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Dental plaque microbial profiles of children from Khartoum, Sudan, with congenital heart defects

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ABSTRACT

Few studies have focused on the bacterial species associated with the deterioration of the dental and gingival health of children with congenital heart defects (CHD). The aims of this study were (1) to examine the dental plaque of children with CHD in order to quantify bacterial load and altered bacterial composition compared with children without CHD; and (2) to investigate the correlation between the level of caries and gingivitis and dental biofilm bacteria among those children. In this cross-sectional study, participants were children (3–12 years) recruited in Khartoum State, Sudan. A total of 80 CHD cases from the Ahmed Gasim Cardiac Centre and 80 healthy controls from randomly selected schools and kindergartens were included. Participants underwent clinical oral examinations for caries (decayed, missing, and filled teeth indices [DMFT] for primary dentition, and DMFT for permanent dentition), and gingivitis (simplified gingival index [GI]). Pooled dental biofilm samples were obtained from four posterior teeth using paper points. Real-time quantitative polymerase chain reaction was used for the detection and quantification of *Streptococcus mutans*, *Streptococcus*sanguinis, and *Lactobacillus acidophilus*. Checkerboard DNA–DNA hybridization was used for the detection of 40 additional bacterial species. CHD cases had a significantly higher caries experience (DMFT = 4.1 vs. 2.3, $p < 0.05$; DMFT = 1.4 vs. 0.7, $p < 0.05$) and a higher mean number of examined teeth with gingivitis (4.2 vs. 2.0; $p < 0.05$) compared with controls. *S. mutans* counts were significantly higher among the CHD cases ($p < 0.05$). Checkerboard results revealed that 18/40 bacterial species exhibited significantly higher mean counts among CHD cases ($p < 0.01$). Correlation analyses revealed that among CHD cases, the detection levels of *Tannerella forsythia*, *Campylobacter rectus*, *Fusobacterium nucleatum* subsp. *vincentii*, *F. nucleatum* subsp. *nucleatum*, and *F. nucleatum* subsp. *polymorphum* were highly positively correlated with GI. CHD cases harbor more cariogenic and periodontopathogenic bacterial species in their dental plaque, which correlated with higher levels of caries and gingivitis.

The human mouth is colonized by a variety of microorganisms, including bacteria, fungi, and viruses. The oral cavity is the second most abundantly colonized system after the gut [1,2]. These microorganisms colonize all oral surfaces, including the tooth surface, forming consortia referred to as oral biofilms [2].

Recent metagenomic analyses have shown that the dental biofilm is comprised of diverse bacterial species that differ significantly between health and disease [3,4]. There is a growing body of evidence demonstrating that the shifts in the balance of these bacteria contribute to both local and systemic diseases [4–6]. The dental biofilm associated with oral health (non-pathogenic dental biofilm) is mainly composed of commensal bacterial species that maintain homeostasis and actively resist the colonization or overgrowth of commensal pathogenic species [7]. Dental biofilm microbial homeostasis is maintained by the host immune system, salivary flow, and healthy dietary intake [7,8]. Recent studies suggest that dental and periodontal diseases are caused by shifts in the relative composition of the commensal bacteria, leading to elevated levels of pathogenic bacteria within the plaque microbiota [9,10]. These changes are brought about by factors related to altered local host immune responses such as chronic inflammation, unfavorable host genetic predisposition, and the presence of other environmental alterations that favor overgrowth and shift of endogenous species [11]. These changes can lead to overgrowth of keystone pathogens, resulting in dysbiotic changes that will elevate the pathogenicity of the whole dental biofilm microbiota [9,10]. Underlying systemic conditions that affect the host tissue response, such as obesity and type 2 diabetes, play a role in promoting a shift in the composition and properties of the dental

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 Supplementary data for this article can be accessed here.

bacterial biofilm [12,13]. Congenital heart defects (CHD) are associated with impaired oral health status, and children with CHD have well-documented experiences of higher caries, more severe gingivitis, and increased plaque accumulation compared with children without CHD [14,15]. Additionally, these children develop serious complications related to oral infections [16].

