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Pathogenic bacteria isolated from children and diabetic human tongue according to some characteristics

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ABSTRACT

Tongue microbiota is an important part of oral microbiome affecting on oral and body health. Sixty samples were collected from tongue of different people including 13.1% from children with milk feeding, 11.3% from children with normal feeding, 11.3% and 6.5% from first class - diabetes before and after Listerine respectively, 12.5 % and 9.5 % from first class – healthy before and after Listerine respectively, 10.1 % and 8.3 % from second class – diabetes before and after Listerine respectively, 10.1 % and 6.5 % from second class – healthy before and after Listerine respectively. 16S rRNA sequencing of 167 isolates showed 28 different bacterial species and Streptococcus salivarius (43 %) had the higher frequency followed by Staphylococcus aureus and Klebsiella pneumoniae (6 % for both). Nevertheless, Klebsiella pneumoniae (18.1%) was the predominant species in children with milk feeding while Streptococcus salivarius (42.1%) was the predominant species in children with normal feeding with the appearance of some bacterial species only in one of the two categories but not in the other. There are no significant difference between bacterial frequency in diabetic patients and healthy subject. However, several bacterial species were appeared only in diabetic patients. Thirty- two new type strains were isolated from tongue and 4 bacterial species were isolated for the first time from tongue. Listerine was significantly reduced the bacteria isolated from tongue by In vivo and In vitro. Moreover, Listerine mutagenic effect was recorded in 10 bacterial isolates. Sea gene was detected in 64 (51.2 %) bacterial isolates from tongue.

Keywords: *Tongue-microbiota, 16S rRNA, Diabetic patients, Streptococcus salivarius, Listerine, Sea gene*

INTRODUCTION

Oral microorganisms are important part of the human microbiome and one of the five research priorities (oral cavity, nasal cavity, vagina, intestine and skin), the study of oral microbiota is still a challenge for science because there are many bacterial species in oral cavity are unidentified and it is unclear what role they play in the oral microbial ecology, the mouth harbors more than 700 species of microorganisms identifying by 16S rRNA and distributed on different surfaces of the mouth (Turnbaugh et al., 2007; Jakubovics et al., 2015; Kilian et al., 2016; Staskova et al., 2019). The oral cavity composed of many surfaces. Microbiota of oral cavity were differ according to anatomical sites. Oral microorganisms are contribute to oral and body health, keeping them in balance of important to prevent progression oral diseases and maintaining the systemic health (Tuominen & Rautava, 2021; Giordano-Kelhoffer et al., 2022). Tongue – coating is a unique ecosystem characterized by microbial diversity because the tongue is the largest part of the oral cavity and the most stable environment due to papillary structure, it is essential to understand what constitutes this microbial community, it has been believed to have a serious and harmful imprint on general health (Jenkinson and Lamont, 2005; Moutsopoulos and Konkel, 2018; Lee et al., 2021). Tongue coating - microbiota is affected by many factors that affect on quantitative and qualitative balance in this community including age, hormonal changes at puberty, feeding type and diseases such as diabetes (Zaura &Ten Cate., 2015). Diabetes mellitus is a pandemic and long-term disease affects on person of all ages and causes serious complications that are correlated with the degree of hypoglycemia. People with diabetes are more susceptible to oral problems due to high glucose content in oral fluids contribute to an overgrowth of pathogenic microorganisms (American Diabetes Association, 2020; Balamanikandan et al., 2021). Milk fed, age and environmental can affect on tongue coating – microbiota in infants and throughout childhood. The bacterial species that colonized at this stage were lead to more diverse and stable ecosystem in adulthood (Grönlund et al., 1999; Oba et al., 2020a; Xiao et al., 2020). SEA (staphylococcal enterotoxin A) is a potent gastrointestinal exotoxin synthesized by S. aureus and considered as the main cause of staphylococcal food poisoning, due to its extraordinarily high resistance to proteolytic enzymes and heat treatment. Sea gene was the most common gene in the isolates recovered from food poisoning outbreaks. Moreover, human S. aureus isolates were harbor at least one of enterotoxin genes encoding for staphylococcal enterotoxins (SEs) and this carriage is a major risk factor for transmission from colonization to infection (Cha et al., 2006; Thomas et al., 2007; Argudín et al., 2010). It was necessary to use preventive methods, especially in people who are at risk of developing pneumonia such as elderly and immunocompromised patients due to the silent aspiration of bacteria residing in the oral cavity. Listerine is an antimicrobial mouth rinse, it was beneficial in maintaining the oral health and prevent bacterial pneumonia (Okuda et al., 1998a).

The aim of this study was to determine the frequency of tongue bacterial species in children with different feeding and in healthy or diabetes patients of first and second class. Furthermore, investigation the effect of Listerine on the tongue bacteria, as well as the enterotoxin (Sea gene) in different bacterial species beside S. aureus.

MATERIALS AND METHODS

Samples collection

Sixty samples were collected in the period between 20/10/2021 to 10/4/2022 from Al-Basrah peoples. Including 10 samples from children with milk feeding, 10 samples from children with normal feeding, 10 samples from (adult) first class of age (25 – 44) – Diabetes before washing with Listerine and 10 samples after Listerine, 10 samples from (adult) second class of age (60 - 75) - Diabetes before washing with Listerine and 10 samples after Listerine, and 20 samples from healthy people distributed in two classes according to the above ages. All samples were taken from the middle third of the tongue surface by sterile swab and transferred to falcon tube containing 5 ml of Brain heart infusion broth (OXIOD, U.K.) as a transport medium (Göhler et al., 2018) to culture on blood agar for isolating.

Identification the bacteria by 16SrDNA amplification

The DNA of 167 isolates including 22 isolates from children with milk feeding, 19 isolates from children with normal feeding, 19 isolates from

first class-Diabetes before Listerine and 11 isolates after Listerine, 21 isolates from first class - healthy before Listerine and 16 isolates after Listerine, 17 isolates from second class - Diabetes before Listerine and 14 isolates after Listerine and 17 isolates from second class - healthy before Listerine and 11 isolates after Listerine was extracted according to the procedure of Presto™ Mini g DNA bacteria kit (Geneaid, Taiwan) after refresh the bacterial pure colony in Brain heart infusion broth for 24h. at 37°C. 16S rDNA was amplified according to Miyoshi et al. (2005) using Alpha primers (Promega, USA) including 27F 5-AGAGTTTGATCCTGGCTCAG-3 and 1492 5-GGTTACCTTGTTACGACTT-3 of 1500bp. PCR reaction mixture (50µl) contains 25 µl of Go TaqGreen master mix (Promega, USA), 19 µl of Nuclease Free water (Bioneer, Korea), 2 µl of DNA template and 2 µl from each primers. The Verity thermo cycler (Applied Biosystem, USA) condition for amplification 94°C for 5min. followed by 35 cycles at 94 °C for 30 sec, 55.5° C for 45 sec and 72°C for 1min, the final extension was 72°C for 5 min. Agarose gel electrophoresis was performed (2 % of agarose powder, 100 ml of TBE buffer and 0.2 of Ethidium bromide) with 100bp DNA ladder (Promega, USA) to detect 16S rDNA bands at 1500bp under UV transilluminator (Wisd, Korea).

