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Exploring the Streptococci Variants in Children's Oral Cavity, Its Microbiome Diversity

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ABSTRACT

This study aimed to identify the variants of the Streptococcus genus in the oral cavity of healthy individuals. Streptococcus is one of the normal flora, which is part of the lactic acid bacteria (LAB) in the oral cavity. LAB has the ability to produce antimicrobial agents, regulate the host's immune response, and inhibit the growth of pathogenic bacteria. The presence of LAB in the oral cavity is crucial in maintaining oral health and preventing periodontal diseases. This study used observational cross-sectional design with 40 healthy elementary school children as subjects. Swabs were taken from their oral cavities and sent to the laboratory for PCR and sequencing tests. The results revealed that isolates 3S, 4S, 9S, 10S, 14S, and 15S were identified as Streptococcus dysgalactiae, while isolates 1S, 8S, and 9S were identified as Streptococcus mitis. Additionally, isolates 2S, 5S, 7S, 11S, 12S, and 13S were identified as Streptococcus salivarius, isolate 6S was identified as Streptococcus agalactiae, and isolate 17S was identified as Streptococcus oralis. These findings provide valuable information on the identification of oral microbiota, particularly Streptococcus genus variants, which can potentially serve as an alternative therapy for periodontal diseases.

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Introduction

The oral microbiota is a collection of genomes in microorganisms that reside in the oral cavity, so it is important to know the characteristics of each oral microbiota to understand oral health and systemic disorders that may occur. In general, the oral cavity and nasopharyngeal area are ideal environments for the growth of microorganisms. The oral cavity has a stable temperature and pH so that bacteria can easily live in this environment, saliva also keeps microorganisms, especially bacteria, hydrated and facilitates the transportation of nutrients to bacteria [1].

Microorganisms, especially bacteria in the human oral cavity, coexist with one another, both pathogenic and beneficial so that homeostasis is maintained. Changes in environmental conditions in the mouth result in increased potential for pathogenic conditions and diseases in the mouth [2]. Various types of oral microbiota can benefit and help the digestive system and help the body's immune system because they produce various chemical compounds that are beneficial against pathogenic germs. The composition of the oral microbiota is influenced by several factors such as host factors, diet and antibiotic use. Other factors that affect the balance of the bacterial population in the mouth are oral hygiene, systemic disease, periodontal disease, and various lesions in the mouth. Saliva also serves a protective function against population balance in the mouth [3].

One of the normal flora in the oral cavity is (Lactic Acid Bacteria) LAB which has the ability to inhibit the growth of pathogenic bacteria so that it is expected to have an impact on oral health [4]. The characteristic of LAB is to produce lactic acid as its main product because it has the enzyme lacto dehydrogenase which can ferment lactose [5]. Microbes from the LAB group are probiotics because they have the ability to maintain health and improve host health. Bacteriocins produced by LAB have antimicrobial effects on pathogenic bacteria so that they can be used as an alternative therapy for a disease [6].

Streptococcus species are classified by the type of hemolytic properties. Alpha-hemolytic species cause oxidation of iron in hemoglobin molecules in red blood cells, resulting in a greenish color when grown on Blood Agar media. Beta-hemolytic species break red blood cells when grown on Blood Agar media so that it looks clear around the bacterial colony. Gamma-hemolytic species do not cause hemolysis. Beta-hemolytic streptococci are further classified by the Lancefield grouping (serotype classification) which describes the specific carbohydrates present in the bacterial cell wall. In medical science, the most important groups are Streptococcus viridans, S. pneumoniae alpha-hemolytic and Streptococcus beta-hemolytic Lancefield groups A and B (also known as "strep group A" and "strep group B") [7]. The Streptococcus genus is not only a pathogenic germ in human life, but also many benefits from

metabolic compounds released by Streptococcus, for example Streptococcus salivarius bacteria produce bacteriocins of the type Salivaricin A, Salivaricin B, Salivaricin G32, Salivarin 9 and Streptin [8]. The study aims to determine the variants of the oral microbiota of the Streptococcus genus using the Polymerase Chain Reaction (PCR) and sequencing methods.

