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Molecular characterization of oral bacteria isolated from human saliva

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ABSTRACT

Periodontitis is an inflammation of gums and bones that supporting the teeth which caused by *Staphylococcus intermedius.* The saliva from a patient of periodontitis or suspect to periodontitis will have higher levels of *Staphylococcus intermedius.* Hence, human saliva is clinically informative in diagnosing oral disease and the oral health of an individual. In this study, oral bacteria in human saliva were identified using 16S ribosomal RNA. 16S ribosomal RNA (rRNA) genes from the isolated colonies were amplified through the colony Polymerase Chain Reaction (PCR) method. 16S rRNA genes were used to determine species identity by sequencing and generating the phylogenetic tree. The results showed that *Streptococcus sp.* and *Staphylococcus sp.* were the most prevalent oral bacteria found from all the saliva samples, while *Lactobacillus sp.* was found from two samples. From the constructed phylogenetic trees, bacteria strains B1 and B2 clustered with the *Staphylococcus sp.* database. Bacteria strains B9 and B10 were categorized as *Streptococcus sp.* as the confidential level between *Streptococcus sp.* database is 100% in Neighbour-Joining tree. Sample B15 and B16 clustered with *Lactobacillus sp.* database. Oral bacteria species typically associated with periodontitis was detected in all saliva samples. Therefore, it is important to fully understand the nature of the oral bacteria before further research on drug design and administration of oral treatment is executed.

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INTRODUCTION

The oral cavity was comprised by two types of surfaces for bacteria colonization, the soft tissue and the hard tooth exposed root surfaces. These two surfaces are coated with an excess amount of bacteria forming biofilm, supporting the growth of different bacterial communities (Özdabak *et al.*, 2012). These microorganisms form a complex ecology community in the oral cavity that indirectly influences the oral and systemic health of individuals (Zhang *et al.*, 2018). According to the Global Burden of Disease Study in 2016, 3.58 billion of people were estimated to suffer from oral diseases. Saliva fulfilled the environment criteria for microorganisms to form a complex ecology community, where its buffers the pH and humidity for the growth of many oral bacteria (Marsh *et al.*, 2015).

According to Bodiba *et al.* (2018), 60%–90% of school children and nearly 100% of adults have dental cavities, and they often suffer in pain and discomfort. There are some severe oral diseases such as periodontitis caused by Staphylococcus intermedius (Lovegrove, 2004), Oral Lichen Planus (OLP) and Oral Cancers. Choi *et al.* (2016), proposed that saliva from OLP patients containg ingivitis/periodontitis-associated bacteria (ex. *Staphylococcus sp.*).

Identification and characterization of disease-causing bacteria using the molecular method will be beneficial in curing oral diseases and to produce new antibacterial drugs that can inhibit and kill the disease-causing bacteria. Furthermore, by characterizing the beneficial bacteria, antibacterial drugs can be made based on the protein that had been produced by the beneficial bacteria to maintain oral health. For example, *Streptococcus salivarius* is a common bacterium found in saliva and also been shown to have considerable potential as probiotic candidates (Burton *et al.*, 2011).

In 2016, Human Oral Microbiome Database pointed out that human saliva contains more than 700 bacterial species. Among these species, some bacterial are beneficial while some are oral disease-causing bacteria towards the human oral cavity. Hence, molecular characterization of bacteria in human saliva samples is needed to identify the taxonomy of the bacteria species, indirectly help in solving this problem. Therefore, our study aimed to isolate and characterize the oral bacteria using biochemical and molecular characterization techniques.

MATERIALS AND METHODS

Sample Collection

Human saliva samples were collected from volunteers. Each of the volunteers was contributed approximately 5 mL of saliva sample, and the sample was collected in a 15 mL falcon tube. The parameters of the volunteers were: non-smokers and were not under any usage of medications (especially antibiotics). The volunteers were advised to abstain from foods or drinks for minimum of one hour before collecting the saliva samples. The sample were stored at 4°C refrigerator.

