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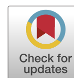
Original article

Isolation and identification of the oral bacteria and their characterization for bacteriocin production in the oral cavity



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ABSTRACT

Oral cavity is a diverse ecosystem which harbors immense diversity of microorganisms like fungi, virus and bacteria. Some of these microorganisms are involved in causing multiple infections. Oral flora is continuously changing due to connection with the external environment and produce bacteriocin against each other to compete for nutrient in this mini ecosystem. Current study was aimed to explore and compare the bacterial fauna of both healthy and non-healthy dental samples, by isolation and identification with biochemical tests to characterize the bacteriocin production. During study 120 swabs were taken from both healthy and unhealthy subjects. Samples were collected from the dental clinics of Makkah City, in sterile eppendorfs containing 1 ml nutrient broth, and were incubated overnight using shaking incubator. Bacteria were isolated following identification through Gram staining, microscopy and biochemical test. Total 15 strains of bacteria were isolated during the study amongst which 8 strains were gram positive and 7 strains were gram negative. The most dominant species of the gram positive strains was *Streptococcus pneumoniae* (n = 26). On the other hand, *Escherichia coli* (n = 26) was the prominent specie amongst the gram negative strains. Overall, the dominated family was Enterobacteriaceae (19.36%) followed by Streptococcaceae with 13.83% abundance. One of the most cariogenic strain *Klebsiella pneumoniae* (n = 14) was also isolated. The bacterial strain diversity between these two type of ecosystem was approximately the same, with slight variation in Shannon (HS:2.627187, NHS:2.653594) and Simpson diversity (HS:0.923461, NHS: 0.92684) index. The current research revealed that bacteriocin production in the *Enterobacter species* was prominent against *Escherichia coli* and *Klebsiella pneumoniae*. Apart from this other strains like *Klebsiella pneumoniae* and *Exiguobacterium spp* were also able to produce bacteriocin against *Enterobacter species* and *Bacillus cereus* respectively.

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1. Introduction

The oral cavity is an essential and a mini ecosystem of the human body (Albandar et al., 1999). The oral health of an individual depends on the presence of healthy indigenous micro flora on surface of gums, teeth and linings of oral cavity (Gerald et al., 2013). It is composed of variety of the organisms including virus, fungi, and bacteria. With the advancement of the molecular and microbiology techniques, more than 700 species have been discovered so far and still there are more waiting to be discovered (Albandar et al., 1999). The inhabitant organisms would have less probability of surviving in the environment which is pathological to the hosts. This type of situation where resident microbes loses homeostasis leads to onset of several oral diseases such as caries and periodontitis (Sharma et al., 2018; Albandar et al., 1999; Lim et al., 2020; Costalonga and Herzberg, 2014; Gotsman et al., 2007).

Furthermore, very little research has been carried out to understand the composition of oral flora and its component bacteria. Normally, the microorganisms are present on surface tissues of all human beings, like oral cavity for example. The number and type of these microbes varies with age, diet and personal hygiene levels of a person (Sharma et al., 2018). These oral bacteria are responsible for causing numerous systematic infections like bacterial endocarditis, respiratory pneumonia, osteomyelitis in children, preterm low birth weight, and cardiovascular disease (Jørn et al., 2005). In normal oral cavity, various species of the genus Streptococcus, Lactobacillus, Lactococcus, Enterococcus, Staphylococcus,

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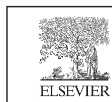

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Corynebacterium, Veillonella and Bacteroids are prominent (Rogers, 2008; Wang et al., 2012). Bacteriocin are long chain of the peptides produced by the bacterial ribosomes that are used to inhibit or kill another bacterium in order to compete in the ecosystem. Bacteriocin production by different members of oral community accounts for biodiversity and ecological suitability of microbes. Different species of natural inhabitant of oral cavity produced bacteriocin through quorum sensing and thus regulate the formation of oral flora (Kreth et al., 2005; van der Ploeg et al., 2005). Bactericion are further categorized into two major groups e.g. class I lantibiotics and class II non-lantibiotics. (Nes et al., 2013). Both Gram-positive and Gram-negative bacteria produce bacteriocin. The bacteriocin produced by Gram-positive bacteria are shorter containing 60 amino acids but they have broad spectrum. Amongst the gram positive bacteria, genus *Enterococcus, Lactobacillus, Lactococcus,* Leuconostoc, *Melissococcus, Oenococcus, Streptococcus, Weissella, Tetragenococcus and Vagococcus etc* produced bacteriocin. (Preciado et al., 2016). Keeping in view the above information, the current study was designed to explore and compare the bacterial fauna of both healthy and non-healthy dental samples, by isolation and identification in order to characterize the bacteriocin production.