The appearance of more severe disease in the CHD population seems to be due to more than poor oral hygiene [17–19]. For instance, these children often have nutritional difficulties during their first years of life that might interfere with normal growth [17] and which require frequent feeding and night meals [17]. Nonetheless, oral health monitoring is frequently ignored because of the child's medical problems [18]. Medication for heart disease also contains sucrose, which may contribute to the higher caries risk among those children [20]. In addition, the use of diuretics induces hypo-salivation, resulting in an increased risk of caries and other oral problems [18,19]. Saliva has many functions, including buffering and washing actions, and the saliva contains immunological components derived from salivary glands or the serum [21].

In order to address the treatment options for these patients better, there is a critical need for more complete information on the bacterial species associated with deterioration of dental and gingival health in children with CHD. Previous reports suggest that children with CHD harbor specific HACEK microbes (*Haemophilus parainfluenzae*, *Aggregatibacter aphrophilus*, *Aggregatobacter actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella*) to a greater extent than healthy children do [22,23]. HACEK microbes have also been shown to cause infective endocarditis in children with CHD, along with viridans streptococci and *Staphylococcus* ssp [24].

In children with CHD, *Streptococcus mutans* is positively correlated with the number of decayed teeth [25]. In addition, a marked reduction in the level of *S. mutans* is evident following preventive treatment in children with CHD [26]. However, other studies report no differences in the bacterial counts of *S. mutans* and *Lactobacillus* ssp. between children with CHD and healthy controls [27,28]. In all of these studies, candidate bacterial species were targeted for investigation. A thorough examination of the bacterial composition in the children with CHD remains to be accomplished. In an earlier study, Sudanese children with CHD were reported to have greater susceptibility (higher odd ratio values) to oral diseases (caries and gingivitis) compared with controls, even after controlling for confounding factors [29]. These findings prompted the authors to conduct further investigations into bacterial profiles of dental

plaque in these children. This study therefore reports the oral bacterial profiles of children with CHD compared to healthy controls. In addition, the presence of cariogenic bacteria (*S. mutans* and *Lactobacillus acidophilus*) is targeted, as well as bacterial species associated with gingival disease and bacterial infective endocarditis.

The aims of this study were (1) to examine dental biofilm samples from children with CHD (CHD cases) compared to children without CHD (controls), and (2) to investigate the correlation between the level of caries and gingivitis with the dental biofilm bacterial profiles/counts.

Methodology

This was a cross-sectional study. Samples were collected as part of a previous epidemiological study conducted to evaluate oral clinical parameters and oral health–related background factors of CHD cases compared to healthy controls [29,30]. Information about the inclusion and exclusion criteria and demographic characteristics have been reported previously [29,30].

Study subjects

The study comprised 80 cases (CHD) and 80 controls. CHD cases were recruited from the Ahmed Gasim Cardiac Centre located in Khartoum north. The center receives patients from three districts (Khartoum City, Khartoum North, and Omdurman) of Khartoum State, as well as patients referred from outside Khartoum. Only patients from Khartoum State were included in the present study, since all controls were locally recruited from schools and kindergartens in the same three districts of Khartoum State. Children in both groups (CHD cases and healthy controls) were matched (frequency matching, since the exact numbers of CHD cases and controls were not available) for age, sex, and the use of antibiotics. CHD cases and controls were subdivided into two age groups of 40 subjects per group: age group 1 (3–7 years old, with primary teeth) and age group 2 (8–12 years old, with permanent teeth). CHD cases were further divided into cyanotic and acyanotic CHD cases. Demographics are presented in Table 1.

The extent of caries was determined using the World Health Organization criteria and scoring system. Caries was registered as lesions in the pits and fissures, on a smooth tooth surface with an unmistakable cavity, undermined enamel, or detectable softened floor or wall, destroyed crown, and temporary fillings and permanent fillings with secondary caries [31]. Decayed, missing, and filled teeth (DMFT) for primary teeth and DMFT for permanent teeth indexes were recorded. Gingivitis was measured

	CHD cases (*n* = 80)	Controls (*n* = 80)	*p*-Value	Cyanotic (*n* = 37)	Acyanotic (*n* = 40)	*p*-Value
Age	7.6 ± 2.9	7.5 ± 3.0	0.769	7.8 ± 2.8	7.5 ± 2.8	0.661
Sex (males), *n* (%)	40 (50.0%)	38 (47.5%)	0.752	18 (48.6%)	17 (42.5%)	0.651
Antibiotic (+ve)	39 (48.6%)	36 (45.6%)	0.749	17 (45.9%)	21 (52.5%)	0.642

