WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.074
Volume 7, Issue 04, 335-348. Conference Article ISSN 2277–7105

THE CULTURABLE ORAL MICROFLORA OF HEALTHY INDIVIDUALS AND PATIENTS WITH DENTAL INFECTIONS

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Article Received on
03 Jan. 2018,
Revised on 24 Jan. 2018,
Accepted on 13 Feb. 2018
DOI: 10.20959/wjpr20184-10787

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ABSTRACT

Background: It has been recently shown that microbial life plays a key role in regulating a broad range of systems, including the human body. Studies on the characterization of healthy versus unhealthy human microflora in different body sites and disease condition have demonstrated a high potential for microbial community profiling techniques in the diagnosis and treatment of diseases. **Objective**: To study culturable oral microflora of healthy individuals with no dental infections and patients with dentals infections. **Materials and Methods:** Samples such as teeth swab, tongue swab and oral wash were collected from individuals with no dental infections and patients with dental. Standard microbiological procedures were followed in the isolation of cultures from samples. Conventional biochemical methods were used to identify the microorganisms. The characterization of isolates involved in the determination of biofilm forming potential and assessment of synergistic antimicrobial action of some herbal extracts against the oral species. **Results:** This study evaluated the prevalence of *Lactobacillus, Micrococcus, Staphylococcus and Candida* species in the oral samples. Microbial diversity of tongue swab samples was higher as compared to other samples. Amongst the organisms isolated from various samples, *Candida spp*. showed highest production of biofilm. Herbal extracts were tested for biofilm inhibition and disruption assays and were seen to inhibit 30 to 70% and disrupt 26 to 72% of biofilm formation. **Conclusion:** The present study focused on studying the culturable oral flora of healthy individuals with no dental infections and patients with

dental problems along with a suggestion for use of herbal extracts for non-formation of biofilms.

KEYWORDS: Culturable oral flora, Biofilms, Susceptibility test.

INTRODUCTION

The normal microbiome of oral cavity consists a variety of species of bacteria, fungi, yeast and possibly even viruses.[1] The flora of healthy and unhealthy individuals depends upon their lifestyle, type of food ingested and hygiene. The oral microbiome is particularly important to health as it can cause oral and systemic diseases. The microorganisms found in the human oral cavity are referred to as the oral microflora, oral microbiota, or the oral microbiome. The human oral cavity contains different habitats such as the cheeks, hard and soft palates, teeth, tongue and tonsils, colonized by bacteria fungi and yeast.

Many species of *Candida, Enterococcus, Streptococcus and Staphylococcus* are responsible to cause various oral infections. During the past two decades, there has been a significant increase in prevalence of fungal infection caused by *Candida* spp.[2] These microorganisms have an important virulence property, which is having the ability to form biofilms known as dental plaques on tooth surfaces and an etiological agent in dental caries. Biofilms are microbial derived sessile communities which have cells that are irreversibly attached to a substratum or to each other. The phenomenon of biofilm formation is a multi-step process dependent on environmental conditions, contact surface properties and extracellular polymers secreted by bacteria.[3] Attachment of bacteria to a polymer is the initial step for biofilm formation where the cell surface hydrophobicity greatly modulates the attachment properties of the organisms.[4] Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesions and presence of surfactants are some factors which influence biofilm formation. Microorganisms growing in the biofilms are intrinsically more resistant to antimicrobial agents. High concentrations of antimicrobial are required to inactivate organisms growing in a biofilm.[5]

In present study, the culturable oral microflora of healthy individuals with no dental infections and patients with dental problems was studied along with their biofilm forming capability. Herbal extracts were used to inhibit and disrupt their biofilm.[6]

MATERIALS AND METHODS

Healthy individuals, with no dental infections and patients with dental infections were selected for the isolation and identification of oral microflora. Samples were collected as teeth swab in 1ml sterile phosphate buffered saline (PBS) pH 7.4, tongue swab in 1ml PBS, and oral wash with 10 ml sterile saline.

Samples were then centrifuged at 8000 rpm for 10 minutes at room temperature. The resulting supernatant was discarded and the pellet was suspended in 2.5ml of PBS. 100µl of this suspension was then spread on Nutrient agar (NA) and Sabauroud dextrose agar (SDA) plates and incubated at 37°C for 24h.

