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Diversity of aerobic and facultative anaerobic bacteria forming the supragingival biofilm in healthy children

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ABSTRACT

Aims: The aim of this study was to reveal the aerobic and facultative anaerobic bacterial diversity in the supragingival biofilms of healthy caries-free children.
Methodology and results: Biofilm sampling was performed by a specialist in the morning at least 12 h after tooth brushing from 6-10 years old children who came to Suleyman Demirel University Pedodontics Clinic. Samples were cultured on selected media. Purified isolates were identified according to 16S rRNA gene sequences. Totally 180 bacterial isolates had obtained. These isolates were identified as 36 different bacterial species belonging to Firmicutes, Proteobacteria and Actinobacteria. Species with the highest number of isolates were *Lactobacillus* and *Staphylococcus*.
Conclusion, significance and impact of study: In this study, a total of 180 bacterial isolates had obtained. These isolates were identified as 36 different bacterial species belonging to 3 bacterial phyla (Firmicutes, Proteobacteria and Actinobacteria) and 9 bacterial genera. Although our study has certain limitations because it is dependent on culture, since there is no study previously conducted in Turkey to our knowledge, it contains important findings. The research findings reported in this paper will serve as the foundation for studies to be conducted to understand the oral colonization and biofilm dynamics of children and to take protective measures accordingly.

Keywords: Bacteria, biofilms, children, supragingival

INTRODUCTION

The oral cavity is the second place after the intestine, which has the most microbial load in the human body. It has been hosting more than 700 species with the contribution of the variety and quantity of food it contains (Dewhirst *et al*., 2010). The microflora of the gastrointestinal system begins to form in the first few years of life. Oral microflora is gained by the colonization of bacteria during birth and in the next few hours (Rosenblatt *et al*., 2015). The first colonization has been shown to be associated with the delivery method and the mother's vaginal microbiota (Li *et al*., 2018). The first colonizers of this system which includes the oral cavity, are aerobic and facultative anaerobic bacteria (Könönen, 2000). Through the degradation of the carbohydrates by these microorganisms, the formation of organic acids such as lactic, acetic and formic acid causes the development of acidic and anaerobic conditions. Changing environmental conditions support the development of different types of microorganisms (Simón-Soro *et al*., 2013). With the beginning of the first dentition period, the number of places where oral microorganisms can colonize is increasing. These are places and regions such as teeth, tongue, oral mucosa, palate, intra-oral lesions and periodontal pockets (Huang *et al*., 2011). Although researchers have focused on bacteria in the planktonic phase in previous years, nowadays, it is known that oral bacteria are organized as biofilms. Biofilm is defined as a unity formed by the polysaccharide matrix enclosing the microbial cells, which are fastened onto in a way that cannot be removed by gentle washing of the surface (Donlan, 2002). It is a structure in an extracellular matrix of host and microbial origin, formed by many different types of microorganisms. The first colonizers to form biofilm need the host's saliva glycoproteins. The first step is the formation of the pellicle layer from the saliva glycoproteins on the clean tooth surface. After conformational changes, the pellicle becomes ready for bacterial adhesion, which is the second step. At this stage, Gram-positive bacteria such as *Staphlycoccus*, *Streptococcus* bind to the pellicle. The extracellular polysaccharides (EPS) released by these bacteria provide the adhesion of other bacteria (e.g., *Actinomyces* spp.).

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The environmental conditions of the biofilms matured by the coaggregation of different kinds of microorganisms become favourable for the development of anaerobic bacteria. Subsequent colonizers of maturing biofilm include *Fusobacterium nucleatum*, *Treponema* spp, *Tannerella forsythensis*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (Marsh, 2004; Marsh and Percival, 2006; Huang *et al*., 2011; Sampaio-Maia and Monteiro-Silva, 2014). It has been shown that pathogenic bacteria that form oral biofilm cause caries and periodontitis which cause tooth loss. Specific pathogens that cause these diseases are the cornerstone of specific plaque theory. Major species that cause caries include *Streptococcus mutans*, *Lactobacillus acidophilus*; periodontal pathogens are *Tannerella forsythensis*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Treponema denticola*. Understanding the dynamics of early oral colonization and oral biofilm formation and development are important in terms of early protective applications' development.

