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ORIGINAL RESEARCH

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Assessment of oral bacteria potentially associated with the mobile microbiome in children with congenital heart disease

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Abstract

In this case-control study, we aimed to investigate the specific oral pathogens potentially associated with the mobile microbiome in children with congenital heart disease (CHD). Caries, oral hygiene and gingival indices were evaluated in 20 children with CHD and a healthy control group, and venous blood samples and saliva were collected. Using quantitative polymerase chain reaction (qPCR), blood samples were analyzed for the presence of bacterial DNA to determine the mobile microbiome, and saliva samples were analyzed to identify and quantify target microorganisms, including *Streptococcus mutans* (*Sm*) and its serotype *k* (*Smk*), *Fusobacterium. nucleatum* (*Fn*), *Porphyromonas gingivalis* (*Pg*), *Scardovia wiggsiae* (*Sw*) and *Aggregitibacter actinomycetemcomitans* (*Aa*) and its JP2 clone (*JP2*). The findings were analyzed by Mann Whitney U, chi-square, Fisher's exact and Spearman's Correlation tests. Bacterial DNA was identified in two blood samples. No significant differences were found between the groups regarding the presence and counts of bacteria in saliva. However, the CHD group exhibited significantly lower caries and higher gingival index scores than the control group. The presence of *Pg* and *Aa* were significantly associated with higher gingival index scores. *Sm* and *Smk* counts were significantly correlated with caries experience. A positive correlation was found between *Fn* and total bacteria counts. In conclusion, the mobile microbiome, which has been proposed as a potential marker of dysbiosis at distant sites, was very rare in our pediatric population. The counts of target microorganisms which are potentially associated with the mobile microbiome did not differ in children with CHD and healthy children.

Keywords

Mobile microbiome; Oral microbiome; Congenital heart disease; qPCR

1. Introduction

The concept of the human microbiome was first proposed by Joshua Lederberg, a Nobel Laureate geneticist, as "signifying the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" [1]. The oral cavity harbors one of the most diverse microbial populations in the human body, with 26% of oral bacteria unidentifiable with traditional culture-dependent techniques [2]. However, technological advancements owing to the development of molecular biology techniques has led to the identification of diverse unculturable microbial species. In recent years, the utilization of techniques that detect specific bacterial DNA sequences has become highly valuable in the fields of basic and translational dentistry. These methods have gained prominence due to their ability to identify specific oral pathogens accurately and rapidly, owing to their specificity and sensitivity. The quantitative polymerase chain reaction (qPCR) method has been successfully employed over several years as a robust and reliable approach in oral microbiology for the detection and quantification of specific oral pathogens [3].

Despite the extensive increase in microbial and biochemical information on the oral microbiota in recent years, the translation of these data to clinical practice, including as diagnostic or prognostic biomarkers for oral and systemic diseases, has remained very limited [4]. Therefore, there is still a need for further studies to be able to establish a substantial causal relationship between the oral microbiome and oral/general health.

The term "mobile microbiome" defines the oral bacteria in extra-oral infections and inflammations *via* blood circulation [5]. Although microbial colonization of specific body sites, such as the oral cavity, gut, vagina and lung, has been well-described so far, a relatively new concept supporting the presence of microbial populations in "healthy" human blood has gained interest by researchers in recent years [6–8].

Microorganisms in a healthy blood microbiota may originate from those of various body sites. Therefore, it has been suggested that the microbiome of the relevant body sites (*e.g.*, oral cavity, gut or vagina) and the blood microbiome should be tested in parallel to elucidate the potential origin of the microorganisms [8].

Several researchers have documented the role of oral bacteria in various systemic conditions, including cardiovascular diseases, obesity, inflammatory diseases, cancer, diabetes, and neurologic diseases [9–11]. Congenital heart diseases (CHDs) are the most common types of birth defects. A recent study using the Global Burden of Disease 2017 database based on the findings of 195 countries and territories reported that the prevalence of CHD was approximately 18 cases per 1000 live births [12]. Children with CHD have an increased risk of infective endocarditis (IE). Bacteremia can be oral-health-related and may develop not only in dental procedures but also in routine oral activities, such as toothbrushing, dental flossing and chewing, if the patient has a poor oral hygiene [13]. Several studies have shown that oral bacteria such as *Streptococcus spp.*, *Veillonella spp*, *Fusobacterium* and *Prevotella* have increased in patients with CHD [14].