Amplification of Sea gene

Sea gene was amplified by PCR for 125 bacterial isolates (including all bacterial isolates from children with different feeding, bacterial isolates appeared in only before Listerine using and only after Listerine using) according to Omoe et al. (2005). The sequence of primers Forward: 5-CCTTTGGAAACGGTTAAAACG-3, Reverse: 5-TCTGAACCTTCCCATCAAAAAC-3. 25µl of PCR reagent mixture contains 12 µl of Go Taq Green master mix (Promega, USA), 2 µl of DNA template, 1 µl from each primers (Macrogen, Korea) and 9 µl of Nuclease Free water (Bioneer, Korea). The Verity thermo cycler was used with conditions for amplifying one cycle at 94°C for 5min. followed by 35 cycles at 94 °C for 35 sec. 55C for 35 sec. and 72°C for 1 min. Final extension at 72°C for 10 min. The bands at 127bp were detected on agarose gel electrophoresis and photographed under UV transilluminator (Wisd, Korea).

16SrDNA and Sea genes sequencing

Twenty µl of PCR product for 16S rDNA gene of 167 bacterial isolates and for 1 bacterial isolates of Sea gene were sending to Macrogen company " http://dna.macrogen.com" for purifying and sequencing. All the sequencing products were identified by BLAST https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch related to National Center for Biotechnology Information.

Phylogenetic tree

The Phylogenetic tree were drawn by MAFFT (Multiple alignment program for nucleotides sequences) http://mafft.cbrc.jp/alignment/server/ and viewed by forester 1064 after concatenated by comparison the result through Clustal Omega https://www.ebi.ac.uk/Tools/msa/clustalo/.

Detection the bacterial growth before and after Listerine (In vitro)

The present study was discovered this experiment to evaluate the effect of Listerine on bacterial growth directly. One isolate from each species was activate on BHIA at 37°C for 24 h, then a single colony of each isolate was inoculated in 15 ml test tube containing 2 ml of Nutrient broth (OXIOD, U.K.) and incubated at 37°C for 24 h, 0.1 ml from each tube was spread by L shape on plate of Nutrient agar (OXIOD, U.K.). On the other way, 1 ml of Listerine was added to the above NB with bacteria for 2 min, 0.1 ml from each tube containing Listerine was spread by L shape on plate containing NA and incubated at 37°C for 24 h and the differences of growth was determined by comparison between the growth of pre-culture and post-culture and the results were recorded as positive or negative. Lastly, the DNA of the bacterial species that showed low growth after exposed to Listerine were extracted and sequenced for comparison of 16S rDNA.

Comparison of 16S rDNA for isolates before and after Listerine (In vivo & In vitro)

"Clustal omega" has been used to compare between 16S rDNA sequences of isolates from tongue before Listerine and after Listerine to detect the mutations caused by Listerine.

RESULTS

Bacterial identification

16S rDNA for 167 isolates were obtained on agarose gel (1.5%) at a suitable size 1500bp (Figure 1). The identification was Streptococcus salivarius (n=72/43 %), Staphylococcus aureus and Klebsiella pneumoniae (n=10 /6 % for both), Rothia mucilaginosa (n=8/5 %), Neisseria subflava (n=7 / 4 %) , Staphylococcus epidermidis (n=6/4 %), Streptococcus parasanguinis, Neisseria mucosa and Neisseria perflava (n=5/3 % for each), Enterococcus faecalis, Staphylococcus haemolyticus and Lactococcus lactis (n=4/2% for each), Corynebacterium argentoratense, Staphylococcus saprophyticus, Escherichia coli and Neisseria macacae (n=3/2 % for each), Acinetobacter variabilis, Macrococcus caseolyticus and Bacillus cereus (n=2/1 % for each), Staphylococcus hominis, Kluyvera ascorbata, Acinetobacter bereziniae, Leclercia adecarboxylata, Citrobacter freundii, Acinetobacter ursingii, Lactococcus taiwanensis, Lactobacillus plantarum and Enterobacter tabaci (n=1 / 1 % for each) as Figure (2). However, four species (Enterobacter tabaci, Lactococcus taiwanensis, Acinetobacter variabilis, Macrococcus caseolyticus) were isolated for the first time in the world from tongue.

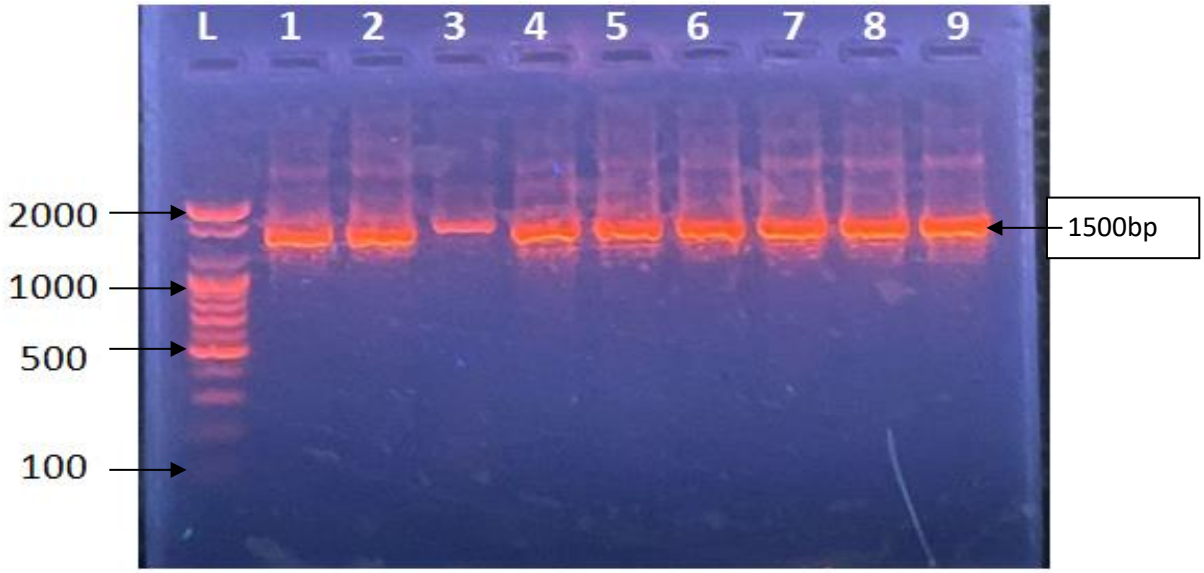


FIGURE 1: Agarose gel (2%) electrophoresis showing PCR product of 16S rRNA (1500bp). Lane L: 100 bp DNA ladder, lane 1-9: a model of 16S rRNA bacterial isolates.

Identification of new bacterial strains

Thirty-two new global strains of bacterial isolates were identified by comparing the nucleotide sequences with their type strains. The new strains databases (from IRQBAS139 to IRQBAS171) were recorded in DNA Data Bank of Japan (DDBJ), published on The National Center for Biotechnology Information (NCBI) and the Gene Bank.