The aim of this study was to reveal the aerobic and facultative anaerobic bacterial diversity in the supragingival biofilms of healthy children. Understanding the oral colonization and biofilm dynamics of children is essential for taking preventive measures.

MATERIALS AND METHODS

Collection of the samples

Biofilm sampling was performed by a professional dentist in the morning at least 12 h after tooth brushing from 6-10 years old children without caries who came to Suleyman Demirel University Pedodontics Clinic. Ethical approval was taken from the Chairmanship of the Ethics Committee of Suleyman Demirel University Medical School (11.02.2013/509). In our study, the principles of the Declaration of Helsinki were followed. During these 12 h, the patients have not eaten and have not used oral hygiene products. The samples taken with transport swabs were brought to Eskisehir Technical University Microbiology Laboratory with a cold chain.

Culturing samples

Solid media selected for culturing samples: Brain Heart Infusion Agar (BHI), DeMan, Rogosa, Sharpe Agar (MRS), Mitis Salivarus Agar, M17 Agar and Blood Agar. After culturing the selected solid media, it was incubated in an incubator containing 5% CO_2. After incubation, colonies of different morphologies were selected and purified. After their purity was checked by Gram staining, they were stored at -80 °C in 20% glycerol.

Molecular methods

DNA extraction, 16S rRNA amplification and sequence analysis are performed by using Bacterial genomic DNA GeneJET genomic DNA purification kit (ThermoFischer Scientific). For 16S rRNA gene amplification, 27F 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′ universal primers were used. Reaction components: 10× TaqBuffer (+KCl-$MgCl_2$), 2.5 µL; 25 mM $MgCl_2$, 2.5 µL; 2.5 mM dNTP mix, 2.5 µL; 2.5 mM 27F primer, 2.5 µL; 2.5 mM 1492R primer 2.5 µL; Taq polymerase (5 U/µL), 0.25 µL; nuclease-free distilled water, 11.75 µL; template DNA, 1 µL. PCR conditions: pre-denaturation at 94 °C for 3 min; denaturation at 94 °C for 30 sec, binding at 55 °C for 1 min, elongation at 72 °C for 2 min, 35 cycle; last elongation at 72 °C for 5 min. 16S rRNA sequence analysis of the resulting PCR products was performed by Macrogen Europe.

BLAST and phylogenetic analysis

The resulting sequences were compared with the other 16S rRNA sequences in the GenBank database on the National Center for Biotechnology (NCBI) website by using the BLAST program. The species-level identification of isolates was determined by percentage similarity. MEGA 7.0.26 program was used for phylogenetic analysis.

RESULTS AND DISCUSSION

In this study, a total of 180 bacterial isolates had obtained. These isolates were identified as 36 different bacterial species belonging to 3 bacterial phylum and 9 bacterial genera. Firmicutes is the most diverse phylum with 5 genera and 27 species. Proteobacteria, which is the only Gram-negative phylum contains 2 genera and 6 species. Actinobacteria is the least diverse phylum with 2 genera and 3 species (*Rothia dentocariosa*, *Rothia mucolaginosa* and *Corynebacteria argentoratense*).

Lactobacillus (49 isolates) in the Firmicutes phylum, *Neisseria* (19 isolates) in the Proteobacteria phylum and *Rothia* (8 isolates) in the Actinobacteria (8 isolates) phylum were the most dominant genera. Figure 1 shows the distribution of isolates in phylum and genera. Three (3) species in the *Staphylococcus* genera; 4 in the *Enterococcus* genera; 6 in the *Lactobacillus* genera; 5 in the *Neisseria* genera; 6 in the *Bacillus* genera; 2 in the *Rothia* genera; 8 in the *Streptococcus* genera; 1 in the *Klebsiella* genera and 1 in the *Corynebacterium* genera were identified. Table 1 shows the identified species and number of isolates. The 16S rRNA gene regions of the bacteria isolated from different mediums were sequenced. BLAST analyzes of the obtained sequences were made, and similarity was obtained at 97-99%. GenBank access numbers of the sequences are received. GenBank Access numbers: MH337307-MH337315, MH813960-MH813979, MH814728-MH814734, MH817377-MH817392, MH997747-MH997814, MH999421-MH999441, MK024278-MK024280, MK024292-MK024298, MK005921-MK005930, MK007306-MK007322, MK014810-MK014826, MK020457-MK020460, MK024140-MK024144, MK024236-MK024239, MK024243-MK024249, MK026976-MK026978, MK027006-MK027009, MK027020-