2. Materials and methods

2.1. Ethical approval

The study was conducted at the Laboratory Medicine Department, Faculty of Applied Medical Sciences, Umm Al-Qura University, Makkah, Saudi Arabia, in accordance with the declaration of Helsinki and its amendments. The studies involving human participants were reviewed and approved by the Internal Review Board of the local Human Research Ethics Committee of Security Forces Hospital Makkah (SFHM) (Reference No. 0431–280621). The patients/participants provided their written informed consent to participate in this study.

2.2. Collection and isolation of bacteria

During this study, 120 adult subjects were selected for swabs collection including 43 healthy subjects while the remaining were suffering from the multiple infections like Dental plaque, carries and periodontal etc. The samples were collected from the local dental clinics in Makkah city. An inclusion criterion for the study was any random patient visiting dental clinic except children below the age of five or patients with communicable viral diseases. Samples were collected in sterilized eppendrof tubes containing 1 ml of nutrient broth and transferred to Laboratory Medicine Department, Faculty of Applied Medical Sciences, Umm Al-Qura University, where they were incubated overnight at 37C using shaking incubator. The pure bacterial cultures were obtained by inoculating the sample on nutrient agar media plates (contains Beef Extract (0.3%), Peptone (0.5%) and Agar (1.5%) in water). For this purpose, samples were streaked on nutrient agar plates with the help of sterilized inoculating loop. The inoculated nutrient agar plates were then incubated in thermal incubator for 24 h at 37C. After incubation the isolated bacterial colonies were picked from growth plates and quadrant streaking was done aseptically to new plate in order to obtain pure strains of bacterial culture. Four quadrants streaking were done by rotating the plates at 90° anticlockwise at four different areas of plate. This was done by dragging the culture across the agar with the help of sterilized inoculating loop from previously streaked area to new one. The plates were then incubated in thermal incubator at 37C for 24 h. After incubation isolated colonies were picked and cultured again to purify the samples.

2.3. Identification of bacterial strains

All pure bacterial isolates were processed initially using gram staining kit, according to the procedure of Hucker's modification (Hucker, 1921). The prepared slides were examined under the 100x magnification of microscope. Observations were made on the basis of colors and arrangement of bacterial cells. Gram positive bacteria were stained dark purple and those appeared as red or pink were gram negative bacteria. After Gram staining and microscopy, different biochemical tests were performed to identify bacterial strains. The basic biochemical tests used to identify bacterial strains includes Starch Test, Simon Citrate, Oxidase, Catalase, Voges Proskauer, Urease, Indole, Methyl Red and Coagulase Test.

2.4. Bacteriocin assay

Deferred antagonism bacteriocin assay was used to characterize the bacteriocin production according to the method reported by Rahman et al (Rahman et al., 2015). The experimental subjects were stabbed into the THY medium (Todd-Hewitt broth supplemented with 0.2% yeast extract) and incubated for 24-hrs at 37C using a candle jar. After the incubation, along with the producer strains, other strains were spread over the Petri dish and incubated for next 24-hrs and finally the diameter of zone produced around the producer strain was recorded.

3. Results

In the current study, species of different families were isolated including six families of the gram positive bacteria; Leuconostocaceae, Listeriaceae, Streptococcaceae, Staphylococcaceae, Bacillaceae, Corynebacteriaceae, and 5 families of gram negative; Neisseriaceae, Enterobacteriaceae, Pseudomonadaceae, Yersiniaceae, and Moraxellaceae, are shown in Fig. 1. The overall dominated family observed was *Enterobacteriaceae* (19.36%) having *Escherichia coli* as the most prevalent specie (53.06%) followed by *Klebsiella pneumoniae* (28.57%). One aggravating factor in this scenario was the emergence of β-lactamase-producing strains, which constitute the most important mechanism of resistance to β-lactam antimicrobials (Alghamdi, 2021). The main factors for oral contamination by these bacteria were poor hygiene, fecal-oral contamination, self-inoculation with toothbrushes and the use of antibiotics. The oral cavity could serve as a potential reservoir of Enterobacteriaceae, which are spread to the environment and to susceptible individuals through saliva. This fact becomes more important when considering the hospital environment, as most Enterobacteriaceae infections take place in this setting (Koneman et al., 2012; Jorge, 2007). Eating habits (diet) also have great influence on the complexity of the oral cavity community. For example, those infants on breast feed have predominated species of Lactobacillus gasseri in their oral cavity compared to the formula-fed infants (Holgerson et al., 2013; Urbaniak et al., 2012).