Frequency of bacterial species in the tongue Tongue of children with milk and/or normal feeding

Twenty two bacterial isolates were obtained from 10 samples from tongue of children with milk feeding and 19 from 10 samples from tongue of children with normal feeding (Table 1). Klebsiella pneumoniae 4 (18.1%) from children of milk feeding and Streptococcus salivarius 8 (42.1%) from normal feeding had the higher frequency with significant differences at $P \leq 0.05$. Nevertheless, Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus saprophyticus, Macrococcus caseolyticus, Acinetobacter bereziniae, Kluyvera ascorbata, Leclercia adecarboxylata and Escherichia coli were present in children of milk feeding only but not in children of normal feeding. On the other hand, Neisseria macacae, Neisseria mucosa and

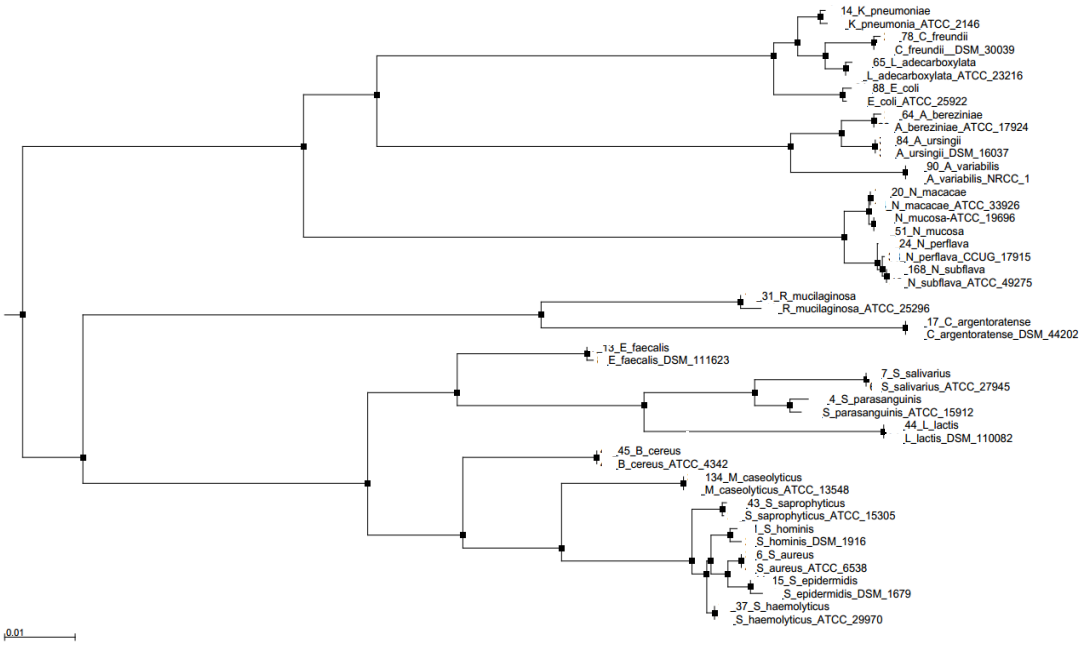


FIGURE 2: Rooted Neighbor Joining phylogenetic tree constructed from concatenated sequences of 16S rRNA with 1308bp and produced by a MAFFT alignment, visualized using forester 1046. This Neighbor Joining tree showing the distribution and Phylogenetic relationships of 24 different bacterial species isolated from tongue of human with their reference strains of several Gen Banks ATCC, DSM, CCUG and NRCC. All horizontal branch lengths were drawn to scale. Bootstrap values after 1000 repetitions areindicated.

*Isolates Lactococcus taiwanensis, Kluyvera ascorbata, Enterobacter tabaci and Lactobacillus plantarum were excluded for their short sequences.

Lactococcus lactis were present in children of normal feeding only but not in children of milk feeding with no significant differences.

TABLE 1 : Comparison of bacterial species from children of milk feeding (10 samples) and normal feeding (10 samples).

No.	Bacterial species	Milk feeding n (%)	Normal feeding n (%)
1	Klebsiella pneumoniae	4(18.1%)*	2 (10.5%)
2	Streptococcus salivarius	3(13.6%)	8 (42.1%) *
3	Staphylococcus haemolyticus	2(9.09%)	1 (5.2%)
4	Enterococcus faecalis	1(4.5%)	2 (10.5%)
5	Staphylococcus aureus	1(4.5%)	1 (5.2%)
6	Rothia mucilaginosa	1(4.5%)	1 (5.2%)
7	Staphylococcus epidermidis	3(13.6%)	0(0)
8	Staphylococcus hominis	1(4.5%)	0(0)
9	Staphylococcus saprophyticus	1(4.5%)	0(0)
10	Macrococcus caseolyticus	1(4.5%)	0(0)
11	Acinetobacter bereziniae	1(4.5%)	0(0)
12	Kluyvera ascorbata	1(4.5%)	0(0)
13	Leclercia adecarboxylata	1(4.5%)	0(0)
14	Escherichia coli	1(4.5%)	0(0)
15	Neisseria macacae	0(0)	2 (10.5%)
16	Neisseria mucosa	0(0)	1 (5.2%)

17	Lactococcus lactis	0(0)	1 (5.2%)
	Total	22 (13.1%)	(11.3%)

*= P≤0.05

Frequency of bacterial species in people of first class (25-44 years) with diabetes before and after Listerine

The effect of Listerine on bacterial growth in mouth was observed, Listerine has two ways of affecting: first, inhibitory effect and Second, lethal effect (bactericidal effect). Since, 19 bacterial isolates were obtained from 10 samples of people tongue with diabetes in first class before Listerine and 11 bacterial isolates after Listerine with significant differences (P≤0.05) as Table (2). Streptococcus salivarius had the higher frequency than other species with significant differences at P≤0.05. Importantly, Rothia mucilaginosa, Staphylococcus aureus, Staphylococcus haemolyticus, Neisseria mucosa and Corynebacterium argentoratense were absent after Listerine using.

TABLE 2: Comparison of bacterial species in people of first class-diabetes before and after Listerine

No.	Bacterial species	no. of sample	before n (%)	after n (%)
1	Streptococcus salivarius	10	9(47.3%)*	7 (63.6 %) *
2	Neisseria perflava		2(10.5%)	1 (9.09 %)
3	Neisseria subflava		1(5.2%)	1 (9.09 %)
4	Acinetobacter variabilis		1(5.2%)	1 (9.09 %)
5	Rothia mucilaginosa		2(10.5%)	0(0)
6	Staphylococcus aureus		1(5.2%)	0(0)
7	Staphylococcus haemolyticus		1(5.2%)	0(0)
8	Neisseria mucosa		1(5.2%)	0(0)
9	Corynebacterium argentoratense		1(5.2%)	0(0)
10	Staphylococcus saprophyticus		0(0)	1 (9.09 %)
	Total		19 (11.3%)*	11 (6.5 %)

*= P≤0.05

Frequency of bacterial species in first class of healthy people (25-44 years) before and after Listerine

Twenty one bacterial isolates were obtained from 10 samples from tongue of healthy people of first class before Listerine and 16 bacterial isolates after Listerine with no significant differences as Table (3). Streptococcus salivarius had the higher frequency with significant differences at P≤0.05. Staphylococcus saprophyticus, Staphylococcus epidermidis, Rothia mucilaginosa, Klebsiella pneumoniae and Streptococcus parasanguinis were absent after Listerine using.