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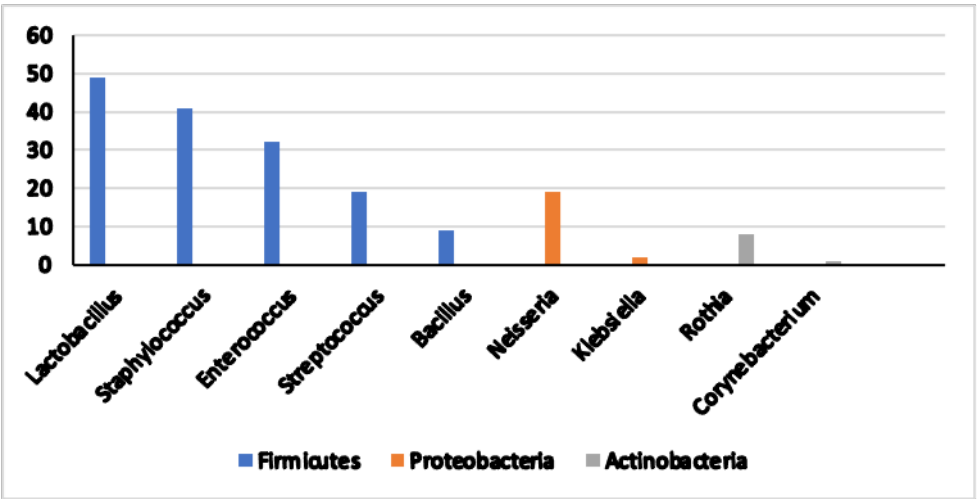

Figure 1: Distribution of bacterial isolates to genera.

Table 1: Identified isolates and their numbers.

Species	Isolate number	Species	Isolate number
Staphylococcus aureus	20	*Streptococcus salivarus*	8
Staphylococcus epidermidis	18	*Streptococcus anginosus*	4
Staphylococcus warneri	3	*Streptococcus lutetiensis*	2
Enterococcus faecium	25	*Streptococcus parasanguinis*	1
Enterococcus faecalis	4	*Streptococcus oralis*	1
Enterococcus lactis	2	*Streptococcus mutans*	1
Enterococcus durans	1	*Streptococcus intermedius*	1
Lactobacillus rhamnosus	23	*Streptococcus sanguinis*	1
Lactobacillus fermentum	11	*Neisseria flavescens*	9
Lactobacillus paracasei	5	*Neisseria subflava*	6
Lactobacillus casei	6	*Neisseria sicca*	2
Lactobacillus plantarum	2	*Neisseria elongata*	1
Lactobacillus salivarus	2	*Neisseria perflava*	1
Bacillus subtilis	2	*Klebsiella pneumoniae*	2
Bacillus amyloliquefaciens	2	*Rothia mucolaginosa*	5
Bacillus licheniformis	2	*Rothia dentocariosa*	3
Bacillus velezensis	1	*Corynebacterium argentoratense*	1
Bacillus pumilis	1		
Bacillus altitudinis	1		

MK027021, MK027245-MK027247, MK024376.

In the conducted studies, it was shown that the most dominant phylum in human oral microbiota is Firmicutes (Peterson *et al*., 2013; Heller *et al*., 2016). The dominant phylum in the microbial composition of oral biofilm was Firmicutes (83.3%); in the phylum, with close representation rates, the dominant genera were *Lactobacillus* (27.2%), *Staphylococcus* (22.7%) and *Enterococcus* (17.7%). *Lactobacillus* sp., the permanent member of the oral microflora, was the most dominant genera isolated from the supragingival biofilms of children. Especially in recent years, these bacteria, which are popular with probiotic properties, have started to be taken with milk and dairy products in daily diets, and this caused an increase in the oral microbiota settlement rate. *Lactobacillus rhamnosus* has been shown to continue colonizing the oral cavity even two weeks after the discontinuation of yogurt consumption (Meurman *et al*., 1994). In addition, using a selective medium in isolation increased the representation rate.