Differences in age between cohorts were determined by Student's *t*-test at a 0.05 level of significance. Sex and antibiotics were compared with a chi-square test with a 0.05 level of significance.

using a simplified form of the gingival index (GI) [32] where six teeth were examined (55/16, 51/11, 65/26, 75/36, 71/31, and 85/46). Visual signs of gingival inflammation and a tendency to spontaneous bleeding was scored as 1, while the absence of these signs was scored as 0. Information regarding the frequency of teeth brushing and the use of fluoride toothpaste among children was obtained from the caregivers in both groups. The responses were dichotomized as frequent (0) for 'several times a day' or 'daily', and not frequent (1) when 'seldom or never' was reported.

Dental plaque sample collection

Dental biofilm samples were collected from the mesio-buccal site of the four posterior first molars or, if not erupted, from the primary molars. Quadrants were isolated with a cotton roll and plaque was sampled using paper points (size 40–60) scraped against the tooth, including the gingival crevices. The collected plaque samples were kept in sterile tubes and stored at −80°C. For the bacterial analysis, samples from the four sites in each participant were pooled.

DNA extraction and purification

MasterPureTM DNA Purification kits (EPICENTRE Biotechnologies, Madison, WI, USA) were used. The procedure is described in detail by Yang et al. [33]. Dental biofilm samples were suspended in 150 μL of TE buffer, and 1 μL of the ready lyse-lysozyme was added. Samples were incubated overnight at 37°C. The next day, 150 μL of 2 × T&C lysis solution was added, together with 1 μL of proteinase K to each sample, and it was incubated at 65°C for 30 min. Samples were cooled at 37°C, placed on ice for 3–5 min, and 175 μL of the protein precipitation reagent were added to each sample. Samples were centrifuged (14–18 × 1000 rcf) for 10 min, and the supernatants were transferred to clean microtubes. Isopropanol (500 μL) was added, and the samples were centrifuged again for 10 min. Isopropanol was removed, and the formed pellets were rinsed with 500 μL of 75% ethanol without dislodging the pellets. The residual ethanol was removed, and the samples containing the purified DNA were re-suspended in 25 μL of TE buffer. DNA quantity and quality in the samples were measured using a NanoDrop UV-Vis spectrophotometer at 260 nm and 280 nm.

Bacterial DNA detection

Bacterial DNA was detected by real-time quantitative polymerase chain reaction (RT-qPCR). PCR kits (genesig PrimerDesignTM Ltd, Millbrook Technology Campus, Southampton, UK) were obtained for detection and quantification of *S. mutans*, *Streptococcus sanguinis*, and *L. acidophilus*. The highly specific glucosyltransferase-1 gene primer for *S. mutans* was GTFB-R 5′: CAG TAT AAG CGC CAG TTT CAT C [34]. The primers and probe have 100% homology, with all reference sequences for *S. mutans* in the NCBI database (AE014133.1, M17361.1, D88654.1, D88651.1, D89977.1, D88660.1, and D88657.1). The target gene for *S. sanguinis* was the highly conserved sequence within the GTFP gene, forward primer sequence: CAA AAT TGT TGC AAA TCC AAA GG; reverse primer sequence: GCT ATC GCT CCC TGT CTT TGA; and probe sequence: AAA GAA AGA TCG CTT GCC AGA ACC GG [35]. The target gene for the detection of *L. acidophilus* was the conserved Recombinase A (recA) gene 5′-AAC TAT CGC TTA CGC TAC CAC TTT GC-3′ [36].

PCR amplification and quantification were performed in optical 96-well plates. The PCR reaction mix was prepared according to the protocol provided by the manufacturer after standardization to the total amount of DNA in each sample. Amplification and detection were performed using Step-One-PLUS, (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Bacterial species detection using checkerboard DNA–DNA hybridization

DNA extracted from the dental biofilm samples was hybridized to DNA probes of 40 bacterial species (Figure S1) using the DNA–DNA hybridization (Checkerboard) technique, following the procedures described by Socransky et al. [37]. DNA extracted from known numbers of bacteria was used as created standard curves for estimated counts, as described by Socransky et al. [37].

The bacterial taxa were arranged in five complexes (red, orange, yellow, green, and purple) for

descriptive purposes; *Actinomyces* spp. were noted separately [38].