Aggregatibacter actinomycetemcomitans (*Aa*), *Fusobacterium nucleatum* (*Fn*), *Porphyromonas gingivalis* (*Pg*) and *Streptococcus mutans* (*Sm*) are well-known oral pathogens, which have been frequently associated with the mobile microbiome in cardiovascular diseases [5, 15, 16]. *S. mutans serotype k* (*Smk*) has been reported to have a high detection rate in patients with bacteremia and infective endocarditis. This serotype has been found to persist in the bloodstream for extended periods, suggesting their ability to circulate throughout the entire body more easily than other serotypes [17]. Moreover, a highly leukotoxic clone of *A. actinomycetemcomitans*, termed the *JP2* clone, has been suggested to be associated with the initiation of periodontal attachment loss and the development of aggressive periodontitis in adolescent patients [18]. Furthermore, *Scardovia wiggsiae* (*Sw*) has been recently identified in caries lesions of children and adolescents [19, 20]. It is crucial to elucidate the specific role of *S. wiggsiae*, as it has been observed to induce caries even in the absence of *S. mutans* [20].

In the present study, we aimed to analyze the specific oral pathogens potentially associated with the mobile microbiome in children with CHD, using qPCR method and compare them with those in healthy individuals.

2. Materials and methods

2.1 Study population

Twenty children aged 5 to 15 years with CHD, recruited from Department of Pediatric Cardiology at Istanbul University Faculty of Medicine, were enrolled in the study. Patients with various severities of CHDs, ranging from mild to complex could participate if they had no additional chronic diseases or syndromes. The control group comprised 20 age-matched healthy children without any systemic disease who were recruited from the Department of Pediatrics Outpatient Clinics. The exclusion criterion was recent antibiotic treatment during the last 3 months. Children exhibiting noncompliance during clinical examination and sample collection were also excluded from the study. The sample size was calculated at an 80% confidence level using the G*Power program (v3.1.9.6, Heinrich-Heine-Universität, Düsseldorf, Germany). According to the analysis result, with a standardized effect size of 0.75 obtained from the previous study [21], the minimum sample size was calculated as 18 per group. We have included 20 patients per group.

2.2 Clinical examination and microbiological sampling

A pediatric dentist (SDA) performed all clinical examinations to assess the caries experience, oral hygiene and gingival health status of the children. The subjects were positioned in a supine position on a dental chair during the examinations.

Caries experience was evaluated using decay-missing-filled teeth/decay-filled teeth (DMFT/dft) indices based on the standards stated in the World Health Organization (WHO) Oral Health Surveys manual [22]. The Simplified Oral Hygiene Index (OHI-S) described by Green and Vermillion [23] was used to determine the oral hygiene status. The OHI-S consists of two components: the simplified debris index (DI-S) and the simplified calculus index (CI-S), which are calculated separately and then combined to obtain the OHI-S score for each subject. The gingival health status was assessed using the Modified Gingival Index (MGI), based on that described by Lobene *et al.* [24].

Unstimulated saliva (5 mL) was collected from each subject. All of the participants were instructed to avoid eating, drinking or brushing teeth for at least 1.5 h prior to sampling. Unstimulated saliva samples were collected based on a protocol used by a previous study [25]. Subjects were instructed to swallow the saliva present in their mouth and keep the mouth slightly open, thereby allowing saliva to accumulate, and subsequently drain into a sterile test tube. Saliva samples were then transferred to 1.5-mL Eppendorf tubes *via* sterile micropipette and stored at −20 °C until further analysis.

Peripheral blood samples of all subjects were collected aseptically by venipuncture into EDTA-containing 2-mL tubes. Blood processing was carried out in accordance with a previous study by Dinakaran *et al.* [21]. The samples were centrifuged for 10 minutes at 6000 g to separate the plasma from the buffy coat layer. The plasma was carefully transferred to a 1.5-mL sterile polypropylene tube, and the separated aliquot was subjected to further centrifugation for 10 minutes at 16.000 g. The top part of the plasma was then transferred to a clear sterile tube and stored at a temperature of −20 °C until DNA isolation.

