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Study on the prevalence of bacterial flora in the oral cavity of healthy individuals in Pondicherry

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Abstract

To determine the bacterial flora in the oral cavity of healthy individuals in Pondicherry. In this present study a total of 200 buccal samples from healthy individuals from age group 35±20 were included in the study. Blood agar was used for the isolation of bacteria from the oral cavity. Identification was based on the Colony morphology, Microscopy, Biochemical tests and 16S rRNA technique. The most predominantly isolated bacteria among healthy individuals was *Staphylococcus aureus* (122 isolates, 56%). 41 isolates of *Micrococcus* sp. (18.8%), 13 isolates of *Lactobacillus* sp.(6%), 12 isolates of *Klebsiella* sp.(5.5%), 8 isolates of *Serratia* sp (3.7%), 5 isolates of *Pseudomonas* sp (2.3%), 3 isolates of *Enterobacter* sp(1.4%), 2 isolates of *Proteus* (0.9%), 1 isolate of *Streptococcus pyogenes* (0.5%), 1 isolate of *Salmonella* (0.5%)and 1 isolate of *Cronobacter sakazakii* (0.5%). The microenvironment of the oral cavity harbours a wide variety of microorganisms. The normal flora of each individual also varied due to dietary changes or poor oral hygiene. Bacteria were isolated from the oral cavity and their prevalence was studied. *Staphylococcus aureus* was the predominant microflora of the oral cavity isolated in this study.

Keywords: Oral cavity, *Staphylococcus aureus*, *Cronobacter sakazakii*, Blood agar and Bacterial isolates.

Introduction

The oral health of the individual is very important since it plays a major role in the well-being of a person, the oral diseases and disorders may have a negative impact throughout the life span of an individual and the scientific research stands critical in decreasing the impact of the oral disease. (National Institute of Health, 2000) Joshua Lederberg coined the term microbiome to represent the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space but have been ignored as determinants of health and disease.(Lederberg andMccray,2001) Now research has been carried out to study the oral microbiome, the peculiar ecology of the mouth and the essential role it portrays in oral and overall health of the individual. Comparing the entire microbiome of the entire human body, the oral cavity which is the mouth contains the most diversified microbiomes constituting over 600 prevalent taxa at the species level with different organisms in different habitats of the oral cavity like the teeth, gingival sulcus, attached gingiva, tongue, cheek, lip , hard and soft palate. At any one time an estimated 20 billion resident microbesare present in the oral cavity. (Dewhirst et al., 2010). Different intraoral surfaces had different proportions of bacterial species (Mager et al., 2003).

The pathogenic bacteria cannot establish themselves in the mouth even though the oral cavity is a gateway of many pathogenic bacteria because the pathogenic bacteria are removed by the normal flora formed in the oral cavity. So most infections are caused by the oral bacteria and are linked with systemic diseases. (Demmer et al., 2006 and Li et al., 2000). The oral cavity is maintained at homeostasis. Each microorganisms colonizes its own ecological habitat

that is, the microbes occupy different surfaces within the mouth and thus the oral cavity is maintained at homeostasis. (Slavkin, 1999) There may be a shift in homeostasis of the oral cavity due to increase in glucose consumption and acidophilic bacteria may cause damage to the teeth, which may result in dental caries. (Burt and Pai, 2001) As the oral cavity is warm and moist and there is a constant intake of nutrients through saliva and food intake, the mouth is a favorable environment for the growth of microorganisms (Loesche, 1986). The study of oral Microbiology is complex and difficult since the normal oral flora constitutes a diverse group which includes bacteria, fungal, protozoa and even viruses. (Jackson et al. 2000; Aas et al., 2005). The normal oral flora mostly comprises of streptococci and anaerobic gram negative bacteria (Aasetal., 2005 and Bagg, 1990). Pneumonia may be caused by Staphylococci and aerobic gram negative bacteria which are transient colonisers of the oral cavity. Alterations in the microenvironment of oral cavity occur due to systemic changes because of disease, Diabetes, pregnancy and the influence the proportion and type of oral flora. (Bagg 1990and Boggess 2006)Prevalence of gram positive and gram negative bacteria are involved in dental diseases and their presence increases with severity of the disease (Yacoubi, 2013) . Among denture wearers, high prevalence of Enterobacteriaceae are seen, when compared to normal population. (Goldberg et al., 1997).