Ethical approvals

Ethical approvals were obtained from Ahmed Gasim Hospital, Federal Ministry of Health Sudan, Research Ethical Committee at the University of Science and Technology, from the Regional Committee for Medical Research Ethics Western Norway (No. 2265), Bio bank (No. 2355), and the State Ministry of Education (Khartoum), the State Ministry of Primary and Pre-school Education in the three districts of Khartoum state: Khartoum city, Khartoum North, and Omdurman. A native language consent form was filled in and signed by all participants' parents or guardians upon agreement to participate (both CHD cases and healthy controls).

Statistical analysis

Data were analyzed using the IBM SPSS Statistics for Windows v22 (IBM Corp., Armonk, NY). Descriptive statistics (means ± standard deviations) are presented. The counts of *S. mutans* and *S. sanguinis* were substantially skewed. Therefore, log transformation of the data was used for normalization of the data and stabilization of variance. General linear models were used to compare the adjusted log-transformed means for both *S. mutans* and *S. sanguinis* controlling for age, sex, antibiotic use, brushing frequency, and use of fluoride. The Mann–Whitney *U*-test was used for the comparisons of the bacterial counts in the Checkerboard assay, with *p*-values adjusted for multiple comparisons. A chi-square test was used to compare differences in proportions between groups. The correlation between *S. mutans*, 16 species of the red and orange complexes, and the level of caries and gingivitis was analyzed with Spearman's rho test, with *p*-values adjusted for multiple comparisons.

Results

Demographics and clinical findings

Participants' characteristics are presented in Table 1. The CHD cases had a mean age of 7.6 ± 2.9 years compared to a mean age of 7.5 ± 3.0 years in the controls. CHD cases had a significantly higher caries experience in both primary (DMFT = 4.1 ± 3.9 in the CHD cases vs. 2.3 ± 2.9 in controls) and permanent (DMFT = 1.4 ± 1.8 in the CHD cases vs. 0.7 ± 1.1 in controls) dentitions. The incidence of gingivitis, quantified as the mean number of examined teeth with gingivitis (GI = 4.2 ± 2.3 vs. 2.0 ± 1.9; $p < 0.05$) was higher in CHD children compared with controls. Most of the children had never visited a dentist. Consequently, there were no fillings or sealants among CHD cases and few among controls. The differences in brushing and fluoride toothpaste use frequencies were not statistically significant between groups ($p = 0.310$ and $p = 0.250$, respectively).

Microbiological profiles

Quantified as present or absent, *S. mutans* was detected in 87.5% of the CHD cases and 88.7% of the controls by q-PCR. The number of *S. mutans* bacterial counts was significantly higher in age group 2 among the CHD cases compared with controls ($p < 0.05$; Table 2). Among the CHD cases, the levels of *S. mutans* tended to be higher in the age group 1 compared with age group 2 (Figure 1). *S. sanguinis* was detected in 97.5% of both CHD cases and controls, and there were no significant differences in the counts of *S. sanguinis* between the CHD cases and controls in either age group (Table 2). Cyanotic CHD cases in age group 1, however, had a significantly higher number of *S. sanguinis* counts compared with acyanotic CHD cases ($p < 0.05$; Table 2). *L. acidophilus* was only detected in one CHD case and one control (data not shown).