2.3 Molecular microbiological analysis of saliva and blood samples

DNA was isolated from saliva samples using the Saliva DNA Isolation Kit (RU45400, Norgen Biotek Corp., Ontario, Canada), and from blood samples using the QIAamp® DNA Blood Mini Kit (cat# 51104, QUIGEN, Hilden, NRW, Germany), according to the manufacturers' instructions. The DNA concentration was calculated by measuring the

absorbance of 1 μL of each sample at 260/280 nm using the NanoDropTM 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Blood samples from patients with CHD and control subjects were analyzed by qPCR to identify the presence of free microbial DNA and establish the total microbial counts to determine the mobile microbiome. Saliva samples from patients with CHD and control subjects were analyzed by qPCR to establish the total bacterial count, and identify and quantify target microorganisms, including *S. mutans* (*Sm*), *S. mutans* serotype *k* (*Smk*), *A. actinomycetemcomitans* (*Aa*), *A. actinomycetemcomitans* JP2 clone (*Aa* JP2), *P. gingivalis* (*Pg*), *F. nucleatum* (*Fn*) and *S. wiggsiae* (*Sw*). Total bacterial count in blood and saliva samples was determined by using universal bacterial primer. All primers used in this study are listed in Table 1. The reaction mixture (25 μL) was composed of 12.5 μL of GoTaq® qPCR MasterMix (2×); 1.25 μL each of 2.5 μM of primer set containing forward and reverse primers; dH2O (5 μL for blood samples, 8 μL for saliva samples) and template DNA (5 μL of blood and 2 μL of saliva). Amplification was carried out in LightCycler® 480 Instrument II (Roche Diagnostics GmbH, Mannheim, BW, Germany). The cycling conditions for qPCR included one cycle at 95 °C for 2 min, 40 cycles at 95 °C for 15 s, 60 °C for 60 s and dissociation curves analysis at 60–95 °C. Detection and quantification were performed for each target microorganism by qPCR analysis. DNA levels in each sample were calculated according to the external standard curves performed on 10-fold dilution series of type-specific amplicons. Data were analyzed with the LightCycler 480 analysis software (v1.2.9.11, Roche Diagnostics GmbH, Mannheim, BW, Germany) and evaluated after log10 transformation.

The sensitivity of the qPCR methods was evaluated using the following reference strains: *S. mutans* ATCC 25175, *A. actinomycetemcomitans* ATCC 29523, *A. actinomycetemcomitans* JP2 clone ATCC 700685, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 25586 (all obtained from the American Type Culture Collection, Rockville, MD), *S. wiggsiae* DSM 22547 (obtained from the Leibniz Institut DSMZ—German Collection of Microorganisms and Cell Cultures), and *S. mutans* serotype *k* LJ23 (obtained from Dr. K. Nakano, Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, Japan). These freeze-dried forms of bacteria were also extracted to serve as positive controls for the species-specific primers or to evaluate the specificity of the primer while sterile distilled water was used as a negative control for each reaction series.

2.4 Statistical analysis

Number Cruncher Statistical System (NCSS) 2007 (NCSS, LLC, Kaysville, Utah, USA) software was used to analyze the data. Normality of data was analyzed with Shapiro-Wilk test. The Mann-Whitney U test was used to compare descriptive statistical methods (mean, standard deviation, median, frequency, ratio, minimum, maximum), as well as the two groups of non-normal distributions in the comparison of quantitative data. Spearman's correlation analysis was applied to evaluate intervariable relationships. Pearson's chi-square and Fisher's exact tests were used to compare qualitative data. The level of significance was set at $p < 0.05$.

3. Results

3.1 Demographic and clinical characteristics of the study population

The CHD group comprised 13 males and 7 females, and the control group comprised 7 males and 13 females. The age of the participants in both groups ranged from 6.5 to 12.7 years. The mean age of the children in the study and control groups was 9.90 (±1.66) years and 10.02 (±1.55) years, respectively, with no significant difference between the two groups.

The distribution of the DMFS/DMFT, dft/dfs, OHI-S and MGI values are given in Table 2. The DMFT and DMFS scores in the CHD group were significantly lower than those in the control group ($p = 0.036$ and $p = 0.045$; $p < 0.05$), while there were no statistically significant differences in dft, dfs, DMFT + dft and DMFS + dfs scores between the groups ($p > 0.05$). The mean OHI-S in the CHD and control groups were 1.24 (±0.84) and 1.47 (±0.72), respectively, with no significant difference ($p = 0.305$) between the two groups. The mean MGI scores in the CHD and control groups were 1.07 (±0.47) and 0.77 (±0.48), respectively. The MGI values in the CHD group were significantly higher than those in the control group ($p = 0.023$; $p < 0.05$).