TABLE 3: Comparison of bacterial species in people of first class-healthy before and after Listerine

No.	Bacterial species	no. of sample	before n (%)	after n (%)
1	Streptococcus salivarius	10	10 (47.6%)*	9 (56.2 %)*
2	Staphylococcus aureus		3 (14.2%)	3 (18.7 %)
3	Lactococcus lactis		2 (9.5%)	1 (6.2%)
4	Neisseria subflava		1 (4.7%)	1 (6.2 %)
5	Staphylococcus saprophyticus		1 (4.7%)	0(0)
6	Staphylococcus epidermidis		1 (4.7%)	0(0)
7	Rothia mucilaginosa		1 (4.7%)	0(0)

8	Klebsiella pneumoniae		1 (4.7%)	0(0)
9	Streptococcus parasanguinis		1 (4.7%)	0(0)
10	Bacillus cereus		0(0)	2 (12.5 %)
	Total		21 (12.5 %)	16 (9.5 %)

*= P≤0.05

The comparison between the isolates of people in first class - diabetes and first class - healthy before Listerine (Figure 3) showed Neisseria perflava, Neisseria mucosa, Staphylococcus haemolyticus, Acinetobacter variabilis and Corynebacterium argentoratense were present in people of first class diabetes but not in healthy, and Lactococcus lactis, Klebsiella pneumoniae, Staphylococcus saprophyticus, Staphylococcus epidermidis and Streptococcus parasanguinis were in healthy but not in patients with no significant differences.

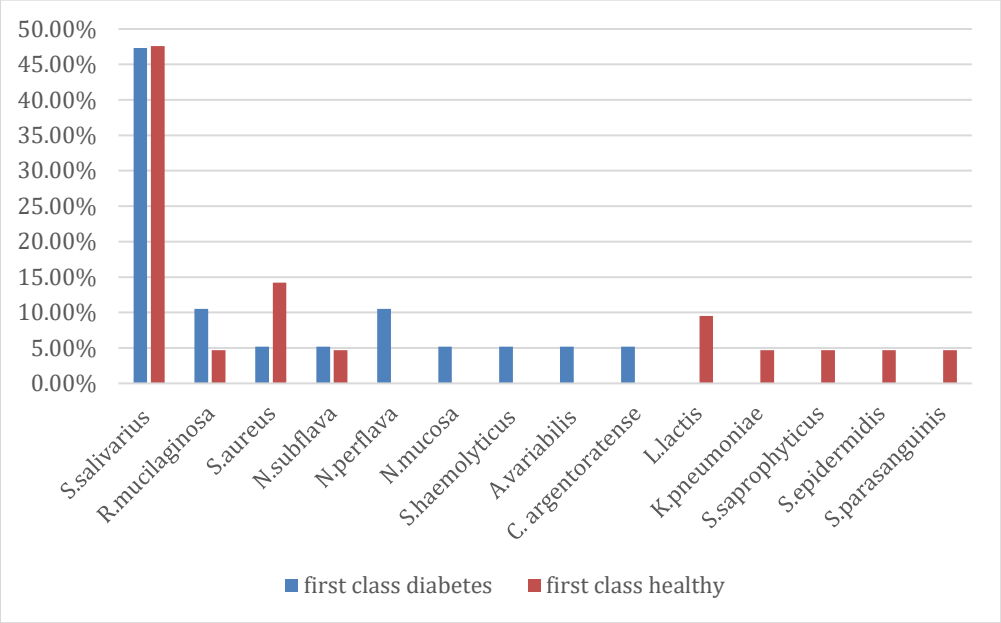


FIGURE 3: Comparison of bacterial species in people of first class - diabetes and first class - healthy before Listerine

Frequency of bacterial species in second class (60-75 years) of diabetes people before and after Listerine

Seventeen bacterial isolates were obtained from 10 samples from tongue of diabetes people of the second class before Listerine and 14 bacterial isolates after Listerine with no significant differences as Table (4). Streptococcus salivarius had the higher frequency with significant difference (P≤0.05) than other species. Rothia mucilaginosa, Enterococcus faecalis and Staphylococcus aureus were found in patients before Listerine but not after, while Neisseria macacae, Lactobacillus plantarum, Staphylococcus epidermidis and Lactococcus taiwanensis were in patients after using Listerine but not before.

TABLE 4: Comparison of bacterial species in people of second class-diabetes before and after Listerine

No.	Bacterial species	no. of sample	before n (%)	after n (%)
1	Streptococcus salivarius	10	6 (35.2%)*	4 (28.5 %)*
2	Neisseria subflava		2 (11.7%)	1 (7.1 %)
3	Klebsiella pneumoniae		2 (11.7%)	1 (7.1 %)
4	Streptococcus parasanguinis		1 (5.8%)	1 (7.1 %)
5	Neisseria mucosa		1 (5.8%)	1 (7.1%)
6	Corynebacterium argentoratense		1 (5.8%)	1 (7.1 %)
7	Escherichia coli		1 (5.8%)	1 (7.1 %)
8	Rothia mucilaginosa		1 (5.8%)	0(0)
9	Enterococcus faecalis		1 (5.8%)	0(0)
10	Staphylococcus aureus		1 (5.8%)	0(0)
11	Neisseria macacae		0(0)	1 (7.1 %)
12	Lactobacillus plantarum		0(0)	1 (7.1 %)
13	Staphylococcus epidermidis		0(0)	1 (7.1 %)
14	Lactococcus taiwanensis		0(0)	1 (7.1 %)
	Total		17 (10.1 %)	14 (8.3 %)

*= P≤0.05

Frequency of bacterial species in second class (60-75 years) of healthy people before and after Listerine

Seventeen bacterial isolates were obtained from 10 samples from tongue of healthy people staged in the second class before Listerine and 11 bacterial isolates after Listerine with significant differences (P≤0.05) as Table (5). Streptococcus salivarius had the higher frequency with significant difference than other species. Neisseria perflava, Neisseria mucosa, Acinetobacter ursingii, Citrobacter freundii and Staphylococcus epidermidis were found only in people before Listerine using and Enterobacter tabaci and Macrococcus caseolyticus were just in people after Listerine using.

