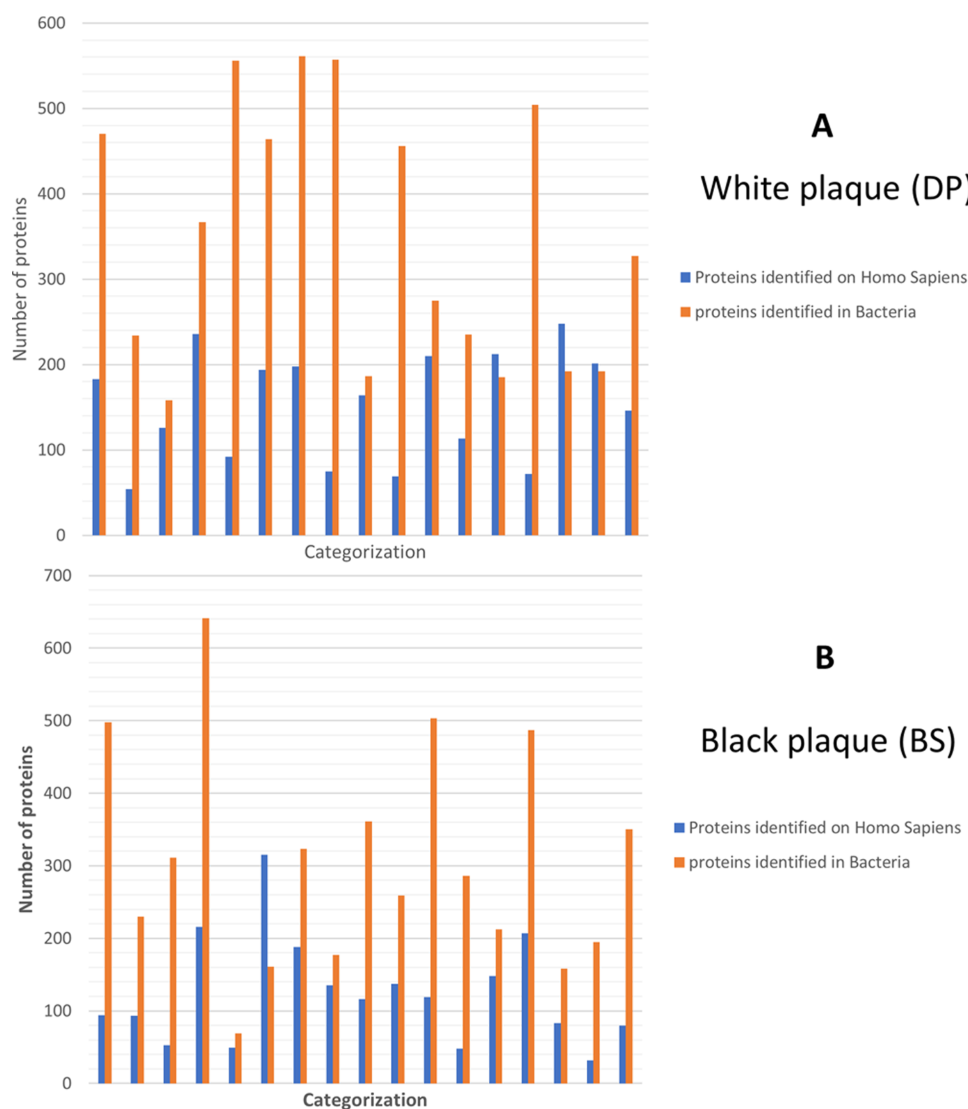

Figure 1. Distribution of proteins identified in human and bacteria in correspondence with selected patients and the protein diversity in each category: DP "A"; BS "B".

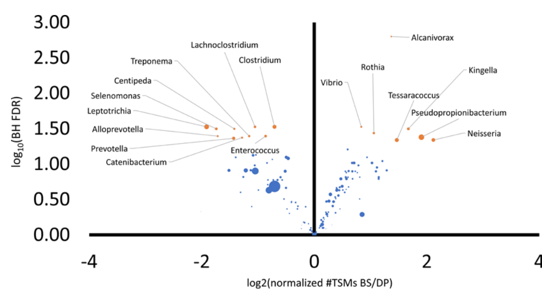

Figure 2. Volcano-plot diagram showing a significant difference of differentially abundant 17 genera "microbiota" in BS and DP categories.

group) in known physiological and path-physiological events' networks and signaling pathways is a powerful approach to get insights into their functions and the respective series of chemical interactions linked to different biological processes. Therefore, it is likely that pathways involved within the DP group can be directly linked to the regulation of biological processes within oral activity/normal dental plaque action including saliva behavior. It is also likely that pathways involved within the BS group can be directly associated with the formation of black tooth stains.