Isolation of bacteria

Human saliva sample of an individual (100μl) was streaked on three different types of agar plates. According to different types of agar plates, the incubation period was different. The saliva samples cultured on Mannitol Salt Agar plate (Oxoid, Hampshire, UK) and MitisSalivarius Agar (MSA) plate (HiMedia, Mumbai, India) were incubated for 24 hours at 37°C. For the saliva sample cultured on Rogosa Agar plate (Laboratories Conda S.A., Madrid, Spain), the plates were incubated for 5 days at 30°C. After incubation periods, different types of colony were sub-cultured into respective selective medium in four quadrate streak plate technique to isolate a single colony.

Biochemical characterization

Isolated oral bacteria were subjected to various biochemical tests. These tests facilitated the identification and characterization of isolated oral bacteria. Biochemical tests used in this study were: Gram staining, catalase test, urease test, citrate test, methyl red (MR) test, and triple sugar iron (TSI) test.

PCR amplification of 16S rRNA gene and purification of PCR products

A single colony for each bacteria strain was collected from a solid agar plate and used for Colony PCR. PCR was carried out to isolated 16S rRNA gene from different strains of bacteria using universal 16S rRNA primer pair (Forward: 5' TGG AGA GTT TGA TCC TGG CTC AG 3' and Reverse: 5' TAC CGC TGC TGG CAC 3') designed by Rahman *et al.* (2015). In thermocycler programme, the samples were preheated at 95°C for 5 mins, followed by amplification under the following conditions: denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 1 min. A total of 35 cycles are performed; and followed by a final extension step at 72°C for 5 mins and infinity store of PCR products at 10°C until the products were collected. The quality and quantity of amplified sequences were analyzed by agarose gel electrophoresis and NanoDrop (Thermo Scientific, Massachusetts, US). The sequences were subjected to homology search using Nucleotide BLAST (BLASTN) against NCBI 16S ribosomal RNA sequence database. The BLAST results along with biochemical tests results were used to determine the species of isolated strains.

Phylogenetic Analysis

The primer set was able to amplified about 614 base pairs of sequences. All the query (Table 1) and subject sequences were analyzed with multiple sequences alignment (MSA) method using SATé. Neighbour-Joining and Maximum-Likelihood with 1000 bootstraps was constructed using MEGA-7 tool.

Pairwise Analysis

Based on Multiple Sequence Alignment, the pairwise distance was calculated through MEGA-7 tool. Heatmap of a matrix was developed through Euclidean method by using Heatmapper.

RESULTS AND DISCUSSION

Biochemical characterization of isolated bacteria strains

The colony morphology of the isolated colonies and the result of the biochemical test were recorded in Tables 2 and 3, respectively.

Phylogenetic relationship among isolated bacterial strains and the homologous bacteria.

Table 1: Query coverage of each bacterial strain sequence

B1		
Description of subject	Accession	Query coverage
Uncultured microorganism clone	JN911710.1	96%
Staphylococcus aureus strain	MG971399.1	95%
Uncultured bacterium clone	GQ467552.1	98%
Staphylococcus aureus strain	MF805760.1	98%
B2		
Description of subject	Accession	Query coverage
Staphylococcus sp. strain	MN315426.1	96%
Staphylococcus aureus strain	KU851228.1	96%
Staphylococcus simiae strain	KF933768.1	96%
Staphylococcus lugdunensis strain	FJ434207.1	96%
B9		
Description of subject	Accession	Query coverage
Streptococcus sp. oral clone	AB121908.1	97%
Uncultured bacterium clone	EF404014.1	98%
Uncultured Firmicutes bacterium clone	GU957427.1	97%
Streptococcus salivarius strain	NR_042776.1	98%
B10		
Description of subject	Accession	Query coverage
Streptococcus salivarius strain	MK330589.1	97%
Streptococcus sp. strain	MH683098.1	97%
Streptococcus salivarius strain	KY038193.1	97%
Uncultured *Streptococcus sp*.	LT677887.1	97%
B15		
Description of subject	Accession	Query coverage
Lactobacillus paracasei subsp. paracasei	HQ697633.1	98%
Lactobacillus zeae strain	KT630827.1	97%
Lactobacillus rhamnosus	LC177236.1	97%
Lactobacillus casei	LC064894.1	97%
B16		
Description of subject	Accession	Query coverage
Lactobacillus casei strain	KF445087.1	98%
Lactobacillus paracasei strain	HQ697645.1	98%
Lactobacillus casei strain	NR_115322.1	97%
Lactobacillus paracasei strain	MH698355.1	97%