3.2 Microbiological analysis of blood samples

According to the qPCR analysis of venous blood samples in both groups, using universal primer pair targeting bacterial *16S rDNA* genes, bacterial DNA was detected with high confidence in one child (number of copies: 32.8) in the CHD group and one child (number of copies: 87.51) in the control group. In all other plasma samples, bacterial DNA levels were calculated at varying levels of 1–12 copies; these findings were interpreted as false positive and were not taken into consideration. Therefore, bacterial DNA was positive in only one plasma sample per group.

3.3 Microbiological analysis of saliva samples

Assessment of the incidence of each target bacteria in saliva samples obtained from children in both groups are shown in Fig. 1.

The percentage of individuals with the presence and absence of each target bacteria and the total counts of each microorganism in the two groups are presented in Table 3. The results showed no significant differences in the incidence of individuals with the targeted bacteria, nor in the total number of each microorganism between the two groups ($p > 0.05$).

The correlation of each target bacteria with the caries status and oral hygiene levels is shown in Table 4. The results showed that the *Sm* counts were significantly correlated with the DMFT/dft and DMFS/dfs indices (r: 0.422 $p = 0.007$; r: 0.461; $p = 0.003$). Additionally, there was a significantly positive association between *Smk* counts and DMFT/dft (r:

TABLE 1. Primers used in the qPCR assay of saliva and blood samples of children in CHD and control groups.

Target microorganisms	Primers (5′ → 3′)	AL (bp)	Reference
Aggregatibacter actinomycetemcomitans	CTAGGTATTGCGAAACAATTTG CCTGAAATTAAGCTGGTAATC	262	[26]
Aggregatibacter actinomycetemcomitans JP2 clone	GCCGACACCAAAGACAAAGTCT GCCCATAACCAAGCCACATAC	686	[27]
Fusobacterium nucleatum	AGAGTTTGATCCTGGCTCAG GTCATCGTGCACACAGAATTGCTG	360	[28]
Porphyromonas gingivalis	AGGCAGCTTGCCATACTGCG ACTGTTAGCAACTACCGATGT	404	[29]
Streptococcus mutans	GGTCAGGAAAGTCTGGAGTAAAAAGGCTA GCGGTAGCTCCGGCACTAAGCC	282	[30]
Streptococcus mutans serotype k	ATTCCCGCCGTTGGACCATTCC CCAATGTGATTCATCCCATCACz	294	[31]
Scardovia wiggsiae	GTGGACTTTATGAATAAGC CTACCGTTAAGCAGTAAG	172	[32]
Universal bacterial primer	TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTAATCCTGTT	466	[33]
Human β-Globin gene	GGTTGGCCAATCTACTCCCAG TGGTCTCCTTAAACCTGTCTTG	226	[34]

AL: Amplicon length; bp: Base pair.

TABLE 2. Evaluation of caries experience, OHI-S and MGI scores in CHD and control groups.

Caries experience	CHD group (n = 20)	Control group (n = 20)	[a]*p*
DMFT			
Min–Max (Median)	0–8 (3.0)	0–14 (4.0)	0.036*
Mean ± SD	2.85 ± 2.18	5.20 ± 3.87	
DMFS			
Min–Max (Median)	0–18 (4.5)	0–32 (7.5)	0.045*
Mean ± SD	5.55 ± 5.22	10.15 ± 8.59	
dft			
Min–Max (Median)	0–10 (4.0)	0–15 (2.0)	0.859
Mean ± SD	4.10 ± 3.55	4.50 ± 4.37	
dfs			
Min–Max (Median)	0–35 (10.5)	0–36 (6.0)	0.785
Mean ± SD	11.35 ± 11.37	12.55 ± 13.20	
DMFT + dft			
Min–Max (Median)	0–15 (6.0)	2–15 (10.0)	0.068
Mean ± SD	6.95 ± 3.82	9.70 ± 4.38	
DMFS + dfs			
Min–Max (Median)	0–44 (13.5)	4–44 (21.5)	0.180
Mean ± SD	16.90 ± 11.93	22.70 ± 13.05	
OHI-S			
Min–Max (Median)	0–3 (1.0)	0–3 (1.4)	0.305
Mean ± SD	1.24 ± 084	1.47 ± 0.72	
MGI			
Min–Max (Median)	0.3–1.8 (1.1)	0.2–1.5 (0.5)	0.023*
Mean ± SD	1.07 ± 0.47	0.77 ± 0.48	