Apart from these significant findings, Streptococcaceae shows 13.83% abundance in two species only, the prominent species of which was *Streptococcus pneumoniae* (74.2 %) followed by *Streptococcus pyogenes* (21.5%) from both healthy and non-healthy oral cavities. Streptococcaceae can cause multiple infections including meningitis, bacterial pneumonia, endocarditis, erysipelas and necrotizing fasciitis. However, many streptococcal species are not pathogenic and form part of microbiota in the mouth, skin, intestine and upper respiratory tract (Patterson et al., 1996).

Fig. 1. Hierarchical Classification of the Isolates.

3.1. Healthy vs non-healthy oral cavities

The overall diversity index (Shannon and Simpsons) of healthy and non-healthy subjects were approximately the same as shown in Table 1. The data revealed no significant difference in the oral flora of two ecosystems i.e. healthy and non-healthy subjects. The reason behind the similarity can be attributed to the same geographic location and setting (clinic based) of the sample collection. Beside major similarity, there were still slight differences as 133 out of 253 of the pure isolated bacteria samples retained crystal violet staining; hence they are Gram positive bacteria. Amongst these 133 g positive strains, 59 strains were isolated from the healthy oral cavity and the remaining 74 strains were isolated from unhealthy subjects' oral cavity. In the healthy subject oral cavity, the dominated gram positive bacteria were *Weissella* confusa (20%), followed by the *Listeria monocytogenes* (17%) and *S. pneumoniae* (15%). Contrary to this, very less abundance of *S. pyogenes* (5 %) was observed for the healthy subjects.

In non-healthy subjects, the most dominant specie was *S. pneumoniae* (23%) followed by the *Exiguobacterium spp* (18 %) and *W.* confusa (14 %). Overall, the dominant species of gram positive bacteria in healthy and non-healthy oral cavity was *S. pneumoniae* (20%). Table 1 enlists the details of the identified bacteria. Total gram negative isolates were 120 out of 253, in which the most dominated species in both healthy and infected subjects was *E. coli* (22%) followed by the *Yersinia pestis* (19%). Amongst the healthy subjects oral cavity, the *E. coli* appeared to be a dominated specie (27%) followed by the *Y. pestis* (22%). On the other hands, amongst the infected subjects oral cavity, *Y. pestis* (20%) appeared as a dominant specie followed by the *E. coli* (17%).

3.2. Bacteriocin production

The Bacteriocin peptide produced by the strains against each other is given in the Table 2. Only limited number of bacterial strains was involved in the production of the Bacteriocin, amongst which the prominent activity was shown by the *Enterobacter* spp. (SS25) against the *K. pneumoniae* (SS32) and *E. coli* (SS28). Bacteriocin was also produced by other strains like *Exiguobacterium spp* (SS4) against *B. cereus* (SS30), *S. pyogenes* (SS39) against *S. pneumoniae* (SS1) and *K. pneumoniae* (SS32) against *E. coli* (SS28).

Table 1
Isolated species from Healthy and non-healthy subjects along with Shannon and Simpson index.