Table 2: Colony morphology on different agar plate of the isolated colonies

Accession No	Colony Morphology
Mannitol Salt Agar plate	
B1	Yellow, circular, opaque
B2	Yellow, circular, opaque
MitisSalivarius Agar plate	
B9	Clear (no pigmentation), undefined (lawn), translucent
B10	Clear (no pigmentation), undefined (lawn), translucent
Rogosa Agar plate	
B15	White, circular, opaque
B16	White, circular, translucent

Table 3: The summarised result of biochemical analysis of isolated colonies.

Accession No.	Gram Stain	Catalase Test	Urease Test	Citrate Test	MR Test	TSI Test
B1	+	+	+	-	+	K/A
B2	+	+	+	-	+	K/A
B9	+	+	+	-	+	K/A
B10	+	-	-	-	+	K/A
B15	+	-	-	-	-	K/K
B16	+	-	-	-	-	K/K

+ : Positive Result (Gram-positive in case of Gram Stain);
- : Negative Result (Gram-negative in case of Gram Stain).

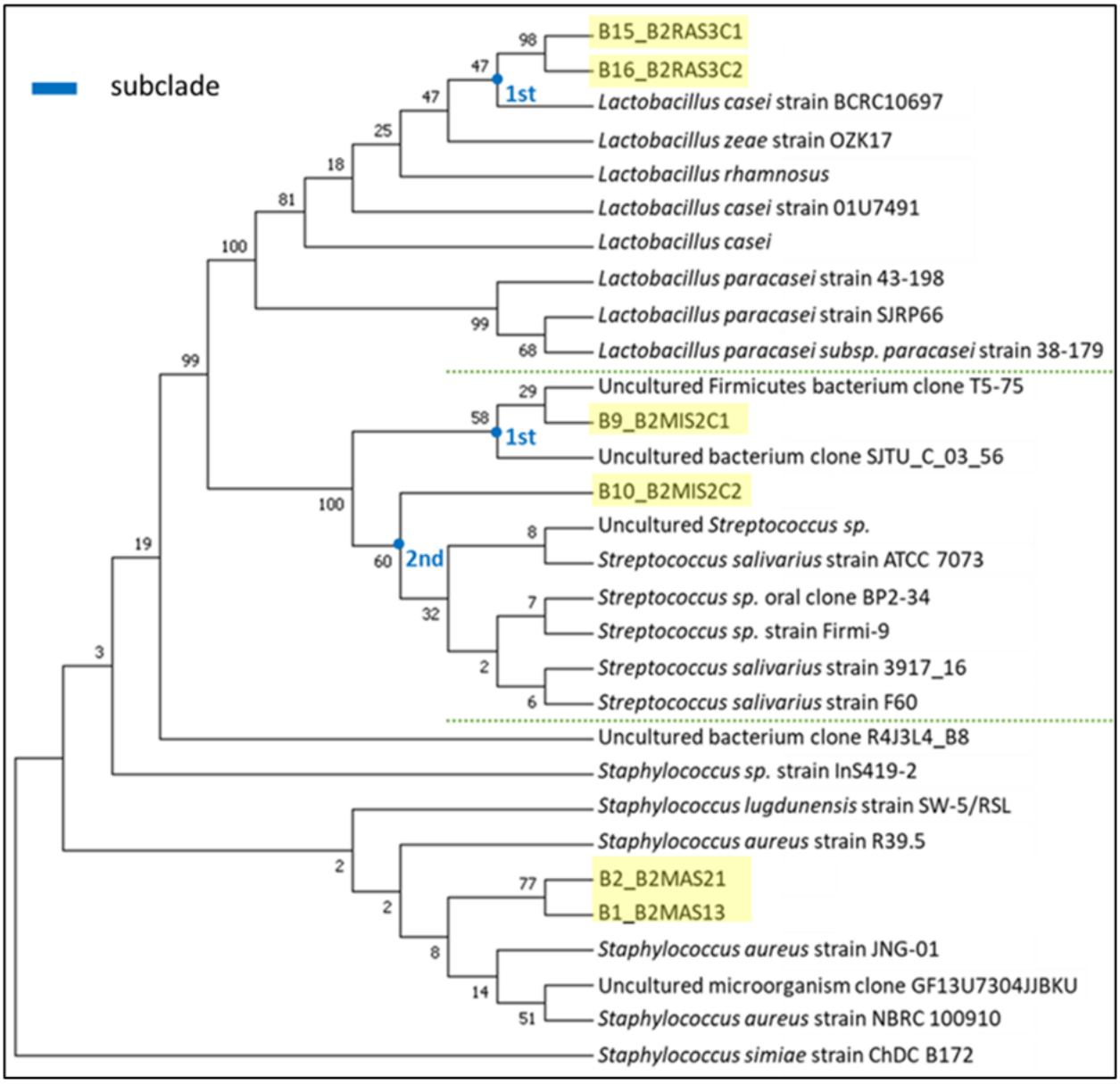

Figure 1: The phylogenetic tree was inferred using the Neighbour-Joining method with 1000 bootstraps. The analyze was conducted in MEGA-7