[a]*: Mann Whitney U Test; DMFT: decayed, missed, filled permanent teeth number; DMFS: decayed, missed, filled permanent teeth surface; dft: decayed, filled primary teeth number; dfs: decayed, filled primary teeth surface; OHI-S: simplified oral hygiene index; MGI: modified gingival index; SD: Standard deviation.*

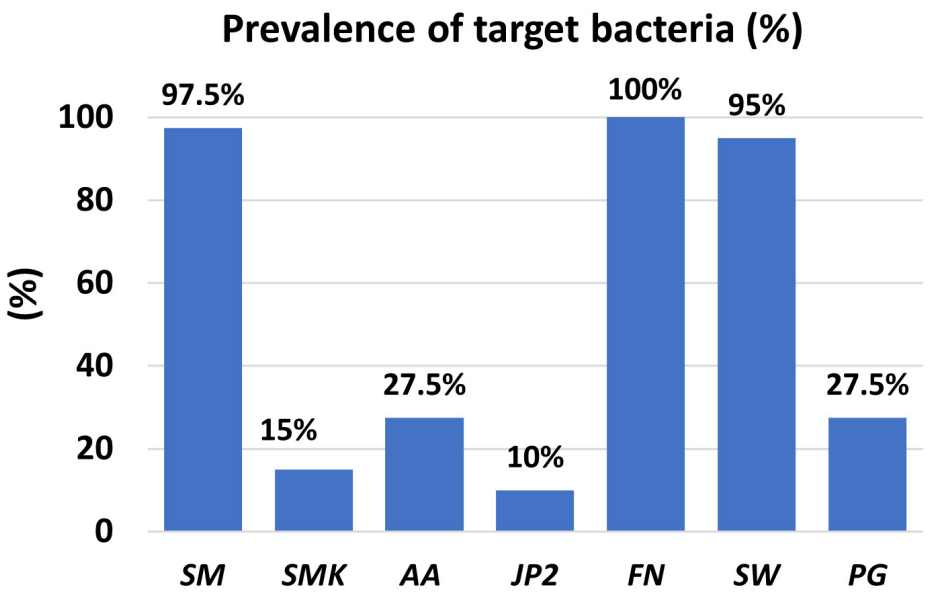

FIGURE 1. Prevalence of target bacteria in saliva samples obtained from all 40 children. *SM*: *S. mutans*; *SMK*: *S. mutans* serotype k; *AA*: *A. actinomycetemcomitans*; *JP2*: *A. actinomycetemcomitans* JP2 clone; *FN*: *F. nucleatum*; *SW*: *S. wiggsiae*; *PG*: *P. gingivalis*.

0.880; $p = 0.021$), as well as between the *Fn* counts and OHI-S (r: 0.327; $p = 0.039$). Moreover, the total bacteria count showed significant positive correlations with both DMFS/dfs (r: 0.383; $p = 0.015$) and OHI-S (r: 0.333; $p = 0.036$).

4. Discussion

The aim of the current study was to assess the specific oral pathogens potentially associated with the mobile microbiome in children with CHD using qPCR, and to analyze the caries experience, gingival health, and oral hygiene levels of the patients.

The DMFT and DMFS scores of the CHD group were significantly lower than those of the control group. Although the dft, dfs, DMFT + dft and DMFS + dfs scores of the children in the control group were higher than those in the CHD group, none of these differences were statistically significant. Our results are in agreement with those of Saraç *et al.* [35], who found no significant difference in the caries experience score among cardiac and healthy patients. However, contrary to our finding, a recent systematic review reported a higher caries prevalence in children with CHD compared to healthy children in seven out of nine included studies, although the difference was significant in only three [36]. This discrepancy may be explained by the fact that present study included patients with all severities of CHD, from mild to complex, unlike some other studies that only included those with complex disease severity. It has been proposed that children with complex CHD show a higher caries experience than healthy children [36].