3. DISCUSSION

BSs are a specific type of external tooth discoloration and are part of the acquired pellicle on the tooth surface. BS are commonly found in children beyond the line representing the cervical third of the crown (dark lines parallel to the gingival margin) and/or lingual side of teeth. Indeed, BS are induced by oral bacteria as well as by products of saliva (e.g., proteins); however, there is controversy about the nature of bacteria involved.[18] A chemically different content with higher expression of calcium, phosphate, sulfur, and copper−iron has been advanced by some researchers. However, others illustrated the origin of the black pigment which is probably in relation with ferric sulfide induced by the reaction between hydrogen sulfide generated by bacteria and iron in saliva or gingival fluid.[19] If the link with several lifestyle factors such as smoking or consuming drinks containing tannins (tea, red wine, coffee, etc.,) can be excluded in children, certain iron-related drug treatments are a potential etiology; however, in these types of black extrinsic staining, the thin films are not limited to the cervical third of the

https://doi.org/10.1021/acsomega.1c04770
ACS Omega 2022, 7, 8258−8267

Table 2. TSMs per Taxa Assigned for the 17 Genera "within BS Versus DP Groups" at Various Taxonomical Levels Including (Phylum, Class, Order, Family, and Genus) are Listed

genus	family	order	class	phylum
Alcanivorax	Alcanivoracaceae	Oceanospirillales	Gammaproteobacteria	Proteobacteria
Clostridium	Clostridiaceae	Clostridiales	Clostridia	Firmicutes
Lachnoclostridium	Lachnospiraceae	Fusobacteriales	Fusobacteriia	Fusobacteria
Leptotrichia	Leptotrichiaceae	Vibrionales	Negativicutes	Actinobacteria
Vibrio	Vibrionaceae	Selenomonadales	Betaproteobacteria	Bacteroidetes
Centipeda	Selenomonadaceae	Neisseriales	Actinobacteria	Spirochaetes
Selenomonas	Neisseriaceae	Micrococcales	Bacteroidia	
Kingella	Micrococcaceae	Bacteroidales	Bacilli	
Rothia	Prevotellaceae	Lactobacillales	Spirochaetia	
Alloprevotella	Enterococcaceae	Spirochaetales	Erysipelotrichia	
Enterococcus	Spirochaetaceae	Propionibacteriales		
Treponema	Propionibacteriaceae	Erysipelotrichales		
Pseudopropionibacterium	Erysipelotrichaceae			
Catenibacterium				
Prevotella				
Tessaracoccus				
Neisseria				

crown.[2] Nevertheless sex, age, oral hygiene, socio-economic status, and dietary habits have often been discussed. Thus, the main challenges are still those associated with saliva and the microbiota. Concerning the salivary modifications in BS patients, there is a consensus on the higher buffering power, higher pH, and increased content of calcium, inorganic phosphate, copper sodium, and total protein content, but less glucose compared to children without staining.[20] Also, a significant decrease in the salivary flow rate has also been reported in children affected by BS. Consequently, investigating the microbiota should be performed at the earliest stage to understand the aetiology of BS as a selective attachment of bacteria to the tooth surface.

This study presents the comparison "for example, differential bacterial genera" between BS versus DP for samples collected from a cohort of children using a novel metaproteomics approach.

Multiple logistic regression used on clinical data (Table 1) through "BS and DP" displayed a significant difference ($p < 0.05$) for DMFT. The DMFT in children presenting BS (4.8 ± 4.6) was lower than that in DP children (6.6 ± 3.9). These results correlate with earlier DMFT results conducted by Koch et al., 2001,[21] where mean DMFT was 0.49 for children with BS and 0.97 for children with DP "without BS" ($p < 0.05$). Both of these studies' results may suggest a linkage between BS and decreased caries prevalence.