Methods

Tools used, vortex shaker, waterbath, Eppendorf tube, Eppendorf tube rack, microtube, GD colom, collection tube, MPW-260R centrifuge, stopwatch, micropipette (BIOHIT Proline PlusSingle channel 0.5-10µl; 10-100µl; 20-100µl) (Biorad Single channel 100-1000 µl), filter tips, reagent bottles, 250 ml Erlenmeyer, analytical balance, DNA thermal cycler (Applied Biosystem), gel documentation, 40C refrigerator. The materials used were distilled water, lyzosyme, buffered salts, proteinase, absolute ethanol, ethidium bromide, TAE buffer, Presto mini gDNA Bacteria Kit (100 Preps), GB buffer solution, wash buffer solution, label paper, marker (Smart leader SF), 2% agarose gel, loading dye solution, de Mann-Ragosa-Sharpe (MRS) medium, 2X Kapa 2G Fast Ready Mix. Genomic DNA extraction was performed following the DNA extraction protocol contained in the Geneaid PrestoTM Mini gDNA Bacteria Kit. DNA amplification was by PCR method. The primers used to detect bacterial genes of the genus Streptococcus are:

Forward Primer = Str1: 5'-GTACAGTTGCTTCAGGACGTATC- 3'

Reverse Primer = Str2: 5'-ACGTTCGATTTCATCACGTTG- 3'

PCR Product Detection was by Electrophoresis. 1.5% agarose gel was made by mixing 1.5 grams of agarose powder into 100 mL TAE Buffer in Erlenmeyer then heated in the microwave for 2 minutes to boil, then added 8 µL Ethidium Bromide. The gel liquid was then cooled at room temperature. After cooling slightly, the gel liquid was poured into an electrophoresis gel mold using a gel comb with a comb number of 14 wells. Each 5 µl of amplification product was inserted into the wells of a 1.5% agarose gel submerged in a tank containing TAE Buffer. Next, electrophoresis was run for 50 minutes with a constant voltage of 100 volts. After 50 minutes the electrophoresis was stopped and the gel was removed to be observed under UV light. The result is positive if there is a DNA band aligned with the 198 bp marker and negative if there is no band on the gel. PCR products from samples that show positive electrophoresis results are continued to the DNA sequencing stage. DNA samples were carried out by 1st Base through PT Genetika Indonesia. The DNA sequencing process was carried out using the Sanger deoxy method.

Results and Discussion

PCR (Polymerase Chain Reaction) is a technique of multiplication (amplification) of pieces of DNA in vitro in specific regions bounded by two oligonucleotide primers. PCR method

can multiply a fragment of DNA molecules into DNA molecules (110 bp / 5 x 10-19) by 200,000 times.

Identification of bacterial isolates based on 16S rRNA with primer pair Str1 (5'-GTACAGTTGCTTCAGGACGTATC-3') and Str2 (5'-ACGTTCGATTTCATCACGTTG- 3'). 16S rRNA of each isolate has been amplified with an annealing temperature of 50 ° C using PCR and obtained the results of the size of the DNA band visible on a 1.5% agarose gel with a length of 198 bp (Fig. 1), then the PCR result sample is sequenced to ensure that the DNA fragments amplified in the process belong to the genus and strain that belongs to Streptococcus bacteria (Fig. 2).

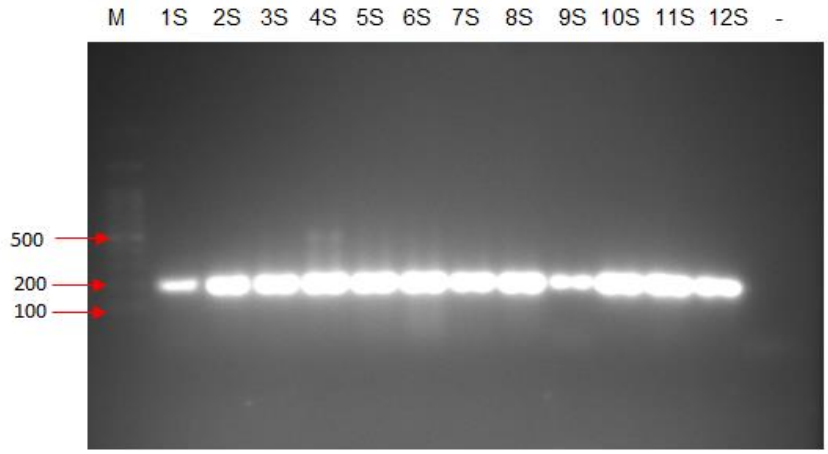
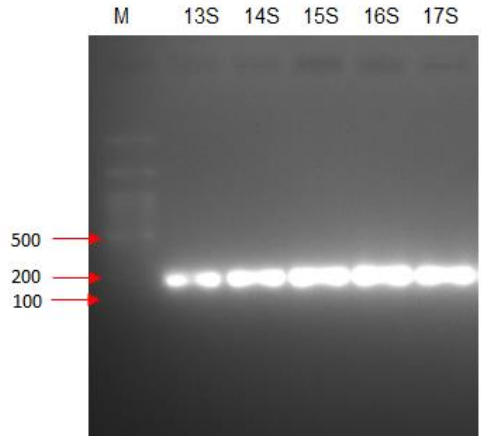