The mean plaque values were not significantly different between the CHD and control groups. Although this finding differs from those of Schulz-Weidner *et al.* [37] and Saraç *et al.* [35], who reported higher plaque values in the CHD group compared to healthy group, it is consistent with those of Pourmoghaddas *et al.* [38] and Bozdogan *et al.* [39]. The similar oral hygiene statuses of the CHD and healthy patients may be attributed to the fact that enrolled patients were in close follow-up in a pediatric cardiology unit and the children and their parents were being referred to the pediatric dentist at a young age; thus, parental awareness regarding the importance of oral care in prevention of infective endocarditis was achieved.

Although the plaque values did not differ significantly between the CHD and healthy groups, the gingival index scores were significantly higher in the CHD group compared to the control group, indicating the susceptibility of patients with CHD to periodontal diseases. These findings align with a study conducted by Mohamed Ali *et al.* [40], which also observed elevated gingival index scores in children with CHD. Moreover, various meta-analyses have demonstrated a significant correlation between periodontal disease and the risk of developing cardiovascular disease in the future [41, 42].

Mobile microbiome analysis of CHD and control group children detected bacterial DNA with high confidence in only two plasma samples: one from the control group and one from the CHD group. In all other samples, only low amounts of bacterial DNA, ranging from 1–12 copies, were detected. As bacterial DNA detected in low quantities may be derived from contamination during DNA isolation, qPCR, or in the production of the chemicals used, these low quantity DNA samples were considered as false positives. A study by Nadkarni *et al.* [33], which used the same primers as the present study, demonstrated that the false positive limit that could result from contamination could be up to 48 copies, depending on the chemicals used. Additionally, although skin disinfection is performed during the blood withdrawal procedure, the possibility that the bacterial DNA detected in the samples may be caused by skin contamination cannot be excluded.

TABLE 3. Distribution of the incidence and total counts of each target bacteria and the total bacterial counts isolated from saliva samples according to the groups.

	CHD group (n = 20)	Control group (n = 20)	*p*
Sm; n (%)			
−	1 (5)	0 (0)	[c]1.000
+	19 (95)	20 (100)	
Sm count (n = 39)			
Min–Max (Median)	17.6–6480 (1430)	60.2–30,500 (2470)	[a]0.384
Mean ± SD	2269.19 ± 2136.98	4447.46 ± 6856.84	
Smk; n (%)			
−	18 (90)	16 (80)	[c]0.661
+	2 (10)	4 (20)	
Smk count (n = 6)			
Min–Max (Median)	982–8500 (4741)	1070–28,700 (2595)	-
Mean ± SD	4741.00 ± 5316.03	8740.00 ± 13,362.34	
Aa; n (%)			
−	16 (80)	13 (65)	[b]0.288
+	4 (20)	7 (35)	
Aa count (n = 11)			
Min–Max (Median)	6870–72,200 (17,850)	11,200–150,000 (57,600)	-
Mean ± SD	28,692.50 ± 29,568.28	63,357.14 ± 45,919.56	
JP2; n (%)			
−	17 (85)	19 (95)	[c]0.605
+	3 (15)	1 (5)	
JP2 count (n = 4)			
Min–Max (Median)	99.7–437 (99.7)	99.7–99.7 (99.7)	-
Mean ± SD	212.13 ± 194.74	99.70 ± 0	
Fn; n (%)			
−	0 (0)	0 (0)	[c]1.000
+	20 (100)	20 (100)	
Fn count (n = 40)			
Min–Max (Median)	18,900–1,550,000 (353,500)	23,000–2,750,000 (532,000)	[a]0.185
Mean ± SD	491,785.00 ± 43,953,417	751,750.00 ± 670,349.07	
Sw; n (%)			
−	1 (5)	1 (5)	[c]1.000
+	19 (95)	19 (95)	
Sw count (n = 38)			
Min–Max (Median)	1.8–1150 (80.5)	3.6–710 (131)	[a]0.715
Mean ± SD	220.22 ± 294.54	180.23 ± 213.63	
Pg; n (%)			
−	14 (70)	15 (75)	[b]0.723
+	6 (30)	5 (25)	
Pg count (n = 11)			
Min–Max (Median)	1.1–24,300 (7085)	4050–32,500 (11,200)	[a]0.273
Mean ± SD	8609.75 ± 9469.84	15,972.00 ± 11,651.98	
Total bacteria count			
Min–Max (Median)	131,000–16,600,000 (4,195,000)	181,000–22,600,000 (5,170,000)	[a]0.317
Mean ± SD	4,900,400.00 ± 4,229,398.15	7,319,950.00 ± 6,491,781.55	