In fact, human proteins play an essential role in the development of BS and they are also involved within the biological processes associated with DP. These proteins can be categorized into different classes resulting in various pathways (based on their physiological "DP" or pathological "BS" roles) which have various implications regarding BS and DP. By focusing on the current results, it is clearly shown that a relatively higher number of proteins (289) is exclusively characterized/identified within the BS category compared to in the DP (264) category, indicating that each category of proteins can have a direct correlation with BS and DP; particularly, there is a relatively huge number of proteins (318) which are commonly identified in both BS as well as DP assuming that these pairs can have the same role for both groups (BS vs DP).

Relevant canonical pathways by ingenuity pathway analysis were observed for DP and BS groups in Figures S2 and S3. The pathways involved within the DP group can be directly linked to the regulation of biological processes within oral activity/normal dental plaque action including saliva behavior. The pathways involved in the BS group are probably associated with the formation of black tooth stains.

It was reported that pathways related to glycolysis, gluconeogenesis (Figure S2), and starch and sucrose metabolism were more abundant in the BS category. The results indicated a more active metabolic state in teeth with BSs. The deposits on the teeth may provide bacteria nutritional substances, and the metabolic products may participate in the formation of BSs (Zhang et al., 2017).[22] Furthermore, it was shown that the formation and maturation of phagosomes (Figure S3) can be generally considered as a key mechanism within innate immunity against bacterial infection, and additionally, it was shown by (Sasaki et al., 1990)[23] that cementoblasts frequently extended broad cell events with phagosomes including collagen fibrils into the dentinal tubules exposed to resorption lacunae, and it was suggested that during detachment of the periodontal ligament concomitant with root resorption, several fibroblasts phagocyted mature collagen fibrils and amorphous material.

Our results show that salivary acidic proline-rich phosphoprotein (PRP, which is considered as one of the main components of parotid and submandibular saliva in human proline-rich proteins) is exclusively identified in the white (DP) group as described by Proctor et al., 2005[24] and Kauffman and Keller, 1979.[25] This family of protein included acidic, basic (the most effective in complexes formation with condensed tannin),[26] and glycosylated proteins. The protein expression of acidic proline-rich proteins is known to be under complex genetic control.[27]

Moreover, the acidic proline-rich proteins can strongly bind calcium, which suggests that they may play a pivotal role in maintaining the concentration of calcium ions in saliva.[28] Additionally, they can inhibit the formation of hydroxyapatite (which forms the teeth matrix and give the teeth rigidity), whereby growth of hydroxyapatite crystals on the tooth surface in vivo could be avoided, because they represent salivary proteins which exhibit high affinities with hydroxyapatite

https://doi.org/10.1021/acsomega.1c04770
ACS Omega 2022, 7, 8258−8267

Table 3. List of the 17 Bacterial Genera that were Found Significantly Modulated between BS and DP Groups and Their Relevant Characteristics[a]