Materials and Methods

Collection of buccal swabs

Approval for the present study was provided by the Institutional Ethics Committee of Madras Diabetes Research Foundation. The study was carried out in collaboration with Dr. Mohan's Diabetics and Endocrine Specialities, Pondicherry.

All the subjects used in this study had voluntarily signed the informed consent. A structured questionnaire was developed and provided to the subjects for gathering information on demographics like age and gender, local factors like denture status, oral hygiene and smoking. Blood samples were used to measure the fasting blood glucose level.

Buccal swabs (Al- Muala et al., 2014) were obtained from 200 healthy subjects chosen randomly (127 females and 73 males with the age range 18 to 55 yrs) They were volunteers from staffs working in the clinics, students, lab assistants and mostly attendees accompanying the diabetic individuals to the clinic.

Initially oral examination was done, then the buccal swab was got from healthy individuals. Samples were collected from 6.30 to 9.00 am when the individuals had fasted.

Processing of Specimens

The buccal swabs were streaked onto Blood Agar (AL- Muala, 2014) and incubated at 37^0C for 24 hrs. After 24 hrs the plates were observed for hemolysis since blood agar is a differential medium and results were recorded as hemolytic and non hemolytic organisms.

Phenotypic Characterization of Bacteria

Phenotypic characterization of bacteria refers to all the expressed features of the bacteria such as morphological, biochemical and phylogenetic properties.

Morphological Characterization of Bacteria included observing the colony morphology of the isolate, Gram staining and Motility.

Biochemical Characterization of Bacteria

Conventional biochemical tests were performed for species identification according to the standard methods described by Holt et al,1994. Collee et al., 1996 and Mac Faddin, 2000.

Catalase, Oxidase, Indole Production, Methyl Red Test, Voge Proskauer test, Citrate Utilization, Urease test. Special test were also performed using selective media using Mannitol Salt Agar,coagulase test, Lactobacillus MRS Agar, Bismuth Sulphite Agar.

Phenotypic and phylogeneticcharacterization of Bacteria

One of the morphological and biochemically identified isolate was further subjected to phylogenetic analysis using 16S rRNA sequencing.

The template DNA was prepared from the pure cultured strain LR-12. Colonies are picked up with a sterilized toothpick, and suspended in 0.5 ml of sterile saline in a 1.5 ml centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet is suspended in 0.5 ml of InstaGene Matrix

(Bio-Rad, USA). Incubated 56℃ for 30 min and then heated 100℃ for 10 min. After heating, supernatant can be use for PCR.

PCR Add 1 µl of template DNA in 20 µl of PCR reaction solution. Use 518F/800R primers for bacteria, and then perform 35 amplification cycles at 94℃ for 45 sec, 55℃ for 60 sec, and 72℃ for 60 sec. DNA fragments are amplified about 1,400 bp in the case of bacteria. Include a positive control (*E. coli* genomic DNA) and a negative control in the PCR.

Purification of PCR products Remove unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing. The purified PCR products of approximately 1,400 bp were sequenced by using the primers (785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT 3'). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

Results

A total of 200 buccal swabs were collected from healthy individuals. The subjects included in the studies (127 females and 73males) belong to the age group 18 to 55 of which 4 had smoking habits 6 were denture wearers, 62 subjects had teeth caries and none of the subjects had oral lesions (Table. 1).

Table .1 Distribution of different factors between male and female subjects.

S.No	Factors	Male	Female
1.	Age range	18 – 55	18 - 55
2.	Caries	18	44
3	Smoking habits	4	0
4.	Lesion	0	0
5.	Fasting Blood glucose level (mg/dl)	75 – 100	75 -100
6.	Denture wearer	1	5

Out of the 200 samples screened, 194 samples showed significant growth on blood agar. A total of 218 isolates were obtained of which 9 isolates did not recover when sub cultured. (Table.3)

Table. 2 Number of bacterial isolates got from buccal cavity

S.No	Bacterial isolate	No. of isolates	% of isolates
1	*Cronobacter sakazakii*	1	0.5
2	*Enterobacter* sp.	3	1.4
3	*Klebsiella* sp.	12	5.5
4	*Lactobacillus* sp.	13	6.0
5	*Micrococcus* sp.	41	18.8
6	*Proteus* sp.	2	0.9
7	*Salmonella typhi*	1	0.5
8	*Serratia marcescens*	8	3.7
9	*Streptococcus pyogens*	1	0.5
10	*Staphylococcus aureus*	122	56.0
11	*Pseudomonas* sp.	5	2.3
12	Unidentified	9	4.1
13	Total	218	100