Since *Staphylococcus* sp. is a natural member of the nasal flora, there is a continuous transition into the mouth. In some studies with adults, colonization in the mouth has been shown to be directly proportional to age (Percival *et al*., 1991; Smith *et al*., 2003). In a study conducted by Ohara-Nemoto *et al*. (2008) on the cultural study of biofilm samples taken from healthy adults, *S. aureus* and *S. epidermis* were found to be the dominant species. These findings are in parallel with our study. In our study, *S. aureus* (11.11%) and *S. epidermidis* (10%) were determined as dominant species in *Staphylococcus*.

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Enterococcus sp. is a natural member of the gastrointestinal system, known as opportunistic pathogens that causes nosocomial infections. It has also been shown to be associated with meningitis, wound and urinary infections (Anderson *et al*., 2016). Oral microbiology is found to be low and temporary. It was seen that the data about the presence of oral microbiota are mostly obtained from patients undergoing endodontic treatment. Sedgley *et al*. (2004) isolated *E. faecalis* from 11% of adults they sampled. In the study, only one of the individuals with *E. faecalis* positive was reported to be healthy in terms of dental and gingiva health. Komiyama *et al*. (2016) have made *Enterococcus* sp. isolation from samples taken from healthy children by mouth rinsing technique. 10% of the samples were evaluated as *Enterococcus* sp. positive, *E. faecalis* was determined as the common species after identification. In our study, the highest isolation rate in *Enterococcus* sp. was *E. faecium* (13.8%) and *E. faecalis* isolation rate was 2.2% in total. *Enterococcus faecalis* complicates the treatment of dental health infections due to its virulence factors and transfer of these by horizontal gene transfer (Anderson *et al*., 2016). According to these data, the low isolation rate of *E. faecalis* in our study is an expected result.

Other members isolated from the Firmicutes phylum include *Streptococcus* sp. (10.55%) and *Bacillus* sp. 5%). Although the number of isolates of *Streptococcus* sp. was low, species diversity was the highest. It is seen that the ratio of *Streptococcus* sp. in oral biofilm is suppressed by the presence of *Lactobacillus* sp. Many studies have reported antibacterial and antibiofilm effects of *Lactobacillus* sp. on *Strep. mutans* (Güngör *et al*., 2013; Wu *et al*., 2014; Lin *et al*., 2015; Lin *et al*., 2017; Ahn *et al*., 2018). *Bacillus* sp. has been reported to be low in oral microbiota (Dewhirst *et al*., 2010). This also coincides with our findings.

Neisseria sp. from the Proteobacteria phylum has the same isolation rate as *Streptococcus* sp. (10.55%). In previous studies, *Neisseria* sp. rate is reported to be low in healthy individuals. For example, Kanasi *et al*. (2010) in their clonal study; Ma *et al*. (2015) in the microarray study have determined early period caries and *Neisseria* sp. in healthy children. It is an interesting finding that *Klebsiella pneumoniae* isolated from adults with caries in previous studies was isolated in our study. This bacterium, which is not naturally found in opportunistic pathogens and oral microbiota, was isolated from 6 adults with caries over the age of 30 in the study by Rajaprabu *et al*. (2018).

From the Actinobacteriaa phylum, *Rothia mucolaginosa* (2.77%) and *Rothia dentocariosa* (1.66%) and *Corynebacterium argentoratense* (0.55%) were isolated. In their microarray study of Ma *et al*. (2015), *Rothia* sp. was detected in healthy children with caries. It is reported that the rate of presence in the caries is higher (Aas *et al*., 2008). In the same study, in the biofilm samples taken from healthy children, *Corynebacterium matruchotii* was detected. In a clonal study on the biofilms with or without caries formed in the primary dentition and in the permanent teeth, *Corynebacterium* sp. was found in both groups.