phyla	genus	Gram+/Gram−	already detected	state 2/state 1 ratio	*t*-test *p*-value	characteristics	reference
Proteobacteria	*Alcanivorax*	Gram−		2.6	0.000	degradation of linear and branched alcanes	Gregson, 2019, 30951249
Firmicutes	*Clostridium*	Gram+	x	0.6	0.001	anaerobic bacilli, can be due to dentist antibiotic treatment	Beacher, 2015, 26404991
Firmicutes	*Lachnoclostridium*	Gram+		0.5	0.001	novel bacteria from gut microbiota	Amadou, 2016, 27493758
Fusobacteria	*Leptotrichia*	Gram−	x	0.3	0.001	lactic acid is the major metabolic end product of *Leptotrichia* that distinguishes it from *Fusobacterium* and *Bacteroides*. *Leptotrichia* is biochemically active. It can ferment amygdalin, cellobiose, fructose, glucose, maltose, mannose, melezitose, salicin, sucrose, and trehalose to produce acid	Eribe, 2008, 18539056
Proteobacteria	*Conchiformibius*	Gram−	x	3.9	0.000	detected in oral microbiome of canines and has been associated with human psoriatic unaffected skin	Chang, 2018, 30185226 &; Dewhirst, 2012, 22558330
Proteobacteria	*Methylococcus*	Gram−	x	2.2	0.001	no associated oral pathologies	
Proteobacteria	*Vibrio*	Gram−	x	1.8	0.000	found in a wide variety of aquatic and marine habitats and can cause infections in humans	Baler-Austin, 2018, 30002421
Firmicutes	*Centipeda*	Gram−	x	0.4	0.001	saccharolytics and generate propionic acid. chronic periodontitis and periodontal health/gingivitis	Rams, 2015, 25037463
Firmicutes	*Selenomonas*	Gram+	x	0.3	0.001	implicated in converting periodontal health to disease, and have also been found in gastric ulcers	Cruz, 2015, 26272608
Proteobacteria	*Kingella*	Gram−	x	3.2	0.001	*K. kingae* is a common colonizer of the oropharynx, can be transmitted from child to child, and can cause outbreaks of infection. *K. oralis* is a common colonizer in the human oral cavity. The organism maybe found in dental plaque, on mucosalsurfaces and in saliva.	
Actinobacteria	*Rothia*	Gram+	x	2.1	0.001	part of the normal flora in the human oral cavity and pharynx. *R. dentocariosa*, a normal commensal of the oral cavity, is a bacterium considered to be of low virulence. Usually associated with dental caries and periodontal disease	Tsuzukibashi, 2017, 28082174
Bacteroidetes	*Alloprevotella*	Gram−	x	0.3	0.002	*Alloprevotella* species are associated with neutral pH in the oral cavity and has been found in cases of caries progression	Ortiz, 2019, 31497256
Firmicutes	*Enterococcus*	Gram+	x	0.5	0.002	related to oral diseases, such as caries, endodontic infections, periodontitis, and peri-implantitis	Komiyama, 2016, 27631785
Proteobacteria	*Methylocaldum*	Gram−	x	2.3	0.002		
Spirochaetes	*Treponema*	Gram−	x	0.4	0.002	widely considered to play important roles in periodontal disease etiology and pathogenesis.	You, 2013, 23578286
Actinobacteria	*Pseudopropionibacterium*	Gram+	x	3.7	0.002	member of the human oral microflora. It has been identified in ancient dental calculus and implicated in conditions such as infective endocarditis	Jersie-Christensen, 2018, 30459334
Firmicutes	*Catenibacterium*	Gram+	x	0.4	0.002	identified in gut microbiota	Garcia-Mantrana, 2018, 29867803
Bacteroidetes	*Prevotella*	Gram−	x	0.4	0.003	black-pigmented anaerobes associated with the initiation and progression of periodontis	Soukos, 2005, 15793117
Actinobacteria	*Tessaracoccus*	Gram+	x	2.8	0.003	frequently found in symptomatic teeth	Lim, 2011, doi: 10.5395/JKACD.2011.36.6.498
Proteobacteria	*Neisseria*	Gram−	x	4.3	0.003	one of the major phyla founded in caries-active dentinal microbiota	Hurley, 2019, 30642327

[a]State 1: condition 1: DP group; State 2: condition 2: BS group.

https://doi.org/10.1021/acsomega.1c04770
ACS Omega 2022, 7, 8258−8267

surfaces.[27] It is worth mentioning that Proctor and his colleagues have described that some salivary proteins, including PRPs have the ability to mediate increased staining of enamel by red wine- and black tea-derived polyphenols.[24] Consequently, there is a direct correlation between less BS formation upon the absence of PRPs. In addition to PRPs, another distinct human protein difference between BS versus DP, which is cathepsin, was reported to be implicated in the formation of caries/pigments leading to the formation of BS. Our findings show also that cathepsin (one of the host-induced enzymes that are intensely involved within caries development) is uniquely identified in BS category.[29] Furthermore, our results show the unique identification of metalloproteinase in BS, and this matches with what was described earlier that metalloproteinase is promoting the formation of caries/BSs.[30]