Isolation of bacteria from buccal cavity specimens

The samples which were streaked onto blood agar were subcultured separately and used for morphological and biochemical characterization. The 218 bacterial isolates were identified based on morphological and biochemical characteristics. The percentage of the isolates obtained is represented in Figure 1. Out of the 218 bacterial isolates 9 isolates did not grow when subcultured and the remaining 209 cultures were identified. The distribution of gram negative and gram positive organisms are 14.67% and 81.19% respectively and 4.1% isolates remain unidentified and in healthy subjects Fig.2. The biochemical characteristics for Gram negative bacteria and Gram positive bacteria are shown in Table 4 and Table 5.

Figure 1. The percentage of the bacterial isolates obtained from Buccal cavity

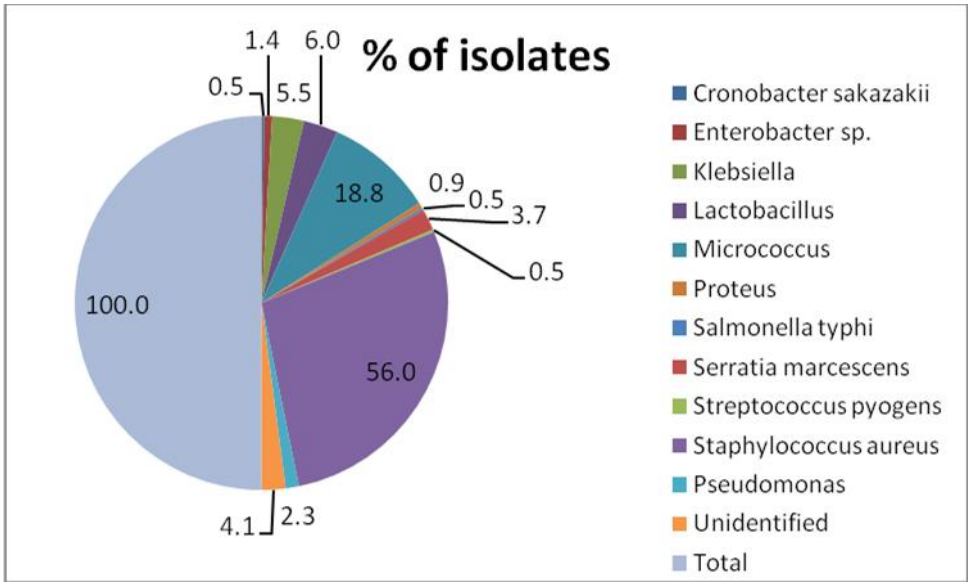


Figure.2 shows the distribution of gram negative and gram positive organisms in healthy subjects