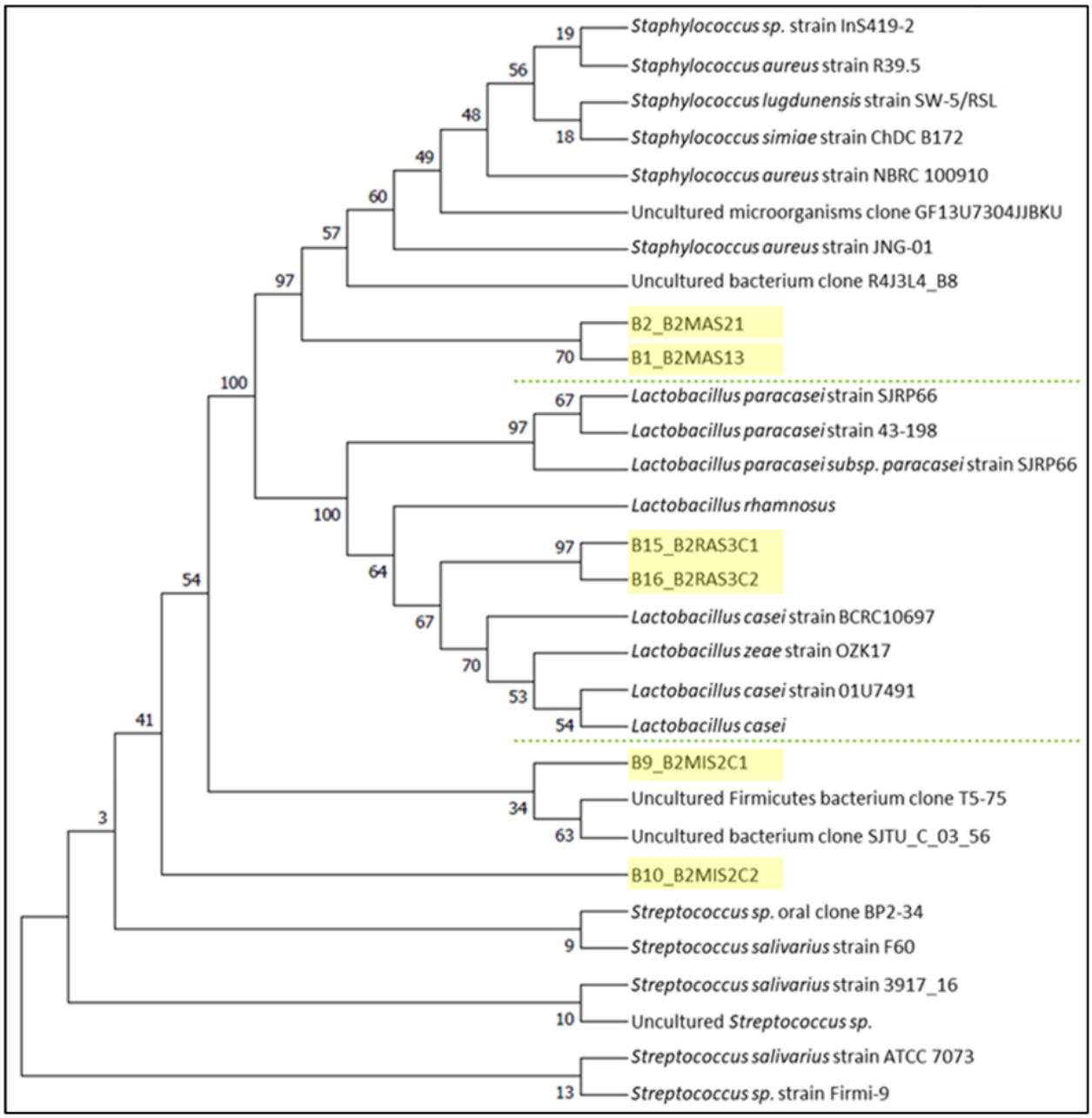

Figure 2: Phylogenetic tree based on MSA using Maximum-Likelihood method with 1000 bootstraps. The analyze was conducted by MEGA-7

A phylogenetic tree was constructed using the isolated bacterial strains with 95% BLAST hits and above. The tree presented their species level identification as well as their phylogenetic relationship with other species. Neighbour-Joining tree (Figure 1) and Maximum-Likelihood tree (Figure 2) both showed bacterial strain B1 and B2 categorized in *Staphylococcus sp.* database, bacterial strain B9 and B10 grouped in *Streptococcus sp.*database, and bacterial strain B15 and B16 classified in *Lactobacillus sp.* database.

In the Neighbour-Joining tree, the confidential level between bacterial strain B1 and B2 is 77%. Bacterial strain B9 have 29% of confidential level with Uncultured Firmicutes bacterium clone T5-75 in the first subclade in *Streptococcus sp.* database, while bacterial strain B10 have 60% confidential level with others *Streptococcus sp.* in the second subclade. The confidential level between these two subclades is 100%. The confidential level between bacterial strain B15 and B16 is 98%, and their confidential level between other homologous bacteria strains is 47% showed in the first subclade in the *Lactobacillus sp.* database.

Compare the confidential level of the bacterial strains in the Neighbour-Joining tree with