The presence of 40 bacterial taxa in the dental plaque samples was determined using DNA–DNA hybridization (Checkerboard). Bacterial species of five complexes (red, orange, yellow, green, and purple) as well as the *Actinomyces* spp. were detected in both CHD cases and controls. Figure S1 presents the mean counts of the 40 bacterial taxa in the plaque samples from the 80 CHD cases and the 80 controls. The results reveal that 18 bacterial species were more frequently detected, with significantly higher mean counts in CHD cases compared with controls, including *Porphyromonas gingivalis* of the red complex and seven species of the orange complex ($p < 0.01$; Figure S1). In age group 1, only six species had higher mean counts in the CHD cases compared with controls (Figure 2), while in age group 2, 18 bacterial species were significantly higher in CHD cases than they were controls, mainly all species of the red complex (Figure 2). Further, within the CHD cases, the bacterial counts of four species (*Campylobacter rectus*, *Fusobacterium nucleatum* subsp. *vincentii*, *Prevotella intermedia*, and *Parvimonas micra*) were significantly higher in age group 2 compared with age group 1, while two species (*Capnocytophaga sputigena* and *E. corrodens*) were significantly higher in age group 1 compared with age group 2 (Figure S2). No differences were recorded in any of the five complexes between cyanotic and acyanotic CHD cases.
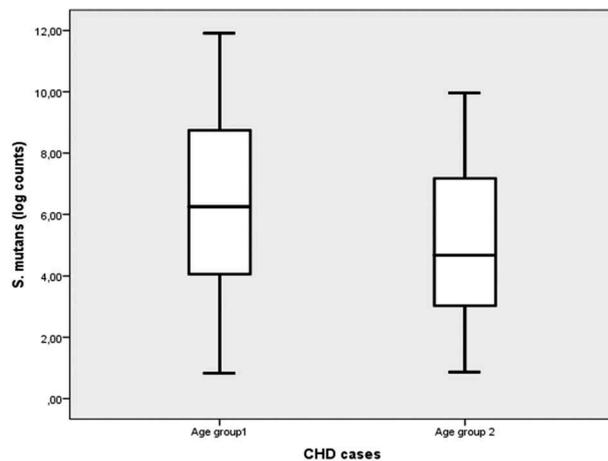
Table 2. *Streptococcus mutans* and *Streptococcus sanguinis* counts in plaque samples of CHD cases and controls.

	S. mutans		S. sanguinis	
	Adjusted mean log counts/µL±SE	p-Value	Adjusted mean Log counts/µL± SE	p-Value
Total (3–12 years)				
CHD cases	5.6 ± 0.4	0.019*	7.6 ± 0.2	0.227
Controls	4.5 ± 0.3		7.9 ± 0.2	
Age group 1 (3–7 years)				
CHD cases	6.3 ± 0.6	0.298	8.1 ± 0.2	0.731
Controls	5.4 ± 0.5		8.1 ± 0.2	
Age group 2 (8–12 years)				
CHD cases	5.0 ± 0.4	0.028*	7.2 ± 0.3	0.151
Controls	3.7 ± 0.4		7.8 ± 0.3	
Total (3–12 years)				
Cyanotic CHD	6.2 ± 0.5	0.116	7.6 ± 0.3	0.869
Acyanotic CHD	5.0 ± 0.5		7.6 ± 0.3	
Age group 1 (3–7 years)				
Cyanotic CHD	6.6 ± 0.9	0.649	8.4 ± 0.3	0.034*
Acyanotic CHD	5.9 ± 0.8		7.5 ± 0.3	
Age group 2 (8–12 years)				
Cyanotic CHD	5.9 ± 0.6	0.067	7.5 ± 0.4	0.396
Acyanotic CHD	4.2 ± 0.7		6.9 ± 0.4	

The adjusted log transformed means of *S. mutans* and *S. sanguinis* counts ± standard error (SE). Comparisons of the bacterial counts made using a general linear model controlling for age, sex, antibiotic use, brushing frequency, and use of fluoride toothpastes.
*$p < 0.05$.
CHD, congenital heart defects.

Figure 1. Presence of *Streptococcus mutans* in age group 1 and age group 2 of cases with congenital heart defects (CHD). The boxplot shows the differences in the distributions of *S. mutans* log-transformed counts between age group 1 and age group 2 CHD cases with higher counts in age group 1. Boxes represent the interquartile range, the median, and the range.

The correlation analyses showed a trend for positive correlation between the *S. mutans* counts and the number of decayed teeth CHD cases (correlation coefficient = 0.26, $p = 0.05$) and among the control (correlation coefficient = 0.13, $p = 0.29$). The level of the red complex species *Tannerella forsythia* was positively correlated with high GI only among the CHD cases (Table 3). Most bacterial species of the orange complex were significantly positively correlated with higher GI among both CHD cases and controls (Table 3). Nevertheless, *C. rectus*, *F. nucleatum* subsp. *vincentii*, *F. nucleatum* subsp. *nucleatum*, and *F. nucleatum* subsp. *polymorphum* were highly positively correlated with GI among CHD cases only (Table 3).