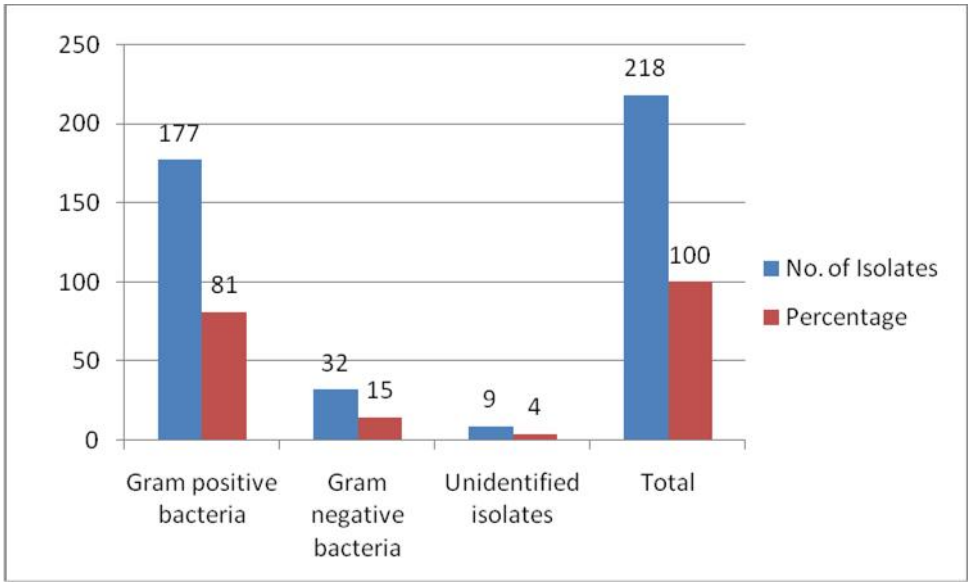
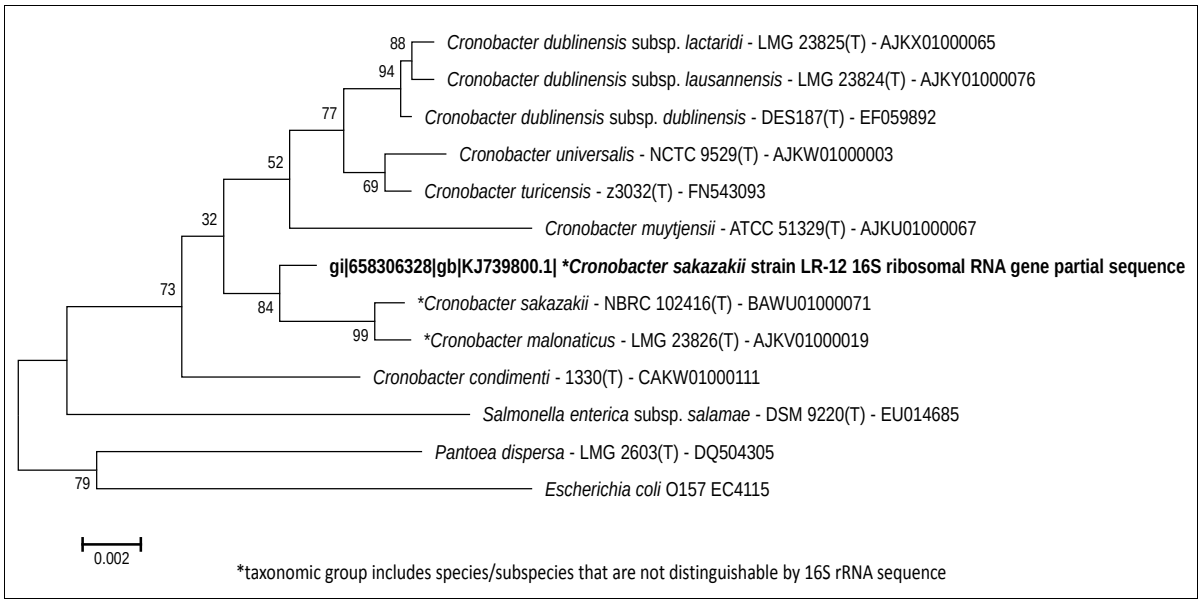


Fig. 3. Showing the phylogenetic re-construction of the strain LR-12, using the neighbor joining phylogenetic tree.



This result also clearly revealed that the isolate could be belonging to a taxonomic group, that may not resolved fully only using the 16S rRNA gene sequence.

Table. 4 Biochemical Characteristics of Gram negative bacteria

Bacterial Isolates	Biochemical tests								
	Gram Staining	Motility	Catalase	Oxidase	Indole	MR	VP	Citrate	Urease
Citrobacter sp.	Gram negative rods	+	+	-	+	+	-	+	+
Enterobacter aerogens	Gram negative rods	+	+	-	-	-	+	+	-
Klebsiella sp.	Gram negative rods	-	+	-	-	-	+	+	+
salmonella typhi	Gram negative rods	+	+	-	-	+	-	+	-
Pseudomonas sp.	Gram negative rods	+	+	+	-	-	-	+	-
Proteus sp.	Gram negative rods	+	+	-	+	+	-	-	+
Strain LR-12	Gram negative rods	+	+	-	-	-	-	+	-
Serratia marcescens	Gram negative rods	+	+	-	-	-	+	+	-

+ = Positive - = Negative

Table.5 Biochemical Characteristics of Gram positive bacteria

Bacterial Isolates	Biochemical tests								
	Gram Staining	Motility	Catalase	Oxidase	Indole	MR	VP	Citrate	Urease
Staphylococcus	Gram positive cocci	-	+	-	-	+	+	-	-
Streptococcus	Gram positive cocci	-	-	-	-	+	-	-	-
Micrococcus	Gram positive cocci	-	+	+	-	-	-	-	+
Lactobacillus	Gram positive rods	-	-	-	-	+	-	-	-

+ = Positive - = Negative

Special tests for confirmation

Staphylococcus aureus was grown on MSA. The culture showed a yellow hallo surrounding the growth All isolates were coagulase positive.