Colony characteristics, gram nature, motility of isolates were studied. Identification of the bacterial isolates was done by carrying out different biochemical tests according to Bergey's Manual of Systematic Bacteriology (2^{nd} edition). Identification of yeasts was done based on the morphology and biochemical characteristics (Lodder, 1970).

Calculation of Diversity index

Simpson's Diversity Index is a measure of diversity which considers the number of species and the relative abundance of each species. As species richness and evenness increases so does diversity. Simpson's Diversity Index can be calculated by the below formula,

$$\text{Simpson's Diversity Index} = 1 - \frac{\sum n(n-1)}{N(N-1)}$$

{n = number of organisms of a particular species}

{N=Total number of organisms}

The value of D ranges between 0 and 1. With this index, 1 represents infinite diversity and 0, no diversity.

Detection of biofilm formation by isolated microorganisms

Staining of biofilm

To qualitatively detect the formation of biofilms, cell cultures were grown in a rich medium, Luria Bertani (LB) broth. The overnight cultures were diluted 1:100 into fresh medium for biofilm assays. 2ml of the dilution was added in tubes and incubated at 37^0C for 4-24h. After incubation the tubes were washed with water to remove the unattached cells and media components. 1.25ml of 1% (w/v) Crystal violet was added to each tube and incubated at room temperature for 10-15 minutes. The dye was then poured off and the tubes were rinsed 3-4 times with water and blot dried on stacks of filter paper to rid the tubes of all excess cells and

dye and allowed to dry for few hours. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube.

Congo red agar (CRA) method

CRA agar medium plates were prepared by mixing Brain heart infusion broth (37g/L), Sucrose (50g/L), Agar (10g/L) and Congo red indicator (8g/L). Concentrated aqueous solution of Congo red indicator was autoclaved separately at 121^0C for 15min and added to Brain heart infusion agar with sucrose at 55^0C. Test organisms were streaked on CRA plates and incubated at 37^0C for 24h aerobically. Black colonies with a dry crystalline consistency on CRA indicated biofilm formation.

Quantifying the biofilm by Crystal violet assay

Overnight grown cultures were inoculated in 1.8 ml Nutrient broth with 1% glucose and incubated at 37°C for 24h. After centrifugation at 150 rpm the supernatant was discarded and the pellet was washed with 2 ml of PBS to remove non-adherent cells. 32.5ml of 96% ethanol was then added to fix the cells. The ethanol was decanted and 1.25 ml of 0.1% (w/v) Crystal violet was added and allowed to remain in the tubes for 15 minutes. After pouring off the crystal violet, tubes were washed with 2.5 ml of distilled water twice and blot dried on stack of tissue paper. For solubalization of the bound dye 2ml of 33% v/v glacial acetic acid was added to each tube and incubated for 10-15 minutes at room temperature. 1.25 ml of solubilized Crystal violet was then transferred to new tubes. The absorbance was measured at 550 nm with 33% glacial acetic acid as blank.

Biofilm Inhibition assay

Biofilm inhibition assay was performed to study the effect of antibiotics and herbal extracts on isolated microorganisms. The organisms were grown in Nutrient broth and cell cultures (OD_{600} = 0.1) were mixed in following combinations in different tubes.

1. Cell culture + LB broth [C]
2. Cell culture + LB + Antibiotic (A) – Azithromycin [C+A]
3. Cell culture + LB +Herbal extracts (HE) - HE1-Green tea extract, HE2-Garlic extract [C+HE1, C+HE2]
4. Cell culture + LB+ Herbal extracts +Antibiotic [C+A+HE1/HE2]
5. Cell culture + LB + Combination of Herbal extracts [C+HE1+HE2]

The tubes were incubated at 37°C for 48h and 0.1% (w/v) Crystal violet was added to each tubes and absorbance recorded at 550 nm.

Biofilm disruption assay

The isolated organisms were grown in nutrient broth (48h old culture) and cell cultures (OD_{600} = 0.1) were mixed in following combinations in different tubes.

1. Cell culture + PBS [C]
2. Cell culture + Antibiotic (A) – Azithromycin [C+A]
3. Cell culture + Herbal extract (HE) – HE1-Green tea extract, HE2- Garlic extract [C+HE1, C+HE2]
4. Cell culture + Herbal extracts + Antibiotic [C+A+HE1/HE2]
5. Cell culture + Combination of Herbal extracts [C+HE1+HE2]

The tubes were incubated at 37°C for 24h and 0.1% (w/v) Crystal violet was added to each tubes and absorbance recorded at 550 nm.