The phylogenetic trees of the 16S rRNA sequences of the isolates, along with the reference sequences taken from the NCBI, were plotted with the 500 bootstrap value by using the Maximum Likelihood method (Figures 2, 3 and 4). The different branching of many of the reference

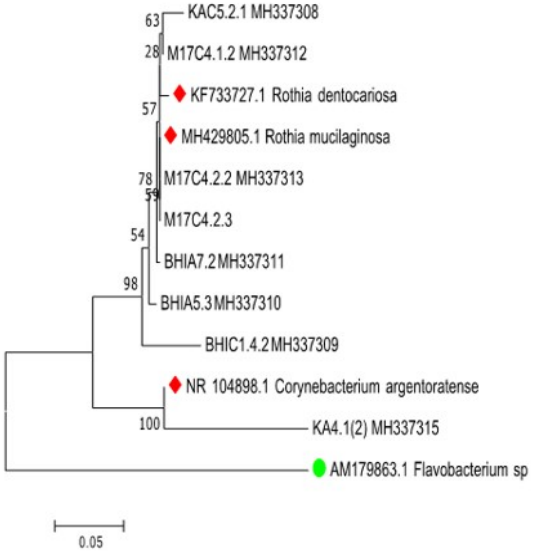

Figure 2: Maximum Likelihood tree of isolates which belongs to Actinobacteria phylum. Reference sequences obtained from GenBank indicated by red diamond, out group indicated by green circle.

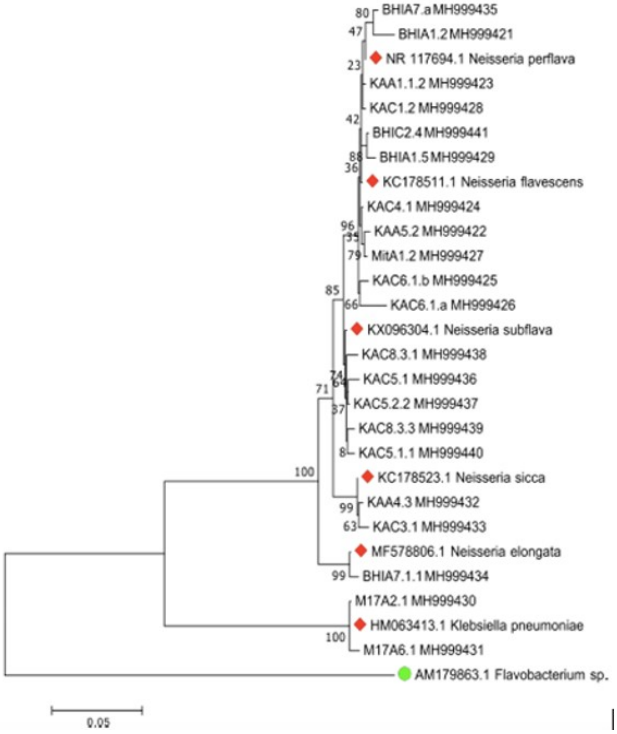

Figure 3: Maximum Likelihood tree of isolates which belongs to Proteobacteria phylum. Reference sequences obtained from GenBank indicated by red diamond, out group indicated by green circle.