Identification of microorganisms present in a sample as well as the estimation of their biomass contributions are essential objectives which should be achieved with the best sensitivity and precision to get a clear overview of the structure and the temporal dynamics of a microbial community. Metaproteomics is a novel approach which allows the estimation of biomass contributions. The phylopeptidomics concept pioneered by Pible et al. (2020) relies on taxonomical information assigned to each MS/MS spectrum and considers both taxon-specific peptides and peptides shared between organisms. The latter are not yet taken into consideration by current metaproteomics pipelines. The resulting relative quantitation of microorganisms is based on taxon-spectra matches and thus is peptide-centric. In this regard, the proposed methodology is basically different from the quantitative approach introduced by Kleiner et al.,[31] that is protein-centered. Indeed, protein inference in metaproteomics is complex due to the large databases required as well as the multiple occurrences of a large ratio of peptide sequences. In the present study, we relied on TSMs (taxon spectra matchings) which is a novel quantitative feature within metaproteomics equivalent of the spectral count for proteins (through which a peptide spectrum matching "PSM" is used) but at each possible taxon level.

Here, we successfully identified 132 genera when considering the whole metaproteomic datasets comprising 34 samples which correspond to 10 phyla, 22 classes, 47 orders and 79 families, while other studies on teeth microbiota identified less bacterial genera. For example, Li et al. identified a total of 10 phyla, 19 classes, 32 orders, 61 families, and 102 genera,[32] using the 16S rRNA gene sequencing method, while 25 samples (10 children with and 15 children without BS) were used in that study. Another study was performed by Chen et al., while 40 samples were used for sequencing analysis and identified 18 phyla, 28 classes, 48 orders, 78 families, and 135 genera using also 16S rRNA technology.[17]

Our results showed the prevalence of *Rothia*, *Nesseria*, *Kingella*, and *Pseudopropionibatcterium* (Figure 2) in children with BS compared to DP; however, it was the opposite for *Selenomonas* as illustrated below: (i) *Rothia* genus is a type of Gram-positive bacteria that is generally linked to infections. *Rothia dentocariosa*, is a type species of the genus, is an aerobic coccoid to rod-shaped, non-motile, catalase-positive Gram-positive bacterium.[33] *R. dentocariosa* was originally isolated from dental plaque as well as caries. However, clinical relevant isolates of *R. dentocariosa* were found in patients having periodontal lesions that could be the source for transient bacteremia leading to systemic diseases, similar to other oral bacteria. Additionally, *R. dentocariosa* rarely induces disease, mainly for patients having dental disease,[34] as in our investigation of the BS group.

Recently, next-generation sequencing studies of saliva as well as DP from subjects under physiological and pathological states have characterized bacteria related to the *Rothia* genus such as ubiquitous members of the oral microbiota.[35] To gain a deeper understanding of molecular mechanisms underlying the chemical ecology of this unexplored group, they performed a genome mining technique that targets functionally crucial biosynthetic gene clusters (BGCs). As a result, a total of 45 genomes which were mined, representing *Rothia mucilaginosa*, *R. dentocariosa*, and *Rothia aeria*, harbored a catechol-siderophore-like BGC, which is associated with BS, due to the toxicity of the catechol molecule,[35−37] (ii) *Neisseria* genus was described to be significantly different "highly abundant" in the BS category compared to the DP/control category;[18] various species at the genus level including *Neisseria* and *Streptococcus* may be main contributors for the formation of BS. It was suggested that alterations in oral microbiome are crucial, (iii) *Kingella* genus was shown/detected to be significantly increased in severe caries group versus non-severe caries group,[38] (iv) *Pseudopropionibacterium* genus has been reported to be linked to dental plaque disease as described by Siqueirajr and Rocas.[39] Microorganisms were found in all cases of root-filled teeth linked to periradicular lesions that strongly support to the assertion that treatment failures are rather of infectious etiology, caused by persistent or secondary intraradicular infections. Others include *Enterococcus faecalis*, three other anaerobic species: *Pseudoalactolyticus*, *Dialister pneumosintes*, and *Filifactor alocis*, and (v) the genus *Selenomonas* was exhibited to be less abundant in BS versus "DP/control group", and it was also a similar case for *Leptotrichia* and *Prevotella* genera.[18] Moreover, at the genus level, Li et al.[32] showed that there was a significant difference between DP and BS for *Prevotella* and *Trepenoma* (which also matches with our findings).