RESULTS

Isolation of microorganisms from samples

Tables 1, 2 and 3 shows the number and types of microbial colonies obtained in samples collected from healthy individuals with no dental infections on NA and SDA in each category of samples. Table 4, 5 and 6 shows the number and types of colonies obtained in samples collected from patients having dental infections on NA and SDA in each category of samples.

Samples from healthy individuals with no dental infections

Table 1: Number and types of colonies on NA and SDA of teeth swab.

Sample	No of colonies (NA) 10^2	No of colonies (SDA) 10^2	Types of colonies (NA)	Types of colonies (SDA)
1	65±3.5	86±4.7	1	2
2	Uncountable	Uncountable	2	3
3	74±4.8	65±3.5	5	1
4	84±5.8	80±5.3	6	1
5	97±6.5	105±6.5	2	3
6	62±4.2	83±4.5	5	1
7	114±6.3	74±3.8	6	3
8	127±6.7	90±5.6	3	2
9	81±5.6	69±4.1	4	1
10	60±4.2	53±3.2	2	1

Table 2: Number and types of colonies on NA and SDA of tongue swab.

Sample	No of colonies (NA) 10^2	No of colonies (SDA) 10^2	Type of colonies (NA)	Type of colonies (SDA)
1	81±5.3	141±6.8	1	2
2	Uncountable	Uncountable	2	3
3	70±6.9	26±1.9	5	2
4	59±4.8	45±5.3	3	2
5	97±5.4	49±5.2	2	3
6	31±2.5	41±3.6	5	1
7	118±6.5	80±5.4	6	3
8	62±6.5	73±6.9	3	2
9	49±3.8	65±5.2	4	1
10	60±5.9	81±5.6	2	1

Table 3: Number and types of colonies on NA and SDA of oral wash.

Sample	No of colonies (NA) 10^2	No of colonies (SDA) 10^2	Types of colonies (NA)	Types of colonies (SDA)
1	8±2.4	21±9.2	1	2
2	Uncountable	Uncountable	2	3
3	67±5.4	----	5	----
4	33±3.7	12±1.7	6	1
5	23±3.5	----	3	----
6	21±2.6	56±4.2	2	3
7	20±1.5	56±4.7	2	2
8	48±3.5	51±5.3	5	2
9	51±5.2	48±3.5	5	2
10	75±6.2	57±4.8	4	3

Samples from patients with dental infections

Table 4: Number and types of colonies on NA and SDA of teeth swab.

Sample	No of colonies (NA) 10^2	No of colonies (SDA) 10^2	Types of colonies (NA)	Types of colonies (SDA)
1	81±5.6	Uncountable	5	2
2	Uncountable	Uncountable	6	3
3	132±6.8	70±6.5	4	2
4	100±5.2	75±6.4	3	3
5	51±4.2	70±5.1	2	1
6	60±4.9	60±6.6	2	3
7	69±4.6	75±5.9	3	2
8	52±4.9	57±4.9	2	4
9	46±3.2	62±4.3	3	2
10	68±3.6	72±5.4	2	1

Table 5: Number and types of colonies on NA and SDA of tongue swab.

Sample	No of colonies (NA) 10^2	No of colonies (SDA) 10^2	Types of colonies (NA)	Types of colonies (SDA)
1	85±4.6	121±6.7	2	3
2	73±5.4	Uncountable	4	4
3	127±6.4	70±5.7	5	1
4	106±5.2	98±5.9	3	3
5	72±4.8	73±4.1	2	2
6	49±3.4	56±3.0	2	2
7	65±5.1	69±5.3	3	3
8	67±4.2	68±5.3	5	4
9	68±4.7	78±5.8	3	2
10	81±5.1	45±3.5	2	1

Table 6: Number and types of colonies on NA and SDA of oral wash.

Sample	No of colonies (NA) 10^2	No of colonies (SDA) 10^2	Types of colonies (NA)	Types of colonies (SDA)
1	79±3.2	69±3.8	3	1
2	121±4.5	140±6.8	2	1
3	83±2.2	68±4.6	4	2
4	65±2.9	77±5.2	5	4
5	77±3.2	78±6.4	2	2
6	64±4.3	61±5.5	1	3
7	62±2.8	57±2.6	3	4
8	uncountable	uncountable	5	1
9	66±6.2	88±4.4	3	5
10	85±3.5	50±1.1	4	2

Identification of microorganisms

Spread plating on NA and SDA led to isolation of 4 bacterial and 1 yeast isolates from the different samples. The bacterial and yeast isolates were designated as I1-I4 and Y1, respectively.