		Healthy subjects							Non-healthy Subjects						G. Total	total
S. No	ID	**Bacteria Strains Gram (+/-)**	**HS1**	**HS2**	**HS3**	**HS4**	**HS5**	**%**	**NHS1**	**NHS2**	**NHS3**	**NHS4**	**NHS5**	**%**	**(HS + NHS)**	**%**
1	SS1	*Streptococcus pneumonia (+)*	2	5	1	0	1	7.89	1	5	4	4	3	12.23	(9 + 17) 26	(10.27%)
2	SS2	*Pseudomonas spp (-)*	1	0	0	2	4	6.1	3	4	4	0	0	7.91	(7 + 11) 18	(7.11%)
3	SS3	*Yersinia pestis (-)*	1	1	1	2	5	8.77	3	4	0	4	2	9.35	(10 + 13) 23	(9.09%)
4	SS4	*Exiguobacterium spp (+)*	0	0	4	1	1	5.26	2	3	4	2	2	9.35	(6 + 13) 19	(7.50%)
5	SS16	*Neisseria spp (-)*	1	0	0	4	2	6.14	3	2	4	1	0	7.19	(7 + 10) 17	(6.71%)
6	SS20	*Weissella* confusa *(+)*	1	1	2	4	4	10.52	2	3	4	1	0	7.19	(12 + 10) 22	(8.69%)
7	SS25	*Enterobacter spp (-)*	1	0	0	2	1	3.50	1	2	1	1	0	3.59	(4 + 5) 9	(3.55%)
8	SS28	*Escherschia coli (-)*	4	4	5	1	1	13.15	2	1	3	3	2	7.91	(15 + 11) 26	(10.27%)
9	SS30	*Bacillus cereus (+)*	4	2	1	0	1	7.01	0	1	1	1	2	3.59	(8 + 5) 13	(5.13%)
10	SS31	*Corynebacterium spp (+)*	1	1	3	0	1	5.26	1	0	2	2	1	4.31	(6 + 6) 12	(4.74%)
11	SS32	*Klebsiella pneumonia (-)*	0	0	1	2	3	5.26	1	2	3	1	1	5.75	(6 + 8) 14	(5.53%)
12	SS33	*Listeria monocytogens (+)*	1	2	2	4	1	8.77	1	1	2	2	2	5.75	(10 + 8) 18	(7.11%)
13	SS34	*Staphylococcus epidermidis (+)*	1	1	0	2	1	4.38	1	2	2	3	1	6.47	(5 + 9) 14	(5.53%)
14	SS35	*Acinetobacter radioresistens (-)*	3	0	0	2	1	5.26	1	1	1	2	2	5.03	(6 + 7) 13	(5.13%)
15	SS39	*Streptococcus pyogens (+)*	0	1	0	1	1	2.63	1	1	0	2	2	4.31	(3 + 6) 9	(3.55%)
		Total	21	18	20	27	28	45.05	23	32	35	29	20	54.94	253	
Applied Statistics			Shannon (H)	2.627187	Simpson (D)	0.923461			Shannon (H)	2.653594	Simpson (D)	0.92684				

(Abbreviation: HS healthy subjects, NHS none-healthy subjects)

4. Discussion

Oral cavity of the human is a mini ecosystem which is comprised of different niche like dorsal and ventral side of tongue, buccal epithelium, hard palate, soft palate and supra-gingival plaque of tooth surfaces which is colonized by immense amount of microorganism including fungi, several types of virus and diverse bacterial fauna. Around 1100 different taxa were discovered from the oral cavity and recorded in the Human Oral Microbiome Database (Chen et al., 2010). The complex community of the buccal cavity principally contain Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes and Fusobacteria with only 4% of species belong to other phyla (Bik et al., 2010) as shown in the Fig. 2 (Ahn et al., 2012). Some of these are very beneficial while other may cause some serious infections. Some of the worthy bacteria may shift their life style from the beneficial to harmful and cause serious oral infections (Ahn et al., 2012). So the current study was aimed to explore the healthy oral cavity diversity in comparison to the oral cavity of non-healthy subjects and ultimately to assess the bacteriocin production among these floras. Since the goal of the present study was to isolate and identify oral bacteria and to characterize them for bacteriocin production. The results acquired clearly demonstrated that 15 oral bacterial strains were successfully isolated and were identified as shown in Table 1. The acquired results also indicate that only limited number of the bacterial strains were able to produced bacteriocin as discussed already. The dominated activity was observed by the *Enterobacter spp*. of family *Enterobacteriaceae*. The occurrence of the *Enterobacteriaceae* in the buccal cavity appears to be unusual. Results of these kinds are very important due to high resistance towards the multi-drugs (antibiotics) (Hariharan et al., 2015). Besides this, the oral cavity has the ability to harbor a wide range of these type of microorganisms and whenever the host (carrier) suffered from any kind of imbalances, bacteria can enter the system and worsen the sickness. Accommodating the high multi-drug resistant enterobacter in the buccal flora can promote high range of disease. This showed that oral cavity can also act as reservoir for these pathogens, which could results in the spread of bacterium to the environment through different ways (Aragão, et al., 2016).