Discussion

The data reveal that caries and gingivitis were both significantly more prevalent in the CHD cases compared with controls with the same age, sex, and history of antibiotic use. The bacterial composition of plaque samples from CHD cases showed higher counts of several bacterial species, including some with high carcinogenicity and periopathogenicity, with significant positive correlations with the level of gingivitis in both CHD cases and controls. The same bacteria (e.g. *S. mutans* and *P. gingivalis)* are also known to cause bacterial infective endocarditis and other systemic complications among children with CHD [16].

The RT-qPCR results revealed that *S. mutans* was detected with significantly higher counts in CHD cases compared with controls, particularly in age group 2 (with permanent teeth). *S. mutans* is strongly implicated as the main species responsible for the initiation and progression of dental caries, together with *Lactobacillus* species [39–41]. These findings are in accordance with the results of several previous studies of CHD cases [25,26,42]. Nevertheless, other studies have revealed no profound differences in the plaque levels of *S. mutans* between CHD and non-CHD children [27,28,43]. Notably, when *S. mutans* was detected [25–28,42,43], it was detected in saliva samples and/or in dental biofilm samples. In the current study, only dental biofilm samples were used for the detection of *S. mutans*, and the analyses were performed using a more sensitive and specific method for bacterial DNA detection (RT-qPCR) than in previous studies. In CHD cases, higher caries and higher *S. mutans* counts in age group 1 compared with age group 2 suggested that plaque bacterial composition and abundance on primary teeth was
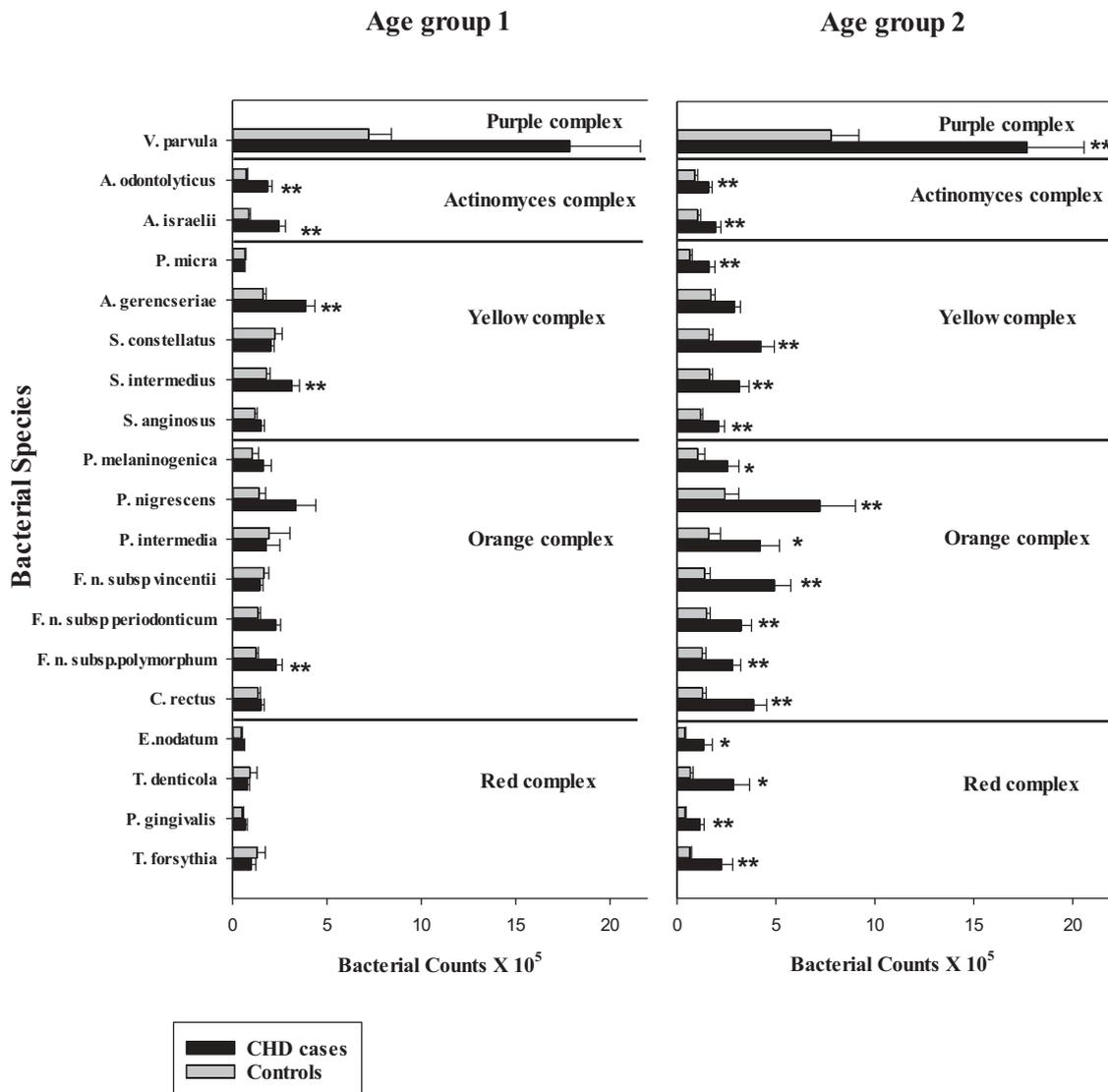
Figure 2. Counts of bacterial species measured in dental biofilm samples from CHD cases and controls in age subgroups (age group 1 and age group 2). Comparisons of mean bacterial counts in dental biofilm samples from age group 1 (3–7 years) of the CHD cases (N = 40) and controls (N = 40) and age group 2 (8–12 years) of the CHD cases (N = 40) and controls (N = 40). The bars represent the mean (±SEM) values. The y-axis shows the names of the bacterial species, and the x-axis shows the count × 10^5. The Mann–Whitney U-test with p-values adjusted for multiple comparisons was used to determine the significance of the bacterial count differences between CHD cases compared with controls (*$p < 0.01$ and **$p < 0.001$). Bacteria grouped using the complexes described by Haffaje and Socransky [38].
<<NB. P. micros should read P. micra>>
<<NB. P. Melaninogenica should read P. melaninogenica>>