Colony characteristics, gram nature, motility of the bacterial and yeast isolates are given in **Appendix I**. Identification of the bacterial isolates was done by carrying out different biochemical tests and confirmatory tests (**Appendix II**) according to Bergey's Manual of Systematic Bacteriology (2^{nd} edition). The bacterial isolates belonged to various genera such as *Lactobacillus*, *Micrococcus* and *Staphylococcus*.

Based on the morphology, biochemical characteristics and confirmatory tests, the yeast isolate was identified as belonging to the genera *Candida* (**Appendix II**).

Calculation for Diversiy Index

Table 7: Diversity indices of oral microflora.

Sample	Simpsons Index of Diversity (1- D)	
	H	P
Teeth swab	0.80	0.41
Tongue swab	0.89	0.39
Oral wash	0.69	0.59

H - Samples from healthy individuals with no dental infections, **P -** Samples from patients with dental infections.

From the above mentioned values, culturable oral microflora of samples from healthy individuals with no dental infections were more diverse than samples from patients with dental infections. The microbial flora of tongue swab sample of healthy individuals with no dental infections was more diverse as compared to other samples since the value of D is more (near 1).

Detection of biofilm formation by isolated microorganism

Staining of biofilm

To qualitatively detect the formation of biofilms by isolates, Biofilm formation assay was performed using staining method. Isolates showed moderate to strong biofilm formation (Figure 1).

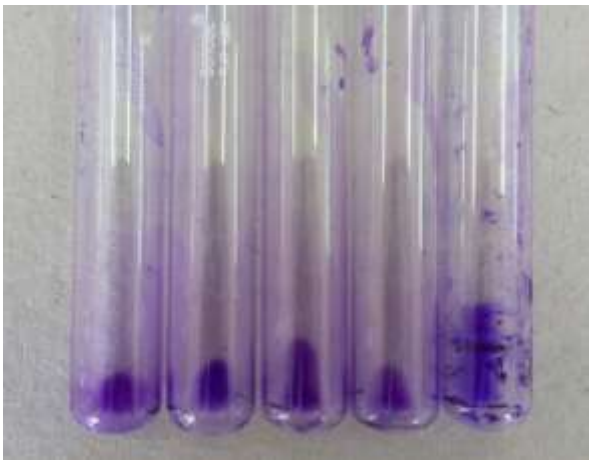


Fig-1: Biofilm formation assay using staining method for isolated microorganisms 1 - ***Staphylococcus spp 1*****, 2 -** ***Lactobacillus spp*** **, 3 -** ***Micrococcus spp*****, 4 -*****Staphylococcus spp*** **2 and 5 -** ***Candida spp*****.**

Congo red agar (CRA) method

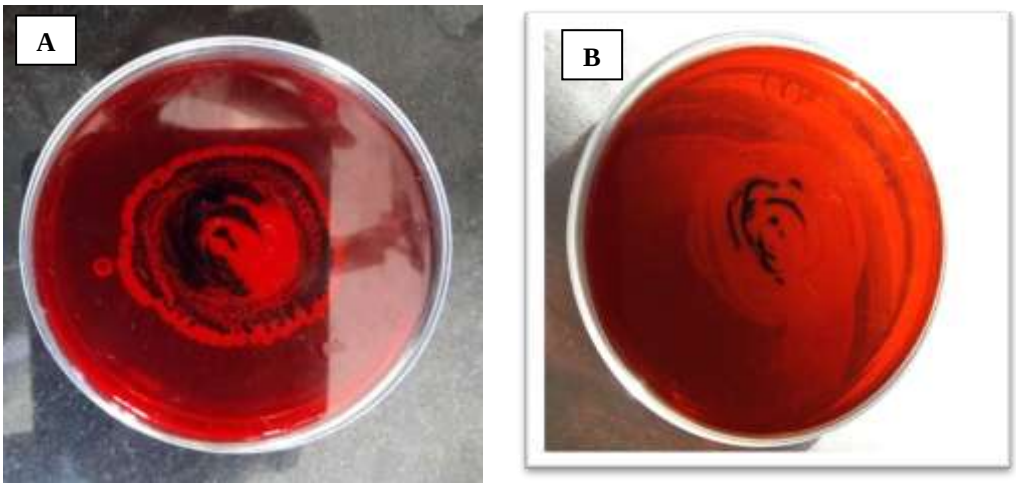


Fig - 2: Biofilm formation on CRA by (A) *Staphylococcus spp* 2 and (B) *Micrococcus spp*. CRA method was also done to detect biofilm formation in isolates. Black colonies with a dry crystalline consistency on CRA were observed which indicated biofilm formation (Figure 2).