[a]: Mann Whitney U Test; [b]: Pearson Chi-Square Test; [c]: Fisher's Exact test; Sm: S. mutans; Smk: S. mutans serotype k; Aa: A. actinomycetemcomitans; JP2: A. actinomycetemcomitans JP2 clone; Fn: F. nucleatum; Sw: S. wiggsiae; Pg: P. gingivalis; CHD: congenital heart disease; SD: Standard deviation.

TABLE 4. The correlation of each target bacteria with the caries status and the oral hygiene levels.

	n	Caries experience			MGI
		DMFT + dft	DMFS + dfs	OHI-S	
Sm count					
r	39	0.422	0.461	0.194	0.135
p		0.007**	0.003**	0.237	0.414
Smk count					
r	6	0.880	0.754	0.116	−0.088
p		0.021*	0.084	0.827	0.868
Aa count					
r	11	0.401	0.375	−0.083	−0.299
p		0.222	0.255	0.808	0.372
JP2 count					
r	4	0.816	0.775	−0.544	0.001
p		0.184	0.225	0.456	1.000
Fn count					
r	40	0.135	0.212	0.327	0.133
p		0.408	0.189	0.039*	0.413
Sw count					
r	38	−0.083	−0.198	−0.293	−0.239
p		0.620	0.234	0.074	0.148
Pg count					
r	11	0.101	−0.150	0.325	0.342
p		0.767	0.659	0.330	0.303
Total bacteria count					
r	40	0.262	0.383	0.333	0.246
p		0.102	0.015*	0.036*	0.126

n: number of samples that were positive for the detection of the target bacteria; DMFT: decayed, missed, filled permanent teeth number; DMFS: decayed, missed, filled permanent teeth surface; dft: decayed, filled primary teeth number; dfs: decayed, filled primary teeth surface; OHI-S: simplified oral hygiene index; MGI: modified gingival index; Sm: S. mutans; Smk: S. mutans serotype k; Aa: A. actinomycetemcomitans; JP2: A. actinomycetemcomitans JP2 clone; Fn: F. nucleatum; Sw: S. wiggsiae; Pg: P. gingivalis.

Considering that inflammation associated with periodontitis, root canal infections, and tissue trauma are the most important factors in the translocation of oral bacteria into systemic blood circulation, the low number of subjects with positive bacterial DNA in the present study may be due to the included children being periodontally healthy.

Our results showed a significant positive correlation between DMFT + dft and DMFS + dfs scores and *Sm* counts in saliva, as well as between DMFT + dft score and *Smk* counts. These findings support those of previous studies, which demonstrated a strong association between caries status and mutans streptococci [25, 43].

Nakano *et al*. [17], reported that although serotype k *S. mutans* strains had lower cariogenicity than other serotypes, they had a higher virulence in blood. Several studies have demonstrated an association between *Smk* strains and a higher risk of bacteremia and infective endocarditis. It has also been stated that serotype k strains are less susceptible to phagocytosis due to a defect in glucose side chains, which allows easier entry into the bloodstream and subsequent spreading throughout the body, as well as extended survival compared to other serotypes [17, 31]. In the present study, *Smk* was detected from the saliva samples of two patients in the CHD group (samples No. 5 and No. 22), one of which (No. 22) also had the highest rate of bacterial DNA among all plasma samples. It is important to identify subjects with serotype k strains as they carry a higher risk for infective endocarditis. Bacterial DNA was also detected in one patient in the healthy group, which should be approached with caution given that *Smk* strains may be associated with the development of cardiovascular diseases [44].