rather different from plaque from permanent teeth, which is also in agreement with previous studies [41,44]. Notably, the presence of elevated levels of caries in the primary dentition suggests an increased likelihood of developing future caries in the permanent dentition, as previously documented [45–47].

A study by Hahn et al. showed that *S. mutans* plays a role in the development of bacterial endocarditis [48]. This is thought to be related to its ability to enter and survive in the blood. *S. mutans* has also been shown to differentiate monocytes to dendritic cells, which can adhere to the injured endothelium

and fibrinogen in blood clots, resulting in the initiation of bacterial endocarditis [48]. The role of *S. sanguinis* is also well-known as a cause of infective endocarditis through the oral route, together with the other viridans streptococci [24,49].

The low detection of *Lactobacillus* was interesting considering the level of caries in this population. This is consistent with the fact that these species are late colonizers, usually present in mature caries associated bacterial communities, and are mostly detected in samples from deep and advanced carious lesion [50,51]. In the present study, the dental biofilm was

Table 3. Correlations between bacterial species counts (red and orange complexes) and gingivitis among CHD cases and controls.

Bacterial species	CHD cases		Controls	
	Correlation co-efficient	p-Value	Correlation co-efficient	p-Value
Red complex				
Tannerella forsythia	0.680	0.001**	0.229	0.041
Porphyromonas gingivalis	0.150	0.183	0.170	0.132
Treponema denticola	0.260	0.020	0.224	0.046
Eubacterium nodatum	0.242	0.030	0.209	0.063
Orange complex				
Campylobacter showae	0.310	0.005	0.311	0.005
Campylobacter rectus	0.367	0.001**	0.149	0.018
Fusobacterium nucleatum subsp. *polymorphum*	0.365	0.001**	0.265	0.018
Fusobacterium nucleatum subsp. *periodonticum*	0.331	0.003	0.245	0.028
Fusobacterium nucleatum subsp. *vincentii*	0.348	0.002**	0.185	0.101
Fusobacterium nucleatum subsp. *nucleatum*	0.373	0.001**	0.213	0.058
Centruroides gracilis	0.314	0.005	0.349	0.002**
Prevotella intermedia	0.222	0.048	0.378	0.001**
Prevotella nigrescens	0.293	0.008	0.401	0.001**
Capnocytophaga ochracea	0.347	0.002**	0.341	0.002**
Selenomonas noxia	0.286	0.010	0.439	0.001**
Prevotella melaninogenica	0.295	0.008	0.217	0.053

Correlations between the bacterial counts (species of the orange and red complexes) and the gingival index GI (number of sites with gingivitis). Spearman's rho non-parametric correlation test was used, and the correlation co-efficients are presented in the table with the significance level (Bonferroni's adjusted p value for multiple comparisons; **$p < 0.003$).

not sampled from carious lesions, and the low detection levels can be explained by the plaque sampling method, as reported by Gross et al. [52].