Quantifying biofilm formation by Crystal Violet assay

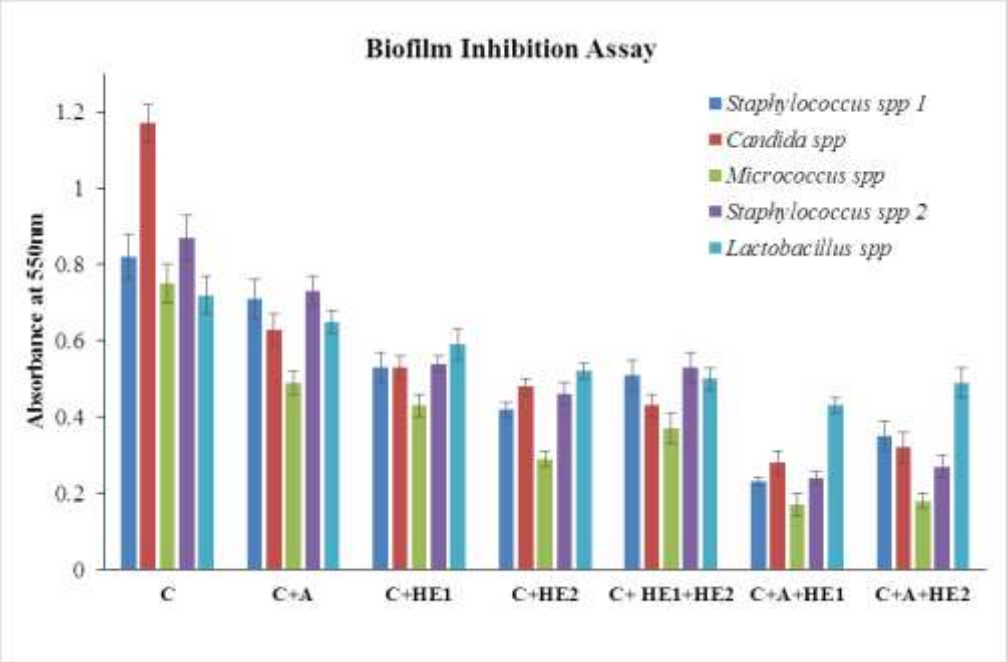
To quantify the biofilm production by isolates, Crystal violet assay was performed. Amongst the organisms isolated from various samples, *Candida spp*. showed highest production of biofilm as seen from Table 8.

Table 8: Quantitative assesment of biofilm production.

Name of microorganism	Absorbance at 550nm
Staphylococcus spp1	0.87±0.06
Staphylococcus spp 2	0.82±0.05
Lactobacillus spp	0.72±0.06
Micrococcus spp	0.75±0.07
Candida spp	1.17±0.12

Biofilm Inhibition and Disruption Assay

The property to form biofilms is one of the important factors for denture plaque formation. Herbal extracts of garlic and green tea along with antibiotic were tested for biofilm inhibition and also for biofilm disruption assays.