TABLE 5: Comparison of bacterial species in people of second class - healthy before and after Listerine

No.	Bacterial spp.	no. of sample	before n (%)	after n (%)
1	Streptococcus salivarius	10	9 (52.9%)*	7 (63.6%)*
2	Rothia mucilaginosa		1 (5.8%)	1 (9.09 %)
3	Streptococcus parasanguinis		1 (5.8%)	1 (9.09 %)
4	Neisseria perflava		2 (11.7%)	0(0)
5	Neisseria mucosa		1 (5.8%)	0(0)
6	Acinetobacter ursingii		1 (5.8%)	0(0)
7	Citrobacter freundii		1 (5.8%)	0(0)
8	Staphylococcus epidermidis		1 (5.8%)	0(0)
9	Enterobacter tabaci		0(0)	1 (9.09 %)
10	Macrococcus caseolyticus		0(0)	1 (9.09 %)
	Total		17 (10.1 %)*	11 (6.5 %)

*= P≤0.05

The comparison between the isolates of people in second class - diabetes and second class-healthy before Listerine (Figure 4) appeared Neisseria subflava, Klebsiella pneumoniae, Staphylococcus aureus, Corynebacterium argentoratense, Escherichia coli and Enterococcus faecalis were present in people of second class - diabetes but not in healthy and Neisseria perflava, Acinetobacter ursingii, Citrobacter freundii and Staphylococcus epidermidis were in healthy only with no significant differences.

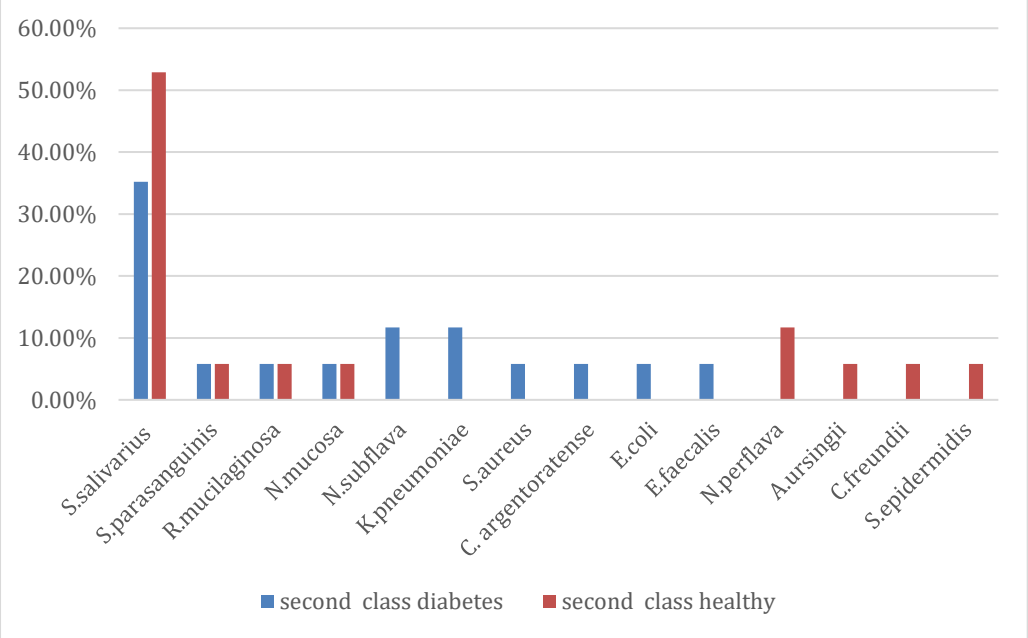
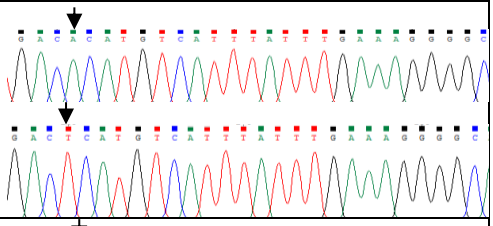
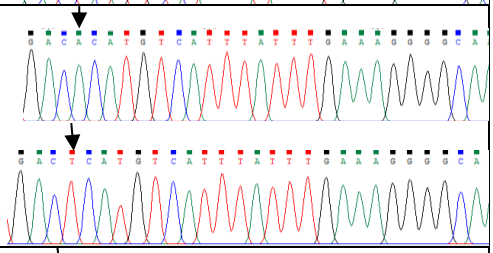
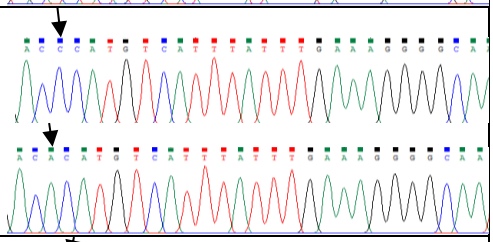
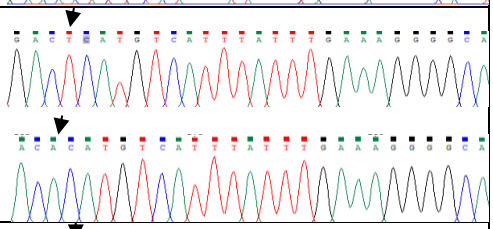
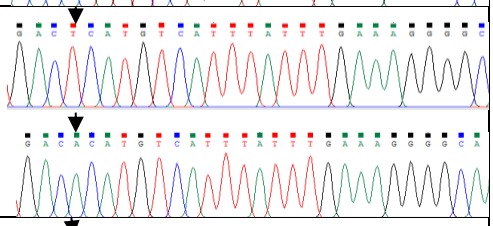
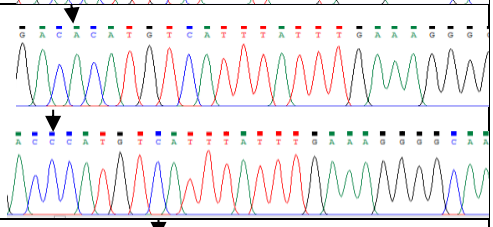
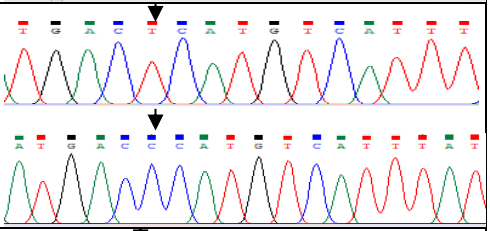
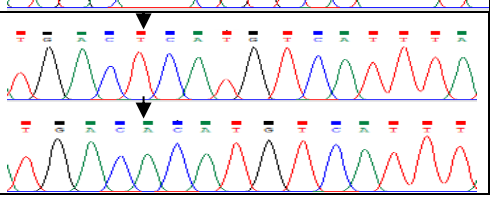


FIGURE 4: Comparison of bacterial species in people of second class - diabetes and second class - healthy before Listerine

The mutations in 16S rRNA gene by Listerine (In vivo)

Forty-two bacterial isolates were obtained from 40 tongue samples of people (healthy and diabetes) in the first and second classes before and after Listerine. In comparison, 32 bacterial isolates (before and after Listerine) were identical in their 16S rRNA gene sequences while 10 showed mutations in the gene after Listerine compared with that of before it (Table 6). Most of bacterial isolates recorded mutations at the same position of 16S rRNA gene sequence as the isolates 104, 118, 7, 100, 159, 139 and 151 was recorded mutation at the position 190bp and 216 bp respectively while the isolates 141 and 114 at the position 190 bp only.