Previous reports on periodontal bacteria among CHD cases are few. Two studies have shown the higher susceptibility in CHD cases to plaque colonization by HACEK bacteria [22,23]. However, direct comparison of results is not possible, since different species were studied.

The bacterial species in the present study were categorized in complexes according to Haffajee et al. [38] for ease of discussion. *P. gingivalis* (red complex) was detected in significantly higher counts among CHD cases compared with controls. *P. gingivalis* is the most widely studied periodontal organism because of its potential virulence factors and presence in >70% of periodontal disease cases [53]. *P. gingivalis* has been shown *in vitro* to escape the immune response of the host and to survive within the gingival epithelial cells [53]. The keystone pathogen hypothesis for periodontal diseases posits that *P. gingivalis* causes an imbalance in the growth of the entire biofilm community and creates plaque dysbiosis that is associated with progressing disease [54]. *P. gingivalis* also has the ability to degrade the intercellular matrix through the production of gingipains, trypsin-like proteases [54]. It has been suggested that these factors are a mechanism for *P. gingivalis* to reach the bloodstream, resulting in systemic bacteremia, a potentially life-threatening condition in CHD cases [54].

Bacterial species of the orange complex exhibited higher counts among the CHD cases, especially in age group 2. According to Socransky et al., increases in bacteria of the orange complex are elevated in sites with gingival redness and bleeding [38]. CHD cases had higher level of gingivitis that correlate with high counts of several orange complex species. Members of the yellow complex, including *Streptococcus anginosus*, *Streptococcus intermedius*, and *Streptococcus constellatus*, were particularly high among CHD cases. These bacteria are known to possess several virulence factors that facilitate their entry and survival in the blood [16]. Members of the *Actinomyces* complex were significantly higher among CHD cases, which may be an indication of poor oral hygiene practices and the accumulation of undisrupted dental biofilm [38].

While these results might be explained by poorer oral hygiene practice in the CHD group, one has to take into consideration that both the CHD and control groups came from similar backgrounds and lower socioeconomic status, and oral hygiene was not good in either group. Other possibilities attribute these local changes to the direct influence of CHD, with significantly lower salivary concentrations of nitric oxide [55], lower serum level of several immunological constituents [56,57], as well as the structural abnormalities of teeth among those children [58].

Collection of dental biofilm using paper points was chosen for this study, since it was less threatening to small children than a metal instrument. This method does not appear to add variability to the present findings, since a previous report comparing samples collected using curettes and paper points showed no detectable differences in results [59]. Although site-specific plaque samples derive more information and may be superior to pooled samples for the detection of *S. mutans*, there are practical considerations in a large field study such as this. Pooled sample analysis

was previously shown to be cost- and time-efficient when considering large numbers of individuals [60,61]. Likewise, the DNA–DNA hybridization technique has drawbacks compared with 16SrDNA next-generation sequencing, since the technique has the risk of cross-reactivity and has lower sensitivity to detect low numbers of bacteria (ranging from 10^3 to 10^4 cells) [62]. However, for a study with relatively large sample size, the technique has the advantages of being relatively easy to handle and has a low cost [63,64], and the cross-reactivity that might occur between the closely related species (e.g. *F. nucleatum* subsp. *nucleatum/periodonticum*, *P. intermedia/Prevotellanigrescens*, and some streptococcus species) had been eliminated, as described by Socransky et al. [37].

Diet and food frequency information, although a potential variable, were not obtained in this study. The constraints of the field study setting, where the visit with children and caregivers included an interview and clinical examination, and sampling was limited to half an hour, precluded obtaining additional information.

In conclusion, CHD cases harbored higher counts of cariogenic and periodontopathogenic bacterial species in both primary and permanent dentitions. These increases correlated positively with caries and gingivitis. Thus, the data point to the critical need to inform caregivers of the increased susceptibility of CHD patients to oral disease and the need for increased attention to oral care in this vulnerable population.

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Disclosure statement

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