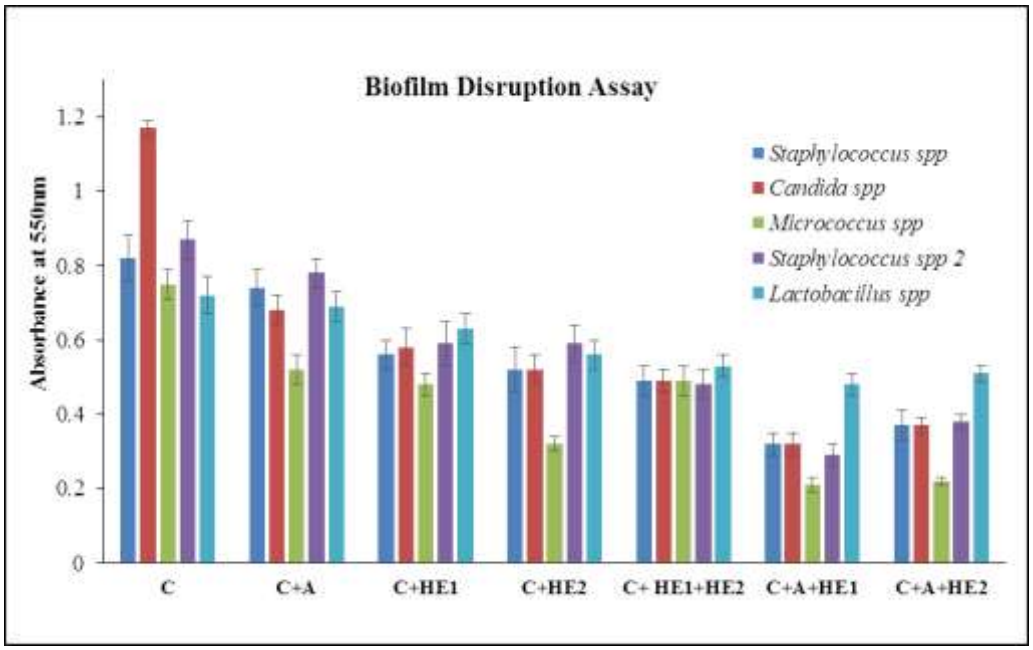


C - Cell culture + LB broth (Control), C+A - Cell culture + LB + Antibiotic (A) - Azithromycin, C+HE1 - Cell culture + LB +Herbal extract HE1-Green tea extract, C+HE2- Cell culture + LB +Herbal extract HE2-Garlic extract, C+HE1+HE2 - Cell culture + LB +Herbal extracts HE1-Green tea extract and HE2-Garlic extract, C+A+HE1 - Cell culture + LB+ Antibiotic + Herbal extract HE1, C+A+HE2 - Cell culture + LB+ Antibiotic + Herbal extract HE2.

Fig 3: Biofilm inhibition assay to study the effect of antibiotics and herbal extracts on isolated microorganisms.

It was observed that combination of both the extracts [Green tea (HE1) and Garlic (HE2)], HE 2 was 63%, 30.6%, 48%, 61.4% and 47.2% effective to inhibit the biofilm of the *Candida spp, Lactobacillus ,Staphylococcus spp1, Micrococcus spp and Staphylococcus spp 2* respectively. Combination of Antibiotic and HE1 was 71%, 76%, 77.4%, 72.5% and 40.3% effective to inhibit the biofilm of *Staphylococcus spp 1, Candida spp, Micrococcus spp, Staphyloccus spp 2* and *Lactobacillus spp* respectively (Figure 3).

Biofilm Disruption assay



C - Cell culture + LB broth (Control), C+A - Cell culture + LB + Antibiotic (A) - Azithromycin, C+HE1 - Cell culture + LB +Herbal extract HE1-Green tea extract, C+HE2- Cell culture + LB +Herbal extract HE2-Garlic extract, C+HE1+HE2 - Cell culture + LB +Herbal extracts HE1-Green tea extract and HE2-Garlic extract, C+A+HE1 - Cell culture + LB+ Antibiotic + Herbal extract HE1, C+A+HE2 - Cell culture + LB+ Antibiotic + Herbal extract HE2.

Fig 4: Biofilm disruption assay to study the effect of antibiotics and herbal extracts on isolated microorganisms.

It was seen that combination of both the extract [i.e. Green tea(HE1) and Garlic (HE2)], Herbal extract 2 was 26.4%, 36.5%, 26.9%, 55.4%, 53.8%and 44.9% effective to disrupt the biofilm of the *Lactobacillus spp, Staphylococcus spp* 1, *Candida spp, Micrococcus spp, and Staphylococcus spp* 2 respectively. Combination of Antibiotic and Herbal extract 1 was 65.9%, 72.6%, 72%, 66.7% and 33.4% effective to disrupt the biofilm of *Staphylococcus spp* 1, *Candida spp, Micrococcus spp, Staphyloccus spp* 2 and *Lactobacillus spp* respectively (Figure 4).

Statistical Analysis

All data are expressed as mean standard deviation (SD) of the triplicate experimental data.