Lactobacillus were grown on MRSA medium as large white colonies.

Salmonella typhi produces black coloured colonies on BSA.

Streptococcus pyogenes grown on blood agar showed beta hemolysis and all were bacitracin sensitive.

The strain that was not identified by the conventional biochemical tests, LR-12 is subjected to phylogenetic identification by sequencing the 16S rRNA gene of that strain (Fig.3). The culture sequence obtained was subjected to BLAST analysis, the phylogenetically similar type strains sequence and other phylogenetic related sequence were selected from the GenBank and they were subjected to multiple sequence alignment and then then align sequences were trimmed to similar length in nucleotides and were subjected to phylogenetic tree (neighbour joining) construction using MEGA 6. In the tree the numbers at the nodes indicate the levels of the bootstrap support [high bootstrap values (close to 100%) meaning uniform support] based on a neighbour-joining analysis of 1,000 re-sampled data sets. The bootstrap values below 50% were not indicated. Bar 0.005 substitutions per site. The phylogenetic analysis clearly showed that the isolate belonged to *Cronobacter sakasaki* and the same has been submitted in the GenBank (Accession No. KJ739800).

Discussion

In the present study the distribution of bacteria in the oral cavity was analysed. The most commonly isolated bacteria were *Staphylococcus aureus* 122 isolates (56%), *Micrococcus* 41 isolates (18.8%) *Klebsiella* 12 isolates (5.5%). Studies have shown that Streptococci, Staphylococci, *Serratia* were among the predominant organisms isolated from the oral cavity in healthy individuals (Sharma et al., 2011).The present study shows that isolates of *Staphylococcus aureus* were 122 in number and constituted to 55%. There has been a lot of research carried out on *Staphylococcus aureus* and coagulase negative Staphylococci whereby little alteration has been paid to the oral cavity as a reservoir for these organs. (Smith et al., 2003 and Jackson et al .,2000). Molecular biological techniques are useful in studying about the numerous non-cultivable organisms and the oral flora contains more than300 known species of bacteria (Wilson et al., 1997). Research suggests that Staphylococci can be isolated in the oral cavity of children (Miyake et al.,1991) the elderly (Bagg et al.,1995) and some groups with systemic disease such as terminally ill(Jobbins et al.,1992) those having rheumatoid arthritis and people with aetiological malignancies. There is a great diversity in the content of the microflora between individuals (Nasidze et al., 2009) and between different oral sites within the same individual (Aas et al.,2005 and Avila et al., 2009).

Host provides nutrients and habitat to the normal bacterial flora of the oral cavity which in turn benefits them. Reports convey that dietary changes combined with poor oral hygiene especially in the old causes a change in the oral flora. (AL- Muala et al., 2014) In one of the previous studies,it is stated that human age has an influence on the type of organism grown (BereZow and Darveau, 2011). In another study, 22% of *Micrococcus luteus* and 11% *Micrococcus lylae* were present in the oral cavity and this is concurrent with the present study where the percentage obtained in the distribution is 18.8%. (Szczerba and Krzeminski, 2002).

In one study, they have described that bacteria in saliva can be detected by culture based diagnostic test because saliva reflect the overall bacteria levels in the oral cavity. About 13 isolates of *Lactobacillus* were identified and it is about 6%. it is not sure whether the isolated *Lactobacillus* are got from caries (Brown et al.,1986, Beighton and Lynch, 1995). This has been isolated in both the cases, where the cavities are present or absent. So there may be a relationship between dental caries and *Lactobacillus*.