The presence of *Aa, Aa* JP2 clone and *Pg* in saliva samples did not differ significantly between the CHD and healthy subjects. Of the 40 children included in the study, 11 (27.5%) were positive for *Aa*, 4 (10%) for *Aa* JP2, and 11 (27.5%) for *Pg*. Aa colonization of the oral cavity with periodontal pathogens during childhood increases the risk of developing periodontal disease in older ages. Therefore, regular long-term follow-ups are recommended for children who are positive for these periopathogenic bacteria, especially the JP2 clone, which is significantly related to aggressive periodontitis [3, 18]. Considering that periodontal probing and pocket measurements in children may give misleading results, especially during the mixed dentition period [45], detailed intraoral clinical examination and radiographic evaluation are important. A recent systematic review investigating the association between the *Aa* JP2 clone and periodontitis reported that the JP2 clone was not consistently detected in specific population groups or in any particular geographical area [18]. In line with the findings of their review, the present study revealed no significant

differences in the detection of the JP2 clone between the CHD and healthy subjects. Notably, in the present study, the *Aa* JP2 clone, which is isolated frequently in North African countries and rarely in Europe, Asia, and the United States [3, 18], was detected at a high rate of 10% of saliva samples. Therefore, there is a need for further studies to identify the spread and prevalence of this clone in Turkey.

Fn, which has also been reported to be frequently isolated in periodontally healthy individuals, facilitates the colonization of periodontal bacteria [46]. In the present study, *Fn* was detected in high numbers from all saliva samples (100%) of both the CHD and control groups. Moreover, our results showed a positive correlation between *Fn* counts and OHI scores ($p = 0.039, p < 0.05$). These data support the knowledge that *Fn* plays an important role in biofilm maturation by acting as a bridge between the primary and secondary colonizing bacteria during the formation of dental plaque [46]. Additionally, *Fn* has been reported to increase cell permeability, not only in its own way but also by facilitating the passage of other bacteria into the bloodstream [47, 48]. Therefore, it is of great importance to regulate oral hygiene habits in children with heart disease, especially those with a high risk of infective endocarditis, and to reduce the *Fn* counts in the oral microbiome.

Sw, a newly characterized cariogenic pathogen, has been associated with early and advanced caries lesions, primarily early childhood caries, in the presence or absence of *Sm* [20, 49]. However, no previous study has reported the prevalence of *Sw* in children with CHD. In the present study, *Sw* was detected in 95% of saliva samples from both groups. The high rate of *Sw* associated with dental caries in the salivary samples of children, in parallel to the high isolation rates of *Sm*, indicates that the pathogen is associated with caries experience and that all children evaluated in this study have a high risk of caries.

In recent years, researchers have discussed the necessity and effectiveness of antibiotic prophylaxis before dental procedures to reduce the risk of infective endocarditis in children with heart disease. It has been emphasized that in the presence of poor oral hygiene, low intensity but frequent bacteremia associated with daily activities is associated with a higher risk of infective endocarditis than dental procedures, and that maintaining optimal oral hygiene is more important than antibiotic prophylaxis. Additionally, there remain serious concerns about antibiotic resistance, which is increasing rapidly worldwide [13]. Considering that increased inflammation-related vascularization in periodontal tissues and increased oral microbial load are the most important factors favoring the translocation of bacteria from the oral cavity into the bloodstream, periodontal health and dental plaque control are particularly important in children with CHD.

The main limitation of this study is the small sample size. A larger sample size could yield more statistically significant results. Another shortcoming of this study is that the severity of the CHDs was not taken into account in the sample population. Moreover, only seven bacterial pathogens possibly related to mobile microbiome were investigated in this study. Currently, there are approximately 700 species listed in the expanded Human Oral Microbiome Database (eHOMD). Complete characterization of all these species through the utilization of next-generation DNA sequencing methodologies, plays a crucial role in assessing the effects of the oral microbiota on cardiovascular disease. Therefore, more extensive research with a larger sample size and more advanced investigation techniques should be implemented to validate our findings.

5. Conclusions

In conclusion, the data obtained in this study suggest that the mobile microbiome is very rare in children. Furthermore, there are no significant differences in the prevalence and counts of selected oral bacteria in healthy children and those with CHD. Because bacteremia risk is often associated with periodontal diseases, it is important that children with early colonization of periopathogenic *Aa*, *Aa* JP2 clone and *Pg*, receive long-term follow-up.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

GK and OA—designed the research study. SDA and NT—performed the research. NT and YG—analyzed the data. SDA and YG—wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Written informed consent was obtained from each parent or legal guardian prior to study enrollment, and the study procedures were approved by the Local Ethics Committee of the Istanbul University Faculty of Dentistry (2016/27-81).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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