Fig. 1. Amplification results of sample DNA with primers Str1



Remark: M: marker; 1S-17S: Bacterial sample DNA

Fig. 2. Amplification results of sample DNA with primers Str2

Based on the visualization results of electrophoresis, it shows that the primers used can be amplified around 198 bp in the 1S-17S sample, the appearance of DNA bands indicates that the 1S-17S sample DNA is amplified with a high concentration so that the band of each sample looks thick. The success of this PCR technique is due to the suitability of primers and

the efficiency and optimization of the PCR process. Non-specific primers can cause other regions of the genome to be amplified that are not desired or vice versa, no genomic regions are amplified. Optimization of DNA annealing temperature in the PCR machine is one of the determinants of the success of the appearance of the desired band [9]. In this study, the optimal temperature for Str1 and Str2 primers was 500C.

DNA sequencing was performed by "Single Pass DNA Sequencing" using the same primer as the Str1 primer gene amplification. The identity of the gene that has been sequenced can be determined by comparing the similarity of the data obtained with the sequence data contained in Genbank, one of which is NCBI. The sequencing data were then analyzed using nucleotide blast at ncbi.nlm.niv.gov. Analysis of blast results provides information and verifies organisms or bacteria that have homologous DNA sequences with the sample so that it can be used to determine the identification of these bacteria.

The acceptable percentage of the results of blast sequencing data is at least 95% unless the sequence data that reads lower is enforced 75%. This proves that all bacterial isolates obtained are included in Streptococcus which belongs to the human oral microbiota. To see the species most closely related to these bacterial isolates, phylogenetic analysis was carried out (Fig. 3).

Score	Expect	Identities	Gaps	Strand
42.1 bits(21)	5e-10	21/21(100%)	0/21(0%)	Plus/Minus

```
Query  1    ACGTTCGATTTCATCACGTTG  21
            |||||||||||||||||||||
Sbjct  158  ACGTTCGATTTCATCACGTTG  138
```

Fig. 3. Results of alignment analysis of DNA sequences of 11S isolates with primer reverts

Fig. 4 presents the DNA sequencing results of 11S isolates from the oral microbiota of children. The figure may show the genetic variations or differences between the isolates, potentially in the form of nucleotide sequences, base pair lengths, or mutation rates. The figure might also display the phylogenetic relationships among the isolates, indicating how closely related they are to each other. The figure is expected to provide important insights into the diversity of Streptococcus genus in children's oral microbiota and may serve as a basis for further analysis and interpretation.