4. CONCLUSIONS

Our results showed that *Rothia*, *Kingella*, *Neisseria*, and *Pseudopropionibacterium* were significantly up-regulated in BS compared to DP, which matches other studies as reviewed in following references (*Rothia*,[33−35] *Neisseria*,[18] *Kingella*,[36] and *Pseudopropionibacterium*[37]). Furthermore, it was also reported that the total number of proteins in caries active subjects is higher than caries free subjects (i.e., salivary proteins play a pivotal role in caries development),[17] which is consistent with our findings. This study showed the importance of the above-discussed metaproteomics approach, which can enable the discovery of novel pathways of BS functional characterization.

5. EXPERIMENTAL SECTION

5.1. Subjects and Study Design. This case-control study is a comparative proteomic analysis of BS samples and standard supragingival plaque in 94 children divided in two groups (n = 47) according to the presence (BS group) or absence (DP group) of black extrinsic tooth stains. Subjects were paired by age ±2 years. A clinician checked that recruited patients had no infectious or systemic diseases and had not taken any antibiotics within the 2-week period before sample collection. For proteotyping microorganisms, we designed a study subgroup of 17 patients for whom we obtained BS and DP from contralateral teeth in sufficient amount and quality for the metaproteomics approach.

All procedures performed in the study were in accordance with the ethical standards of the institutional and national research committees (public health law 09/08/2004, bioethics laws, computer and freedom law and good clinical practices) and with the 1964 Helsinki Declaration and its later amendments. The general experimental workflow is briefly shown in the graphical abstract. The protocol was approved by the South Mediterranean I Human Protection Committee from Marseille, France (Authorization Number 2016-A01496-45). Prior informed consent was obtained from all caregivers (parents or legal guardians) of children included in the study.

5.2. Inclusion/Exclusion Criteria. This case-control study was carried out at the Montpellier Regional University Hospital (CHRU), where children from 3 to 9 years old attending the Dental Hospital (CSERD: Centre de Soins, d'Enseignement et de Recherche Dentaires, Montpellier, France) were included from December 2016 to June 2019. All the children were healthy according to their parents. Children with at least six black-stained teeth were included in the BS group, while children free of BS were categorized in the DP group. The only exclusion criterion was children's signs of precocious puberty, responsible for hormonal variations.

5.3. BS Scores, Dental Plaque Index, and DMFT Index. Through visual inspection by a single investigator, a specialist in paediatric dentistry, black extrinsic stains (BS) were evaluated based on the presence of pigmented dark lines parallel to the gingival margin or an incomplete coalescence of dark dots rarely extending beyond the cervical third of the crown. BS classification used was 1, 2, 3 criteria according to "Gasparetto et al. 2003".[3] Score 1 corresponded to the presence of pigmented dots or thin lines with incomplete coalescence parallel to gingival margin; score 2 corresponded to continuous pigmented lines limited to half of the cervical third of the tooth surface; score 3 corresponded to the presence of pigmented stains extending beyond half of the cervical third of the tooth surface. Dental plaque "DP" index according to a simplified "Silness−Loë" nomenclature was investigated during oral examination to evaluate oral hygiene: "0" refers to no supragingival plaque, "1" refers to supragingival plaque limited to the cervical third of the tooth surface, "2" refers to supragingival plaque extending half of the tooth surface, "3" refers to supragingival plaque extending beyond half of the tooth surface. The decayed, missing or filled teeth (DMFT) index in both group BS and DP was used to assess caries experience according to the WHO criteria (oral survey basic methods World Health Organization 1997). This DMFT score was applied to the whole tooth rather than a specific tooth surface and was determined for each BS or DP subject. The between group comparison of DMFT was performed with the Mann−Whitney test and a 5% level of significance (Stata software, v16.1).

5.4. Sample Collection Procedure. Black extrinsic stains (BS group) and standard supragingival plaque (DP groups) were removed, without bleeding, using a periodontal curette (metallic CK6) from two sites. Site 1: buccal faces of upper first or second deciduous molars, and site 2: buccal faces of maxillary incisors or canines. Both BS and DP were placed in a 0.5 mL Eppendorf (Hamburg, Germany) tube. At least 2 mg of DP and BS were obtained. All samples were immediately frozen at −20 °C and stored at −80 °C until use.

5.5. Sample Preparation for Mass Spectrometry Analysis. Chemicals in this step are provided by Sigma-Aldrich (Saint Louis, USA) (DTT, IAA, Urea, Tris, TFA), Biosolve (Dieuze, France) [formic acid (FA), acetonitrile, water], and Promega (Promega, Madison, USA) for trypsin.