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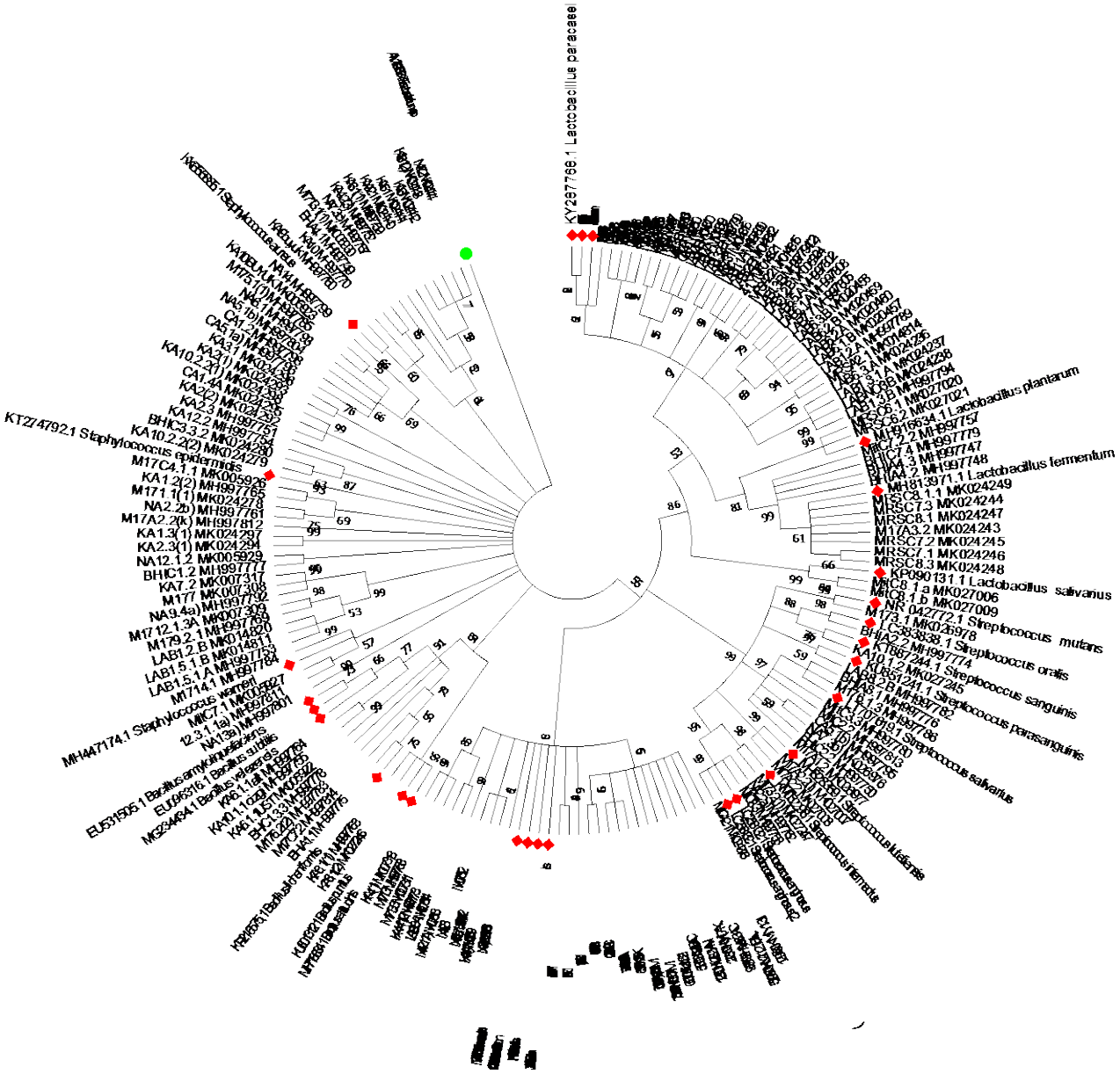

Figure 4: Maximum Likelihood tree of isolates which belongs to Firmicutes phylum. Reference sequences obtained from GenBank indicated by red diamond, out group indicated by green circle. Bootstrap support values below 50% were not included in the figure**.**

sequences indicates that different phylotypes are formed as a result of horizontal gene transfer within the biofilm. Currently, 730 species are defined in the Human Oral Microbiome Database (http://www.homd.org). As a result of increasing genome studies, the number of phylotypes is expected to reach 19.000 (Sampaio-Maia and Monteiro-Silva, 2014).

Since our study is culture-dependent, the microbial diversity it reveals is limited. Incubation conditions are suitable for the growth of facultative anaerobic bacteria that can grow in aerobic and 5% CO_2 conditions. Therefore, *Fusobacterium* sp. and anaerobic bacteria such as members of the phylum Bacteriodes could not be isolated. Since our biofilm samples were obtained from caries-free children, high numbers of *Bacteroidetes* and *Fusobacteria* are not expected (Kalpana *et al*., 2020). However, the inability to isolate these genera at all is related to the incubation conditions. Metagenomic studies of oral biofilm have revealed a wide variety of species (Espinoza *et al*., 2018; Kalpana *et al*., 2020). In this sense, it seems appropriate to expand the study with a metagenomic study. Therefore, it may be prudent in

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diversity studies to use both techniques in combination rather than rely on a single method with many known and unknown biases.

CONCLUSION

In conclusion, in our study, the aerobe and facultative anaerobe bacterial diversity in the biofilm samples taken from healthy children were examined. The presence of isolated and identified species in plaques without caries in children was supported by previous studies. *Lactobacillus* sp., which is known to cause caries and *Staphylococcus* sp. isolated mostly from adult caries were shown to be present in the biofilm samples taken from healthy children. Although our study has certain limitations because it is dependent on culture, since there is no study previously conducted in Turkey to our knowledge, it contains important findings.

The findings reported in this paper will serve as the foundation for the studies to be conducted to understand the oral colonization and biofilm dynamics of children and to take protective measures accordingly.

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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