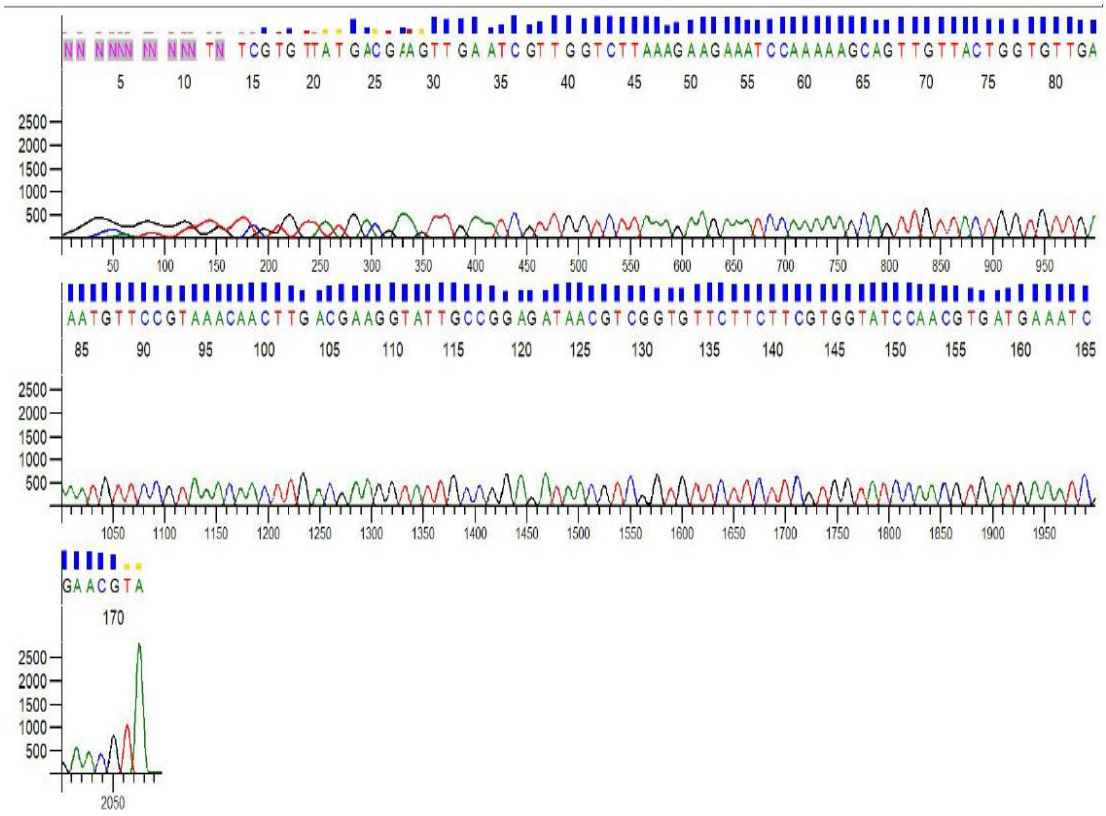


Fig. 4. DNA sequencing results of 11S isolates

Fig. 5 presents the results of DNA sequence alignment analysis of an 11S isolate with Accession LS483366.1. The figure show the degree of similarity or dissimilarity between the two DNA sequences, potentially in the form of nucleotide sequences, base pair lengths, or mutation rates. The figure display the alignments between the two sequences, indicating the specific regions of similarity or difference. The figure is to provide important insights into the genetic relationships between the 11S isolate and Accession LS483366.1, and help to identify any genetic variations or mutations that distinguish them from each other. This information is useful for further analysis and interpretation of the genetic diversity of Streptococcus genus in children's oral microbiota.

Score	Expect	Identities	Gaps	Strand
283 bits(153)	1e-77	158/160(99%)	2/160(1%)	Plus/Minus

```
Query  1677120  ACGTTCGATTTCATCACGTTGGATACCACGAAGAAGAACACCGACGTTATCTCCGGCAAT  1677179
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  160      ACGTTCGATTTCATCACGTTGGATACCACGAAGAAGAACACCGACGTTATCTCCGGCAAT  101

Query  1677180  ACCTTCGTCAAGTTGTTTACGGAACATTTCAACACCAGTAACAACTGCTTTTTGGATTTC  1677239
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  100      ACCTTCGTCAAGTTGTTTACGGAACATTTCAACACCAGTAACAACTGCTTTTTGGATTTC  41

Query  1677240  TTCTTTAAGACCAACGATTTCAACTTCGTCATTAACACGA  1677279
                |||||||||||||||||| |||||||||||| |||||||
Sbjct  40       TTCTTTAAGACCAACGATT-CAACTTCGTCAT-AACACGA  3
```

Fig. 5. Results of DNA sequence alignment analysis of 11S isolate with Accession LS483366.1

Table 1 represents the homologous Streptococcus species identified in samples from the oral microbiota of children. The table includes a sample code for each sample, followed by the name of the identified homologous Streptococcus species, the query cover percentage, identities percentage, and accession number. The query cover percentage represents the percentage of the query sequence (i.e., the DNA sequence obtained from the sample) that was covered by the identified homologous Streptococcus species. The identities percentage represents the percentage of nucleotides that matched between the query sequence and the identified homologous Streptococcus species. This table provides valuable information about the diversity of homologous Streptococcus species present in the oral microbiota of children, which may have important implications for understanding the development of oral health and disease in this population.

Table 1. Blast Data Sequencing Results

No	Sample Code	Homologous Streptococcus species	Query Cover	Identities	Accession
1	1S	*Streptococcus mitis* strain SK637	76%	94.78%	**CP028415.1**
2	8S		87%	99.38%	
3	16S		89%	93.21%	
4	2S	*Streptococcus salivarius* strain NCTC7366	87%	98.68%	**LS483366.1**
5	5S		93%	96.97%	
6	7S		94%	95.91%	
7	11S		99%	98.75%	
8	12S		94%	96.99%	
9	13S		91%	96.95%	
10	6S	*Streptococcus agalactiae* strain TFJ0901	91%	98.17%	**CP034315.1**
11	3S	*Streptococcus dysgalactiae* strain 0722xy	94%	98.79%	**EU595708.1**
12	4S		94%	95.27%	
13	9S		93%	96.36%	
14	10S		89%	98.76%	
15	14S		98%	96.88%	
16	15S		93%	97.01%	
17	17S	*Streptococcus oralis* strain RAG16	99%	96.93%	**KU295730.1**

Based on the blast results of the sequencing data of the 17 bacterial isolates, all isolates belong to the genus Streptococcus with five different species (Table 1). to see the species most closely related to the 17 isolates, a phylogenetic analysis was carried out which aims to precisely construct the relationship between organisms and estimate the differences that occur from one descendant to the next.