Each pellet was digested in a 96-well plate to perform Reduction/Alkylation/Digestion/Clean-up on BRAVO Assay-Map (Agilent Technologies, Santa Clara, USA). 30 μL of denaturation mixture (8 M urea, 20 mM DTT, 100 mM Tris pH 8.5) was added and incubated 1 h at 37 °C with stirring (450 rpm). Alkylation was then performed with 6 μL of alkylation solution (400 mM iodoacetamide, 1 M Tris pH 11) at 37 °C for 30 min (stirred at 450 rpm). The sample was then diluted before digestion (+210 μL of 20 mM Tris pH 8.5 and 2 mM DTT). Trypsin enzyme was added to an overnight digestion at 37 °C with stirring at 450 rpm. 9 μL of a diluted solution at 0.05 μg/μL of trypsin in 1 M Tris pH 8.5 was added.

Digestion was stopped using 3 μL FA addition. Produced peptides were cleaned with C18 tips (Agilent Technologies, Santa Clara, USA). C18 tips were primed with 70% ACN/0.1% TFA, equilibrated with 0.1% TFA, and sample was loaded. Digested sample clean-up was performed including washes with 0.1% TFA followed by elution using 70% ACN/0.1% TFA. Clean peptides were dried on Speedvac (Labconco, Kansas, USA). Samples were resuspended with stirring with 10 μL of 2% acetonitrile/0.1% FA/97.9% water, for 10 min prior to LC−MS/MS injection.

5.6. Mass Spectrometry Analysis. 7 μL of samples were injected on nanoElute (Bruker Daltonics, Massachusetts, USA). NanoFlow LC was coupled to the Q-TOF MS instrument (Impact II, Bruker Daltonics, Massachusetts, USA) through the captive spray ion source (1200 V, dry gas: 3 L/min at 150 °C) operating with nanobooster (0.2 bar of nitrogen boiling in acetonitrile). In the LC part, samples were desalted and pre-concentrated on-line on a PepMap u-precolumn (300 μm × 5 mm, C18 PepMap 100, 5 μm, 100 Angström, Thermo Fisher, Massachusetts, USA). To perform separation, peptides were transferred to an analytical column (75 μm × 500 mm; Acclaim Pepmap RSLC, C18, 2 μm, 100 Angström, Thermo Fisher). A gradient consisting of 5−26% B for 192 min and 90% B for 10 min (A = 0.1% formic acid, 2% acetonitrile in water; B = 0.1% formic acid in acetonitrile) at 400 nL/min, 50 °C, was used to elute peptides from the reverse-phase column.

For peptide identification, data-dependent acquisition was performed with a lock-mass as the internal calibrator (m/z 1222, Hexakis "1*H*,1*H*,4*H*-hexafluorobutyloxy" phosphazine, Agilent Technologies, Santa Clara, USA). Using Instant Expertise software (Bruker Daltonics, Massachusetts, USA), the most intense ions per cycle of 3 s were selected and then active exclusion was used (after 1 spectrum for 2 min unless the precursor ion exhibited intensity higher of 3 times than the previous scan). All MS/MS spectra were extracted, deconvoluted with proteomics parameters (C, H, N, and O) in automatic mode (no charge state restriction) and exported to a .mgf file with deconvoluted peaks as single-charged ions by DataAnalysis software (Bruker Daltonics).

5.6.1. Peptide Identification. All MS/MS spectra were searched against the Nextprot database (2018-01-17) to identify proteins from Homo Sapiens and against the SwissProt database (2017-07-28) with "Bacteria" as Taxonomy by using the Mascot v 2.6.0 algorithm (Matrix Science, http://www.matrixscience.com/) with the following settings: (1) enzyme: trypsin, (2) variable modifications: oxidation (M) and deamidated (N,Q), (3) fixed modifications: carbamidomethyl (C), (4) missed cleavages: 2, (5) instrument type CID: ESI-QUAD-TOF, (6) peptide tolerance: 10.0 ppm, (7) MS/MS tolerance: 0.05 Da,

https://doi.org/10.1021/acsomega.1c04770
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(8) peptide charge: 1+, 2+ and 3+, (9) mass: monoisotopic, (10) C13: 1, (11) minimum peptide length: 5, (12) peptide decoy: ON, (13) adjust FDR [%]: 1, (14) percolator: on, (15) ions score cut-off: 12, and (16) ions score threshold for significant peptide IDs: 12.