TABLE 6: Mutations in 16S rRNA sequences of bacterial species before and after Listerine

Bacterial species	Mutation	Peak
104 Before-Streptococcus salivarius After	Transversion mutation (T instead A) at 190bp & (A instead T) at 216bp	
118Before - Streptococcus salivarius After	Transversion mutation (T instead A) at 190bp & (A instead T) at 216bp	
7 Before - Streptococcus salivarius After	Transversion mutation (A instead C) at 190bp & (T instead A) at 216bp .	
100 Before -Streptococcus salivarius After	Transversion mutation (A instead T) at 190bp & (T instead A) at 216bp	
159 Before -Streptococcus salivarius After	Transversion mutation (A instead T) at 190bp & (T instead A) at 216bp	
139 Before -Streptococcus salivarius After	Transversion mutation (C instead A) at 190bp & (A instead T) at 216bp	
151 Before -Streptococcus salivarius After	Transition mutation (C instead T) at 190bp	
114 Before -Streptococcus salivarius After	Transversion mutation (A instead T) at 190bp	

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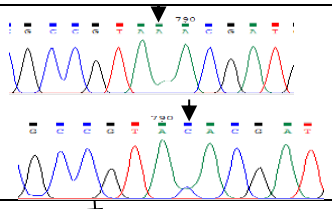
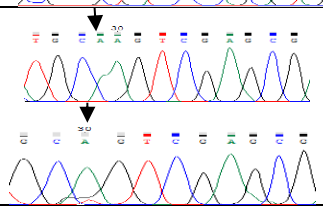
26 Before - Streptococcus salivarius After	Transversion mutation (C instead A) at 789 bp	
6 Before - Staphylococcus aureus After	Frame shift mutation (deletion A) at 29bp	

Growth of bacterial species before and after Listerine (In vitro)

Twenty two bacterial species showed a positive result (No or low growth) when exposed to Listerine for 2 min. (In vitro) as Figure (5). 19 bacterial species were of low inhibiton effect with significant difference at P≤0.05 and three species showed bactericidal effects. On the other hand, six bacterial species showed a negative result meaning they did not show any change in growth before and after exposure to Listerine (Table 7).

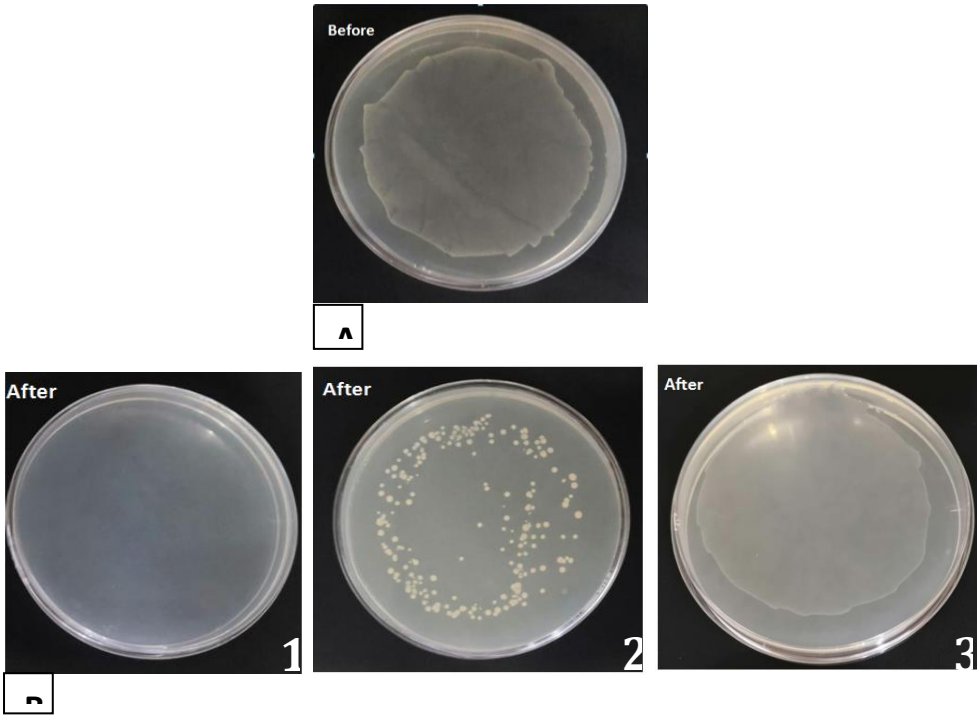


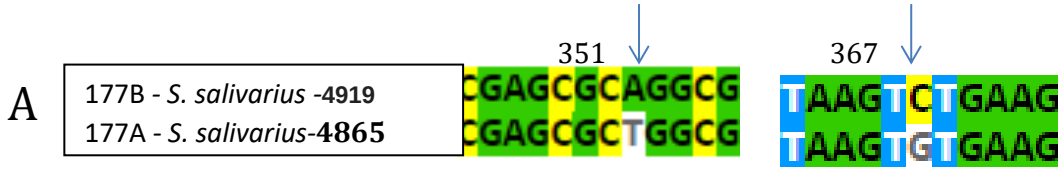
FIGURE 5: A: Growth of bacterial species Before adding Listerine .B: Growth of bacterial species After adding Listerine (1: no growth, 2: low growth or 3: growth).

TABLE 7 : Growth of different bacterial species before and after Listerine (In vitro)

No.	Bacterial species	No growth	Low growth	growth
1	Neisseria mucosa	+	-	-
2	Neisseria perflava	+	-	-
3	Neisseria subflava	+	-	-
4	Rothia mucilaginosa	-	+	-
5	Lactococcus lactis	-	+	-
6	Streptococcus salivarius	-	+	-
7	Streptococcus parasanguinis	-	+	-
8	Lactococcus taiwanensis	-	+	-
9	Klebsiella pneumoniae	-	+	-
10	Corynebacterium argentoratense	-	+	-
11	Acinetobacter variabilis	-	+	-
12	Neisseria macacae	-	+	-
13	Citrobacter freundii	-	+	-
14	Staphylococcus haemolyticus	-	+	-
15	Staphylococcus hominis	-	+	-
16	Kluyvera ascorbata	-	+	-
17	Acinetobacter bereziniae	-	+	-
18	Lactobacillus plantarum	-	+	-
19	Enterobacter tabaci	-	+	-
20	Leclercia adecarboxylata	-	+	-
21	Bacillus cereus	-	+	-
22	Escherichia coli	-	+	-
23	Macrococcus caseolyticus	-	-	+
24	Staphylococcus saprophyticus	-	-	+
25	Staphylococcus aureus	-	-	+
26	Acinetobacter ursingii	-	-	+
27	Staphylococcus epidermidis	-	-	+
28	Enterococcus faecalis	-	-	+
	Total	3 (10.7 %)	19(67.8 %) *	6 (21.4 %)

*= P≤0.05

Sequence of 16S rRNA gene were investigated for only 4 bacterial species (2 from gram negative bacteria and 2 from gram positive bacteria) that showed a positive result (low growth) after exposure to Listerine In vitro. Two bacterial species of gram negative bacteria: Klebsiella pneumoniae and Citrobacter freundii showed identical 16S rRNA gene sequences meaning that they did not induce a mutation in 16S rRNA gene sequence. While two bacterial species of gram positive bacteria appeared a mutation in 16S rRNA gene sequence. Since, 177B (Before Listerine) & A(After) Streptococcus salivarius has transversion mutations (T instead A) and (G instead C) at 351bp and 367bp respectively and 173B&A-Rothia mucilaginosa has a transition mutation (G instead A) and a transversion mutation (T instead G) at 68 bp & 69 bp respectively (Figure 6).