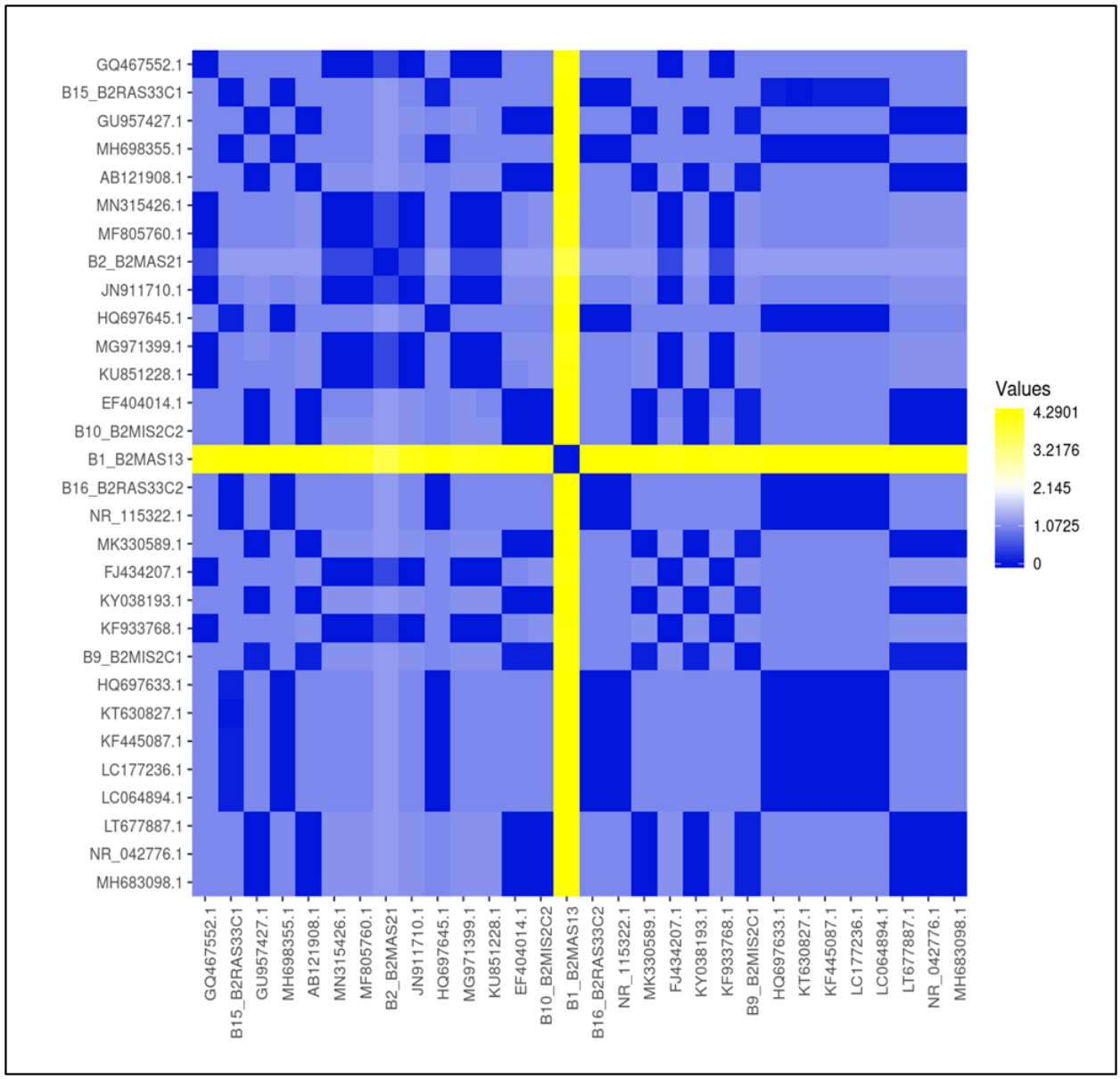

Figure 3: Heatmap of Euclidean distances between query and subject sequences (n = 30). The heatmap was constructed using Heatmapper

Maximum-Likelihood tree; bacterial strains B1 and B2 have a similar confidential level in both trees, which is 70% in the Maximum-Likelihood tree. But the confidential level between others *Staphylococcus sp.* have a large difference, the confidential level between the samples (B1 and B2) with other homologous bacteria strains is 97%. Similar results shown in the *Lactobacillus sp.* database, the confidential level between bacterial strain B15 and B16 is 97%, and the confidential level between other homologous bacteria strains is 67%. In the *Streptococcus sp.* database, bacterial strain B9 have 34% confidential level with Uncultured Firmicutes bacterium clone T5-75 and Uncultured bacterium clone SJITU_C_03_56. Bacterial strain B10 does not have any directly confidential level with other homologous bacteria strains, which indicating B10 undergo own evolution from others *Streptococcus sp.*