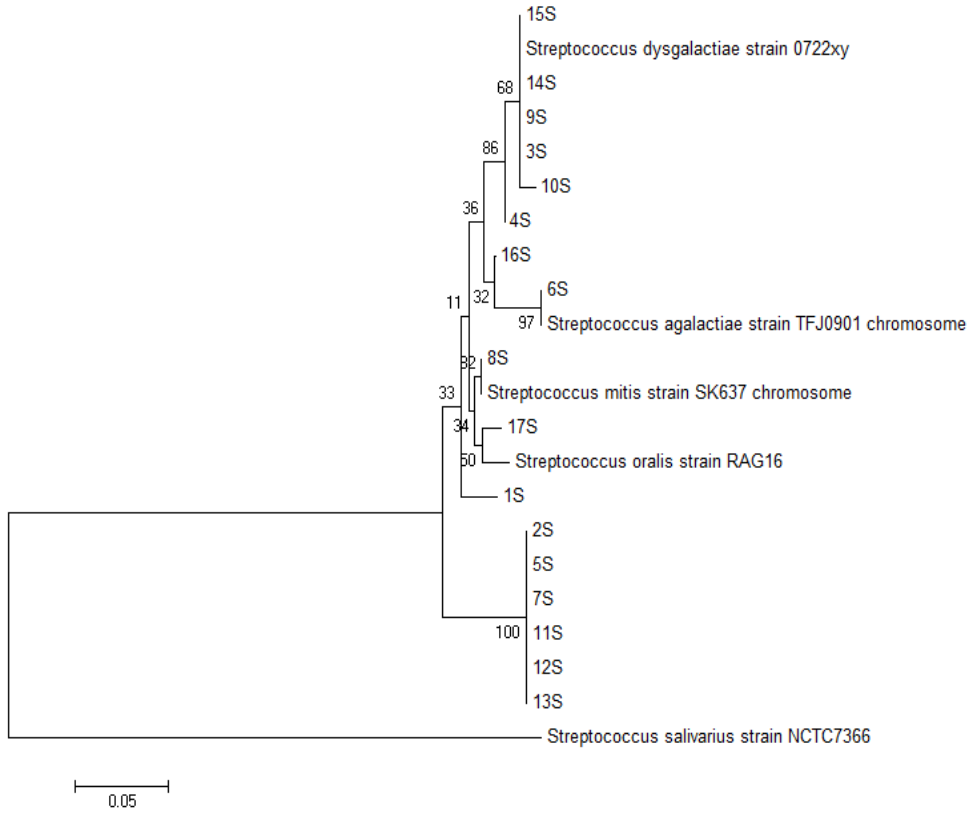


Fig. 6. Phylogenetic tree based on 16S rRNA-coding DNA sequences for Streptococcus from bacterial sample isolates compared to 16S rRNA-coding DNA sequences of Genbank bacteria

The 17 bacterial isolates identified as the genus Streptococcus based on genotypic identification through the 16s RNA sequence of the Streptococcus marker using the Str1 primer are isolates isolated from the tonsils of healthy children. Ref. [10] stated in his research that the genus Streptococus is generally present as normal microflora in the oral cavity, skin surface, upper respiratory tract and digestive tract. Streptococcus mitis, S. salivarius, and S. oralis bacteria belonging to the viridans group are normal oral microbiota found in humans [9].

Conclusion

The conclusion of this study is that there are several oral microbiota of the genus Streptococcus, namely isolates 3S, 4S, 9S, 10S, 14S, 15S are Streptococcus dysgalactiae. Isolates 1S, 8S, 9S are Streptococcus mitis, and isolates 2S, 5S, 7S, 11S, 12S, 13S are Streptococcus salivarius. Isolate 6S is Streptococcus agalactiae and isolate 17S is Streptococcus oralis found in children's mouths. Further research needs to be done on other microbiota species found in adults.

Conflict of Interest

The authors declare that there is no conflict of interest.

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