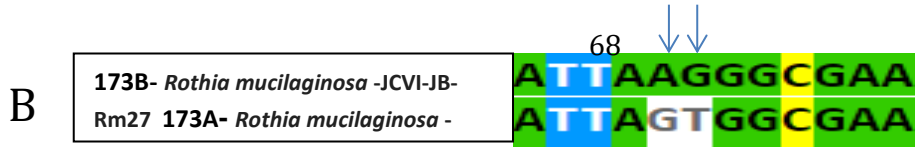


FIGURE 6: A: Transversion mutation, B: Transition mutation & Transversion mutation.

Amplification of Sea gene

The Sea gene bands were detected in 64 (51.2 %) isolates of the 125 tested isolates (Figure 7).

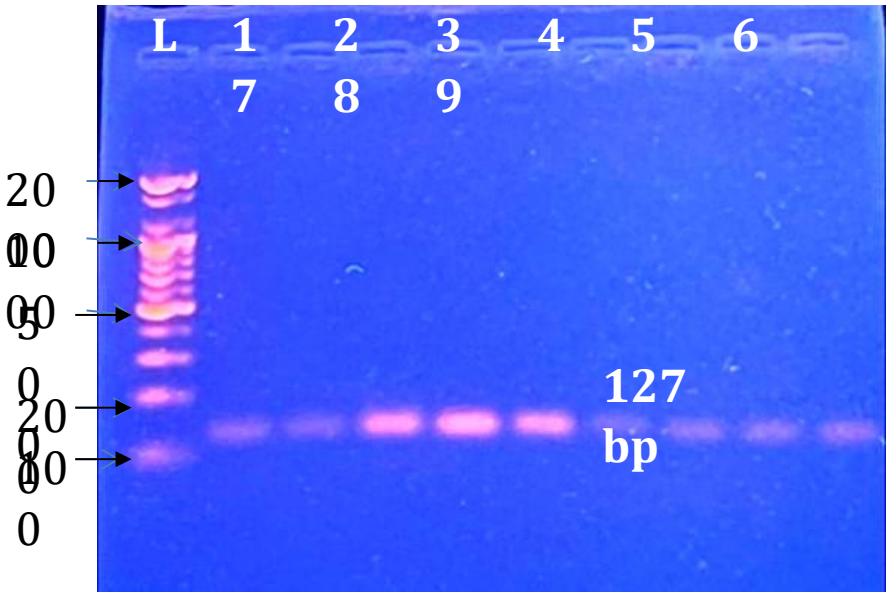


FIGURE 7: Agarose gel electrophoresis (2%) showing amplified Sea gene (127 bp). Lane L: 100 bp Marker, Lane 1-9: Sea gene bands of S. hominis, S. aureus, S. aureus, S. aureus, S. epidermidis, N. subflava, R.mucilaginosa, R.mucilaginosa and N.perflava, respectively.

Frequency of Sea gene among the different bacterial species

The Sea gene was recorded a high frequency in Streptococcus salivarius with significant difference at P≤0.05 (Table 7). Eighteen bacterial species gave a positive result for the presence of enterotoxin (Sea gene) including five species belong to Staphylococcus spp., the presence of Sea gene was previously recorded in these species. On the other hand, the present study was recorded, for the first time in the world, this gene in the other thirteen species (Streptococcus salivarius, Klebsiella pneumoniae, Rothia mucilaginosa, Lactococcus lactis, Enterococcus faecalis, Neisseria subflava, Neisseria perflava, Neisseria macacae, Escherichia coli, Corynebacterium argentoratense, Acinetobacter bereziniae, Acinetobacter ursingii and Citrobacter freundii).

TABLE 7 : Frequency of Sea gene among different bacterial species

No.	Bacterial species	no. of isolates	Sea gene positive n (%)
1	Streptococcus salivarius	45	25 (55.5 %) *
2	Klebsiella pneumoniae	9	4 (44.4 %)
3	Staphylococcus aureus	7	6 (85.7 %)
4	Rothia mucilaginosa	7	6 (85.7 %)

5	Staphylococcus epidermidis	6	4 (66.6 %)
6	Lactococcus lactis	4	3 (75 %)
7	Enterococcus faecalis	4	2 (50 %)
8	Staphylococcus haemolyticus	4	1 (25 %)
9	Neisseria subflava	4	1 (25 %)
10	Neisseria perflava	4	2 (50 %)
11	Neisseria macacae	3	1 (33.3 %)
12	Staphylococcus saprophyticus	3	2 (66.6 %)
13	Escherichia coli	2	2 (100 %)
14	Corynebacterium argentoratense	2	1 (50 %)
15	Staphylococcus hominis	1	1 (100 %)
16	Acinetobacter bereziniae	1	1 (100 %)
17	Acinetobacter ursingii	1	1 (100 %)
18	Citrobacter freundii	1	1 (100 %)
19	Neisseria mucosa	4	0 (0)
20	Streptococcus parasanguinis	3	0 (0)
21	Macrococcus caseolyticus	2	0 (0)
22	Bacillus cereus	2	0 (0)
23	Lactobacillus plantarum	1	0 (0)
24	Lactococcus taiwanensis	1	0 (0)
25	Kluyvera ascorbata	1	0 (0)
26	Leclercia adecarboxylata	1	0 (0)
27	Enterobacter tabaci	1	0 (0)
28	Acinetobacter variabilis	1	0 (0)
		125	64 (51.2 %)

*= P≤0.05

DISCUSSION

All the samples were grown on blood agar, a enriched medium supports the growth of all microorganisms (Zawadzki et al., 2016). 16S rRNA gene sequence is considered "gold standard" for identification and classification of bacterial at the species level (Woo et al., 2008). The variable regions of 16S rRNA gene sequences provide species-specific signature sequences useful for bacterial identification (Mahdi and abd Al-Abbas, 2021). As well as to detect the bacterial type strain as a result to the diversity in the nucleotides of the major gene as 16S rRNA (Chmagh and Abd-Al Abbas, 2019). On the other hand, the phylogenetic tree of tongue isolates didn't have Lactococcus taiwanensis, Kluyvera ascorbata, Enterobacter tabaci and Lactobacillus plantarum because they haven't enough size like other isolates. At the same time, four species recorded in the present study including Macrococcus caseolyticus, Acinetobacter variabilis, Enterobacter tabaci and Lactococcus taiwanensis which are neither present in the Human Oral Microbiome Database (http://www.ehomd.org) nor characterized in previous studies on the oral microbiome. Macrococcus caseolyticus have been found in cattle milk or meat products (Mašlaňová et al., 2018). Lactococcus taiwanensis, a lactic acid bacterium isolated from fresh cummingcordia (Chen et al., 2013). Acinetobacter variabilis have been found in human, animal and environmental spacimens of the hospital (Krizova et al., 2015; Manohar et al., 2019). A possible explanation for this finding might be due to the presence of leftovers in the tongue, while for the child, it may be transmitted from the surrounding environment during the feeding or acquired from the hospital environment.