Appendix I

Morphological characterization of bacterial and yeast isolates

Characteristics	I1	I2	I3	I4	Y1
Size	4mm	2mm	1mm	3mm	3mm
Shape	circular	Cicular	Irregular	Cicular	Circular
Colour	Golden yellow	White	White	Yellow	White
Elevation	elevated	Raised	Raised	Elevated	Elevated
onsistancy	Mucoid	Mucoid	Butyrous	Butyrous	Mucoid
Margin	entire	Entire	Entire	Entire	Entire
Opacity	Translucent	Opaque	Opaque	Translucent	Opaque
Surface	Smooth	Smooth	Smooth	Smooth	smooth
Gram staining	Gram positive cocci	Gram positive cocci	Gram positive rod	Gram positive cocci	-
Motility	Non motile	Non motile	Non motile	Non motile	-

Appendix II

Biochemical characterization of Bacterial isolates

Test	I1	I2	I3	I4
Indole	-	-	-	-
MR	+	+	+	-
VP	+	+	+	+
Nitrate reduction	-	+	-	+
Glucose fermentation (a/g)	+/-	+ /-	+ /-	+ /-
Sucrose fermentation (a/g)	+ /-	+ /-	+ /-	+ /-
Catalase	+	+	-	+
Coagulase	-	+	-	-
Citrate	-	-	-	-
Oxidase	-	-	-	+
Blood agar	β-hemolysis	-	-	β-hemolysis
Probable organism	***Staphylococcus spp* 2**	***Staphylococcus spp* 1**	***Lactobacillus spp***	***Micrococcus spp***

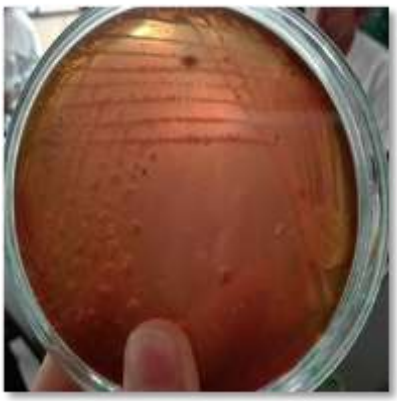
a, acid production; g, gas production; +, positive; -, negative.

Sugar utilization tests for Y1

Tests	Y1
Dextrose	+
Maltose	+
Sucrose	-
Lactose	-
Probable organism	***Candida spp***

(+) = Positive result, (-) = Negative result

Confirmatory tests for *Lactobacillus spp* on De Man, Rogosa and Sharpe agar medium (MRS), *Staphylococcus spp* 2 on Blood agar medium and *Candida spp* on CHROM agar medium.



***Staphylococcus spp* 2 on Blood agar**

***Lactobacillus spp* on MRS agar**

***Candida spp* on CHROM agar**

DISCUSSION

From previous studies the organisms found from the oral microflora were *Streptococcus mutans, Streptococcus sorbinus, Lactobacillus spp, Staphylococcus spp, Candida spp* and *Enterococcus faecalis*. In the present study samples were collected from healthy individuals with no dental infections and patients suffering from dental problems. Here, organisms such as *Lactobacillus, Micrococcus, Staphylococcus and Candida* species were isolated and biochemically characterized. Also, the diversity present in the different categories of samples was calculated and compared using diversity indices. Culturable oral microflora of samples from healthy individuals with no dental infections was more diverse than samples from patients with dental infections. Tongue swab samples showed higher diversity than teeth swab and oral wash.

Isolates obtained were further tested for biofilm formation and a few isolates showed good capability of biofilm formation. The property to form biofilms is one of the important factors for dental plaque formation. Hence some herbal extracts, their combinations and antibiotic were tested for biofilm inhibition and biofilm disruption assay. Combinations of herbal extracts of green tea and garlic were seen to inhibit and disrupt biofilm formation.

CONCLUSION

The oral cavity may serves as a reservoir for bacterial pathogen of medical importance in systemically healthy or disease subject. The occurrence of higher proportion of microorganisms in subgingival samples and oral sites may represent a significant problem in causing and maintaining periodontal infections. Thus the present study focused on studying the oral flora of healthy individuals with no dental infections and patients with dental problems along with a suggestion for use of herbal extracts for non-formation of biofilms. Future work in this area may provide treatment to infections caused by pathogenic organisms by using herbal extracts as well as herbal extracts with combination of antibiotics.

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