About 12 isolates of *Klebsiella* was obtained 5.5% previous studies says that in the oral cavity of healthy persons *Klebsiella* occurs sporadically and in small numbers (Subhash et al.)2 isolates of *Proteus* were obtained (0.9%). A study conveys that the gram negative bacteria that colonize the skin can also inhabit the oral cavity. *Proteus mirabilis* causes 90% of *Proteus* infections. (Gus Gonzalez, 2015) In this study 2 isolates (0.9%) of *Proteus* was cultured and this was isolated in low number of patients when compared to other organisms. (AL-Muala et al., 2014). Studies show that the bad breath is due to the bacterial

metabolism in the oral cavity. Gram negative bacteria are mainly responsible for malodor. In the denture population the prevalence of Enterobacteriaceae in oral cavity is high. *Klebsiella* and *Enterobacter* isolates emit foul odors which resembles bad breath (Goldberg, 1997). About 8 isolates (3.8%) of *Serratia marcescens* was isolated and identified. Previous studies shows 3.7% of healthy subjects harbor *S. marcescens* in the oral cavity. Studies are needed to elucidate the role of bacteria in pathogenesis of periodontal disease. (Barbosa et al., 2006.) Around 5 isolates (2.3% of *Pseudomonas* was isolated. Colonization in the lower respiratory track and subsequent pulmonary infections in cystic fibrosis patients may occur due to isolates of *Pseudomonas aeruginosa*. (Komiyama et al., 1985). One isolate of *Streptococcus pyogenes* (Group A Streptococci) has been isolated in Blood Agar. Bacitracin sensitivity was performed to confirm the organism. This organism have been isolated from children and causes a diverse nature of disease complications. *Cronobacter sakazakii*, one isolate (0.5%) was identified by using 16S rRNA technique. This organism has generally been isolated from dried foods and is also responsible for nosocomial infections in neonates. Oral hygiene was significantly associated with oral infections as reported by Goldberg et al., 1997. Enterobacteriaceae was prevalent in denture wearing population compared with normal non denture wearers.

Conclusion

From the present study, it can be concluded that normal healthy individuals may harbor different oral microorganisms which may be present in the microenvironment of the oral cavity. According to this studies *Staphylococcusaureus* was the predominant organism isolated from the oral cavity of healthy individuals in Pondicherry.

References

Aas J.A., Paster B.J., Strokes L.N., Olsen I and Dewhirst FE.(2005) Defining the normal bacterial flora of the oral cavity. J. Clin. Microbiol4: 5721-5732.

AL-Muala HD., Samy SM and Al Shimirty I. (2014) Comparison between normal oral bacterial flora in healthy and diabetic patients. Journal of Kerbala University 12:257-263.

Avila M, Ojcius DM, and Yilmaz O. (2009) The oral microbiota: living with a permanent guest. DNA Cell Biol.:28:405-411.

Bagg J, Sweeney MP, Harvey-Wood K and Wiggins A. (1995) Possible role of *Staphylococcus aureus* in severe oral mucositis among elderly dehydrated patients. Microb Ecol Health Dis 1995; 8:51-56.

Dongoghue H.D. (1990) Can the colonization resistance of the oral microflora be reduced? Microb. Ecol. Health Dis 3: v-viii.

Barbosa FC Irino K, Carbonell GV and Mayer MP. (2006) Characterization of *Serratia marcescens* isolates from subgingival biofilm, extraoral infections and environment by prodigison production, serotyping and genotyping. Oral Microbiol. Immunol. 21:53-60.

Beighton D and Lynch E. (1995) Comparison of selected microflora of plague and underlying carious dentine associated with primary root caries lesions Carries.Res. 29:154-158.

Berezow AB and Darveau RP. Microbial shift and periodontitis.(2011) Periodontol. 55: 36-47.

Boggess KA.and Edelstein, BL (2006) Oral health in women during preconception and pregnancy: implications for birth outcomes and infant oral health. Matern. Child Health J.10: 169-174.

Brown LR, Billings RJ and Kaster. AG. (1986) Quantitative comparisons of potentially cariogenic micro organisms cultured from non-carious and carious root and coronal tooth surfaces. Infect Immun 1986: 51(3)765-770.

Burt BA. and Pai S. (2001). Sugar Consumption and Caries Risk: A Systematic Review. J. Dent. Educ. 65: 1017 – 1023.

Collee, J.G. Fraser, A.G.Marmion, B.P and Simmons A. (1996). Mackie and McCartney, Practical Medical Microbiology, 14th Edn. Churchil Livingstone, New York.

Roman- Mendez, C. Cecile Badet, Yanez, A. Dominguez, M.L. Giono, S. B. Richard, J. Nancy and Dorignac. G.(2009) Identification of oral strains of Lactobacillus species isolated from Mexican and French children. Journal of Dentistry and Oral Hygiene 1: 009-016.