In general, the formation of oral microbiome during infancy leads to enhanced oral health at later stages of the life. The microbial colonization in the tongue of infants may affected by several factors such as delivery method, age, environmental exposures (such as contacting with hospital equipment, staff and family) and feeding method. Clinical evidence proved, there are differences of microbiota in the tongue between breast-fed and formula-fed infants (Holgerson et al., 2013; Oba et al., 2020b). Breast milk contribute to the colonization of Streptococcus, Staphylococcus and Lactobacillus

in the oral cavity of infants (Al-Shehri et al., 2016; Soeorg et al., 2017). However, lactic acid bacteria isolated from breast milk were able to inhibit the growth of pathogenic bacteria such as *Enterococcus faecalis*, *Salmonella enterica*, *Listeria monocytogenes*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Escherichia coli* by producing Hydrogen peroxide (Uehara et al., 2001; Reis et al., 2016). This explains why the tongue of children with milk feeding is colonized with low rate of Streptococcus spp. leading to the emergence of bacterial pathogens. Since, most of children with formula-fed, mixture of formula and breast milk and a few of them depend on breast-fed. In addition, most of them have a prior history of hospitalization. Moreover, Oliveira et al. (2012) indicates that the presence of Enterobacteriaceae in oral cavity is an indicator to fecal - oral contamination, isolation these species from tongue of children at this age group may be a risk factor for pneumonia , diarrhea, bacteremia and meningitis in infants (WHO, 2005; Rosso et al., 2007; Tavares et al., 2020; Wolde et al., 2021; Zar et al., 2022). On the contrary, when compared with children of normal feeding (solid food), it was noted that the Streptococcus salivarius is the predominant species in tongue of children with normal feeding, its agreement with Milnes et al., (1993). The high rate of Streptococcus spp. due to the absence most of the aforementioned pathological species. The microbial diversity was decreased during childhood which is in agreement with Sampaio-Maia and Monteiro-Silva, (2014). This due to the fact that the bacterial population begin to change from aerobic or facultative gram-positive cocci to anaerobic fastidious gram-negative bacteria (Tanner et al., 2002).

Pathogenic bacteria is a major risk factor, especially in the immunocompromised host. Risk of infection in oral cavity depends on several factors including host defence mechanisms, presence of pathogenic bacteria and absence of normal flora (Haerian-Ardakani et al., 2015a)and host defense mechanisms is decline with the age and may become impaired in the elderly affecting the balance of resident tongue microbiota, the dorsum of the tongue becomes colonized by opportunistic pathogens such as Streptococcus is the major causative pathogen in maxillofacial space infections of diabetic patients (Ljiljana et al., 2008; Rao et al., 2010). *Streptococcus salivarius* is one of opportunistic pathogens involved in the formation of plaque that causes dental caries (Dye et al., 2007; Tahir & Nazir, 2018). In addition, Streptococcus salivarius can colonize the intestinal tract and cause alteration in gut micobiota, it is contribute in the intestinal inflammatory process and associated with severe human infections such as meningitis, endocarditis and bacteremia (Ruoff et al., 1989; Conte et al., 2006; Elsawy et al., 2018; Peng et al., 2022). In general, *Streptococcus* spp. was increases the risk of periodontal diseases and gastric cancer (Wu et al., 2018; Yuan et al., 2022). There was no significant difference between diabetic patients and healthy people in two classes but the following bacterial species Neisseria perflava, Neisseria mucosa, Staphylococcus haemolyticus, Acinetobacter variabilis and Corynebacterium argentoratense appeared only in diabetic patients of first class and the following bacterial species Neisseria subflava, Klebsiella pneumoniae, Staphylococcus aureus, Corynebacterium argentoratense, Escherichia coli and Enterococcus faecalis were appeared only in diabetic patients of second class which are considered as opportunistic pathogens in immunocompromised patients especially in the elderly people and a major risk of pneumonia, gastritis, otitis media, tonsillitis, periodontitis and dental root canals treatment failure (Stuart et al., 2006; Sumi et al., 2006; Rams et al., 2013; Alghamdi & Shakir 2020; Solsi et al., 2020. Eltwisy et al., 2022). Saliva in diabetic patients has low bacterial load with a different composition of the oral microbiome due to the acidification of saliva which allows the growth of anaerobic bacterial species that are more resistant to acids (Inchingolo et al., 2022).

Reduction in bacterial numbers on the tongue after using Listerine for 30 seconds *In vivo* and on bacterial species *In vitro* was observed. This is prove the efficiency of Listerine to maintain the low growth of bacteria on dorsal of tongue which is beneficial for oral cavity health, in addition to eliminate on bacterial pathogens. This results are agreement with several studies(Okuda, et al., 1998b; Sharma et al., 2004; Fine et al., 2005) carried out on bacteria in the saliva, reducing plaque and gingivitis and reduction the number of anaerobic bacteria on tongue that cause halitosis and the mutagenic effect of Listerine

mostly comes from its alcohol content. Mutation was recorded in the same position of 16S rRNA gene indicating to the ability of Listerine to affect on this site of DNA and most of the mutations occurred in the thymine and adenine bases, as they are linked by two hydrogen bonds. These mutations caused by Listerine can cause DNA damage leading to effect on the genetic stability of the cell (Haerian-Ardakani et al. 2015b; Milić et al., 2019).

The present study is the first to be conducted on Sea gene of all bacterial species isolated from tongue. the presence of Sea gene was recorded for the first time in different species that do not belong to the Staphylococcus spp. Oral bacterial communities tend to form biofilms which is important mechanism made them survival in the stress conditions, the processes of horizontal gene transfer and biofilm production are related, since horizontal gene transfer rates are often higher in biofilm communities than planktonic communities (Madsen *et al.,* 2012; Vestby et al., 2020). This explains the result of the present study in possibility of the gene transmission between the bacterial species endemic to the tongue. This is a major risk to immunocompromised paitent causing infections in their host due to the toxic effect and emetic activity of enterotoxin A, in addition to its superantigenic role in stimulating inflammatory cytokines(Ortega et al., 2010).

CONCLUSION

The tongue is a harbor of many bacterial species in addition to four species were isolated for the first time. The tongue of children with milk feeding recorded the highest bacterial frequency and diversity including Klebsiella pneumoniae as the predominant species while Streptococcus salivarius was the predominant species on tongue of children with normal feeding. There are no significant differences in bacterial counts between diabetic patients and healthy subjects but there was an appearance of pathogenic bacteria on the tongue of diabetic patients with different ages. Listerine is efficient in reducing pathogenic bacteria and causing mutations at a specific site in DNA. Sea gene was recorded in high frequency of different bacterial species isolated from tongue threating to cause an abdominal infection.

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