During our study it was revealed that *S. pyogenes* was the least abundant specie amongst the identified with a prevalence of only 5%. In non-healthy subjects the most dominant specie was *S. pneumoniae* with a prevalence of 23 % followed by the *Exiguobacterium spp* (18%) and *W.* confusa (14%). The overall dominant species of gram positive bacteria in healthy and non-healthy oral cavity was *S. pneumoniae* (20% abundance). Detail of the bacteria identified is given in the Table 1. Though the main flora of the buccal cavity appeared to be similar throughout but they did vary in different individuals due to many reasons like variation in habitat/ecosystem and eating habits etc. This argument can be better supported by a study conducted by *Nasidze* and their colleagues in 2009. They collected samples from 120 healthy individuals living in different places across the world and concluded that bacterial species isolated from these samples were highly diverse (Nasidze et al., 2009). Another study conducted in south America revealed that a geographically isolated Amerindian tribe has microbial patterns comprised of 62% of bacterial families and 23% of the bacterial genera and had a lower alpha diversity when compared with non-Amerindians (Contreras et al., 2010).

5. Conclusion

The current research revealed that bacteriocin production in the *Enterobacter species* was prominent against *E. coli* and *K. pneumoniae*. Apart from this other strains like *K. pneumoniae* and

Table 2
Bacteriocin production by bacterial strains against each other.

producer	SS1	SS2	SS3	SS4	SS16	SS20	SS25	SS28	SS30	SS31	SS32	SS33	SS35	SS39
SS1	-	-	-	-		-	-	-	-	-	-	-	-	-
SS2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS3	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS4	-	-	-	-	-	-	-	-	+	-	-	-	-	-
SS16	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS20	-	-		-	-	-	-	-	-	-	-	-	-	-
SS25	-	-	-	-	-	-	-	++	-	-	++	-	-	-
SS28	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS30	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS31	-	-		-	-	-	-	-	-	-	-	-	-	-
SS32	-	-	-	-	-	-	-	+	-	-	-	-	-	-
SS33	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS35	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS39	+	-	-	-	-	-	-	-	-	-	-	-	-	-

(+indicates moderate level of bacteriocin production, - indicates no bacteriocin production, ++ indicates increased level of bacteriocin production)

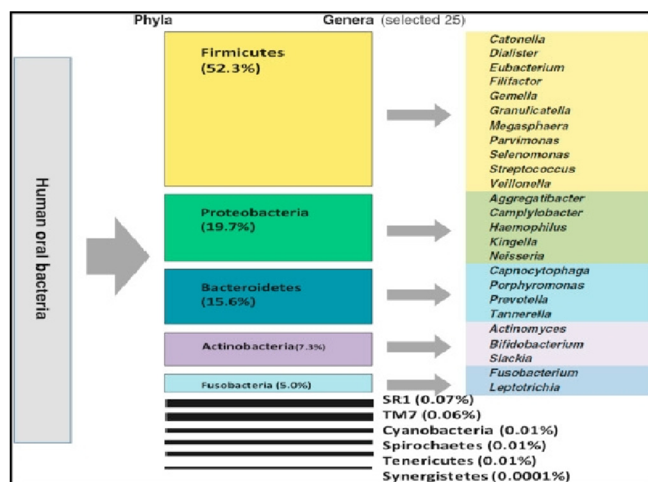

Fig. 2. Composition of Normal Bacterial Flora in Human Oral Cavity (Ahn et al., 2012).

Exiguobacterium spp were also able to produce bacteriocin against *Enterobacter species* and *B. cereus* respectively. Further molecular and biochemical studies are required to explore the diverse and changing community of oral cavity and to understand the chemical and molecular nature of observed bacteriocin. With the data acquired from this research we can also explain the role of disease-associated pathogen in affecting human health. Also if we compare and statistically analyze significant number of samples then it will give us a broader opportunity to explore more precisely the oral micro flora.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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