6.7. Bioinformatic Analysis. QIAGEN Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA) was performed on proteomic data to identify proteins involved in pathways affected by BS. IPA is a commercial tool that is based on a proprietary database to facilitate the identification of biological themes in proteomics or gene expression data. The association between proteins in the data set and canonical pathways in the Ingenuity Pathways Knowledge Base was measured as a ratio of the number of molecules from the mapping data to a pathway divided by the total number of molecules that map to the canonical pathway.

6.7.1. MS/MS Data Interpretation for Proteotyping Microorganisms. Each MS/MS dataset was queried with the Mascot search engine (Matrix science) against a subset of NCBI-nr downloaded on the 3rd of January, 2018 as ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz, with one representative taxon per species (50,080,649 protein sequence entries from 9133 organisms including bacteria genera and *Homo sapiens* taxonomies). For each dataset, a cascade search was carried out: the theoretical proteomes from genera identified in the first stage were used as database for a second search round; the theoretical proteomes from species identified in the second stage were used as database for the third search round. Peptide-to-MS/MS spectrum assignation was done with the following parameters: full trypsin specificity, maximum of one missed cleavage, mass tolerances on the parent ion of 2 ppm for the first search round and 5 ppm for the second and third search rounds, 0.5 Da on the MS/MS, static modification of carboxyamidomethylated cysteine (+57.0215 Da), and oxidized methionine (+15.9949 Da) as dynamic modification. Mascot DAT files were parsed using the Python version of Matrix Science msparser version 2.5.1 with function MS_peptide summary (http://www.matrixscience.com/msparser.html) for selecting Peptide-Spectrum Matches (PSMs) with a Mascot expectation value below 0.3 for the first search round, 0.1 for the second search round, and 0.05 for the third search round using Mascot homology threshold (MHT), and allowing multiple PSMs per MS/MS spectrum. The raw number of PSMs per taxon (further called TSMs for Taxon-Spectrum Matches), the number of matching peptide sequences, the count of specific or unique peptide sequences and corresponding specific PSMs were calculated for the species, genus, family, order, class, phylum, and superkingdom "canonical" taxonomical levels.[12]

6.7.2. Taxonomical Comparative Analysis. For all the identified organisms, their abundances were evaluated by means of their TSMs extracted from the results of the first search stage at the genus level and normalized by the total number of bacterial TSMs per sample. A *t*-test was performed to compare results obtained for black stains "BS" and white dental plaque "DP"samples, with Benjamini-Hochberg multiple tests correction.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04770.

General BS patients' information selected for the comparative metaproteomics study and results of taxonomical comparative analysis (XLSX)

Venn-diagram showing the distribution of identified proteins between DP and BS and QIAGEN Ingenuity pathway analysis results (PDF)

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https://doi.org/10.1021/acsomega.1c04770
ACS Omega 2022, 7, 8258−8267

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Authors have given approval to the final version of the manuscript. C.H.: conceived the study and wrote the manuscript. A.M.: writing of the article and revising the text and figures. E.M.: background theory and clinical sampling and data. O.P.: phylopeptidomic data analysis. R.O.: reviewing and revising the text and figures. J.A.: phylopeptidomic data analysis. V.J.: phylopeptidomic data analysis. C.L.: reviewing and revising the text and figures. G.D.: background theory. A.Y.M.: statistical data analysis. C.D.-R.: statistical data analysis. L.T.: sample biobanking. J.-P.L.: statistical data analysis. P.T.: statistical clinical data analysis. M.-c.G.: scientific advisor. S.L.: scientific advisor. D.D.d.P.: writing of the article and reviewing and revising the text and figures. J.V.: writing of the article and reviewing and revising the text and figures.

Notes

The authors declare no competing financial interest.

Data Availability: The RAW global MS data and the identified protein groups from Mascot have been to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD029245.[40]

■ ACKNOWLEDGMENTS

This work was supported by the Montpellier hospital funding "Appel d'Offre Interne du CHU Montpellier", BlackStains AOI 2016.

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