According to Bull *et al.* (1993), the confidence level of 70% is an indication of support, but without regard for the conditions under which this value was obtained, that is, equal rates of change, symmetric phylogeny and internodal change of 20% or less of the characters. But to meet these two conditions are unrealistic for real phylogenies. Thus, when all of these conditions are not met, bootstrap values of 50% or more may be overestimates of accuracy. This suspected that bootstrap values are a poor measurement to measure the accuracy of the phylogenetic tree as well. This can be proven by the

most significant results from the *Staphylococcus sp.* database in the sixth clade in the Neighbour-Joining tree. Theoretically, *Staphylococcus aureus* strain R39.5 in the second subclade, *Staphylococcus aureus* strain JNG-01 in fourth subclade and *Staphylococcus aureus* strain NBRC 100910 in the fifth clade should undergo the same subclade, but they were allocated in different subclade, and the confidential level between them were extremely low (2% - 14%). Unfortunately, they might also be a possible reason where most of the *Staphylococcus aureus* strain experienced the same evolution speed while *Staphylococcus aureus* strain R39.5 undergo faster evolution until they cannot be recognized by the algorithm as closely related species.

In the Neighbour-Joining tree, *Lactobacillus sp.* and *Streptococcus sp.* were extended from the same branch point. However, in the Maximum-Likelihood tree, *Staphylococcus sp.* and *Lactobacillus sp.* were expanded from the same branch point. A branch point indicates where two lineages diverged (Avissar *et al.*, 2014). Hence, when two lineages stem from the same branch point, there are sister taxa.

The first selection had been done during isolation of bacteria with a specific medium, selecting targeted bacteria. The isolated bacteria were first confirmed through biochemical characterization. Further confirmation was done through molecular characterization, in which phylogenetic trees had double confirmed the species of the samples. For example, in biochemical characterization, the most significant difference between bacterial strain B15 and B16 with another four bacterial strains (B1, B2, B9, B10) is the result of the MR test. Bacterial strain B15 and B16are MR test negative while bacterial strain B1, B2, B9, B10 are MR test positive, confirming B15 and B16 are *Lactobacillus sp.* In both phylogenetic trees, bacterial strain B15 and B16 are categorized in the *Lactobacillus sp.* database.

Molecular characterization results had reviewed that *Streptococcus sp.,* followed by *Staphylococcus sp.* are the highest population of oral bacteria presented in the oral cavity according to the phylogenetic tree and the clustering of the queries with the subjects. *Lactobacillus sp.* are the third-highest population of oral bacteria presented in the oral cavity as it was found from two out of four samples.

Pairwise Analysis

A pairwise distance matrix is used to compare the sequence distances within multiple sequences, between one sequence to another sequence. By comparing the sequences, the interrelations between the sequences can be intuitively grasped. The sequence distances were measured with Euclidean distance matrix, which Euclidean distance measure the true straight-line distance between two points in the Euclidean space. The heatmap indicated the overall similarity between the sequences, with the expectation of a diagonal line from the upper-left to the bottom-right (Figure 3). The diagonal line illustrated the sequences on the y-axis are totally same to the sequences on the x-axis, as the diagonal line is the meeting point of every same sequence.

CONCLUSIONS

Based on biochemical and molecular characterization, the most ubiquitous oral bacteria species that found in human saliva are *Streptococcus sp.*, *Staphylococcus sp.*, followed by *Lactobacillus sp.* These preliminary results will help to further analyze the virulence factors from the oral bacteria samples. The properties of the virulence factors will be useful in solving the problem of oral diseases and drugs invention for oral diseases.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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