Demmer RT and Desvarieux, M. (2006) Periodontal infections and cardiovascular disease. The heart of the matter. J. Am. Dental Assoc. 137: 14-20.

Dewhirst, F.E., T. Chen, T. Izard J. Paster, B.J., Tanner, ACR. Yu, W,-H., Lakshmanan A and Wade WG (2010). The human oral microbiome. J Bacterial, 192:5002:5017.

Goldberg S, Cardash H, Browning H., Sahly H and Rosenburg M (1997) Isolation of Enterobacteriaceae from the mouth and potential association with malodor. J. Dent. Res. 76: 1770-1775.

Gonzalez G (2015) Proteus Infections. Medscape. Oct8, 2015.

Holt, J.G.; Krieg, N.R.; Sneath, H.A.; Stanely, J.T. and Williams, S.T. (1994 and Wilkins, USA). Bergeys manual of determinative bacteriology. 9th ed., Baltimore; Wiliams.

Jackson MS, Bagg J. Kennedy H, Michie J. (2000) Staphylococci in the oral flora of healthy children and those receiving treatment for malignant disease. Microb Ecol Health Dis 12: 60-64.

Jobbins J, Bagg J, Parsons K, Finlay I, Addy M, Newcombe RG. (1992) Oral carriage of yeasts, coliforms and staphylococci in patients with advanced malignant disease. J Oral Pathol Med 21: 305-308.

Komiyama K, Tynan JJ, Habbick BF, Duncan DE, and Lupert DJ. (1985) *Pseudomonas aeruginosa* in the oral cavity and sputum of patients with cystic fibrosis. Oral. Surg. Med. Oral. Pathol. 59: 590-594.

Lederberg, J and Mccray, AT. (2001).'Ome Sweet 'Omics – A Genealogical Treasury of Words. The Scientist 15:8-10

LiX., Kolltveit K.M., Tronstad L and Olsen, I (2000) Systemic diseases caused by oral infection Clin. Microbiol. Rev. 13:547-558.

Loesche, W.J. (1986). Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. 50: 353-380.

MacFaddin, J.E (2000). Biochemical tests for identification of medical bacteria, 3rd edition Lippincott Williams and Wilkins Philadelphia. USA p.912.

Mager, D.L., L.A. Ximenez-Fyvie, A.D. Haffajee, and Socransky SS. (2003) Distribution of selected bacterial species on intraoral surfaces. J. Clin. Periodontol. 30:644-654.

Miyake Y, Iwai T, Sugai M, Miura K, Suginaka H, and Nagasaka N. (1991) Incidence and characterization of *Staphylococcus aureus* from the tongues of children. J Dent Res 70: 1045-1047.

Nasidze I, Li J, Quinque D, Tang K, and Stoneking M. (2009) Global diversity in the human salivary microbiome. Genome Res.19:636-643.

Sharma M., Tiwari S.C., Singh K and Kishore K (2011) Occurrence of bacterial flora in oral infections of diabetic and non-diabetic patients. Life Sci. Med. Res. 32:1-6.

Slavkin, H.C (1999). *Streptococcus mutans*early child hood caries and new opportunities. J. Am. Dent. Asso. 130:1787-1792.

Smith AJ, Robertson,D. Tang, MK, Jackson, MS, MacKenzie, D and Bagg J (2003). *Staphylococcus aureus*in the oral cavity: a three year retrospective analysis of clinical laboratory data. Br.Dent. J.195: 701 -703.

Subhash TS, S.H.Bhandi, MD and KhaiserI (2010) "*Klebsiella* revisited!". Endodontology 22: 92-94.

Szczerba I and Krzeminski Z (2002) Occurrence of bacteria in the mouth from genera of *Micrococcus*, *Kocuria*, *Nesterenkonia*, *Kytococcus* and *Dermacoccus*. Med Dosw Mikrobiol 54: 29-34.

Wilson MJ, Weightman AJ, and Wade WG. (1997) Applications of molecular ecology in the characterization of uncultured microorganisms associated with human disease. Rev Med Microbiol 8: 91-101.

Yacoubi A. (2013) Microbiology of periodontitis in diabetic patients in Oran, Algeria. Ibnosina J. Med. BS 5: 280-287.