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Antimicrobial Activity of Salivary Normal Flora against Bacteria Isolated from Periodontitis

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ABSTRACT

Microbes are ubiquitous, typically undetectable, and present in large quantities on both living and inanimate items. Some microorganisms are helpful to humans, even as others are harmful. The teeth, gingival sulcus, tongue, hard and soft palates, and tonsils are among the several habitats found in the human oral cavity. The human body is protected from unwanted effects by the interaction of various oral bacteria. Severe infection disorders like periodontitis can also occur as a result of an imbalance in the mouth cavity's natural flora. A gradual breakdown of the tissues supporting the tooth is a characteristic

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of periodontitis, an inflammatory disease of the gums. The aim of the current investigation was to ascertain the salivary normal flora's antimicrobial efficacy against periodontitis-related bacteria. The study was carried out at the Microbiology Research Laboratory (MRL), Abasyn University, Peshawar, samples were taken from Khyber Dental Hospital, Peshawar. The swab approach technique was used; 20 clinical samples were taken from individuals with periodontitis for this investigation. The identified bacteria that were responsible for the infection were *Escherichia coli*, *Actinomyces spp.* and *Streptococcus spp.* For, the normal flora isolation, two healthy male and two healthy female volunteers' saliva were used. Two male samples were diagnosed with *Lactobacillus* spp whereas two female samples had *Streptococcus* spp identified as normal flora. Samples of normal flora and periodontitis were identified using Gram staining and several biochemical testing. Nutrient broth media was used to extract secondary metabolites from bacteria isolated from oral normal flora. The flasks were incubated at 37 °C with constant shaking at 120 rpm for 3 days. After incubation, centrifugation was performed to collect the cell-free supernatant, Ethyl acetate was added to extract the organic phase. The organic phase was evaporated to dryness using a rotary evaporator to yield ethyl acetate extracts. The agar well diffusion technique was used to assess the antibacterial activity of salivary normal. The maximum zone of inhibition of salivary normal flora was observed against *Escherichia coli* followed by *Actinomyces spp.* and lastly *Streptococcus spp.* Among normal flora *Streptococcus spp.* showed strong zone of inhibition followed by *Lactobacillus spp.*

Keywords: Antimicrobial Activity, Normal Flora, Periodontitis

INTRODUCTION

Microbes are everywhere, normally invisible, they are abundant on everything from living things to inanimate objects. Although some microbes are pathogenic, there are also some which are beneficial to humans (Casadevall, 2016). The human oral cavity contains a number of different habitats, including the teeth, gingival sulcus, tongue, hard and soft palates and tonsils. Interaction of variable oral microorganisms helps human body against invasion of undesirable outcomes. In the oral cavity most habitats are known to be dominated by *Streptococcus* but these were followed in abundance by *Haemophilus* (Gao *et al.*, 2018). The oral cavity is comprised of many surfaces, each coated with a plethora of

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bacteria, the proverbial bacterial biofilm. Some of these bacteria have been implicated in oral diseases such as caries and periodontitis, which are among the most common bacterial infections in humans. For example, it has been estimated that at least 35 % of dentate U.S. adults aged 30 to 90 years have periodontitis. In addition, specific oral bacterial species have been implicated in several systemic diseases, such as bacterial endocarditis, aspiration pneumonia, osteomyelitis in children, preterm low birth weight, and cardiovascular disease (Aas *et al*., 2005).

The imbalance of the normal flora of the oral cavity can also lead to development of severe infectious diseases like periodontitis. Periodontitis is an inflammatory disease of the gums which is characterized by a progressive destruction of the tissues supporting the tooth (Aas *et al*., 2008). There are epidemiological studies which have shown that periodontitis could be an independent risk factor for mortality, for diabetes, for osteoporosis, for pulmonary infections, for premature low birth weight and for cardiovascular diseases. To prevent periodontitis from being a possible risk factor it should be treated thoroughly (Arweiler and Netuschil, 2016). Saliva, apart of oral cavity is an exocrine secretion consisting of approximately 99 % water, containing a variety of electrolytes (sodium, potassium, calcium, chloride, magnesium, bicarbonate, phosphate) and proteins represented by enzymes, immunoglobulins and other antimicrobial factors, mucosal glycoproteins, traces of albumin and some polypeptides and oligopeptides of importance to oral health. There are also glucose and nitrogenous products, such as urea and ammonia. The components interact and are responsible for the various functions attributed to saliva (Arweiler and Netuschil, 2016). Adult human saliva is reported to contain approximately 6 billion microorganisms per milliliter including *Streptococci*, *Peptostreptococci*, *Veillonella*, *Cornybacterium*, *Neisseria*, *Myocardia*, *Fusobacterium*, *Bacteroides*, *Lactobacilli*, *Actinomyces*, *Spirochetes* (Majumdar and Singh, 2014). The common perception of bacteria in the mouth is that they reside there because of the available warmth, moisture, and protection and they take advantage of the regular input of nutrients from food whilst providing little or no benefit to the host (Majumdar and Singh, 2014).

A major benefit of the oral microbiome to whole body physiology has already been described the nitrite-producing bacteria on the tongue which contribute to nitric oxide production and the lowering of blood pressure. There are likely to be others as more studies explore the oral metabolome in relation to whole body health. Clearly, if there is a

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benefit to whole body health then the body should nurture the oral microbiome. If true, this could explain the recent concept of "resilience" the ability of the oral microbiome to resist pressure to change from antibiotic treatment or overgrowth of one species, into a dysbiotic state often associated with disease. Crucial to the process of maintaining oral commensals is saliva (Arweiler and Netuschil, 2016). Previously, most studies have described the anti-microbial properties of saliva as bacteriostatic with some bacteriocidal properties, which it clearly has, but there is also evidence that it has bacterial growth promoting properties as well. Broadly speaking, the growth-promoting properties can be split into three main sections; nutrients, attachment, and environment (Majumdar and Singh, 2014).

MATERIALS & METHOD

Study Site

All of the samples were collected from Khyber Dental Hospital, Peshawar and the research was conducted at Microbiology Research Laboratory (MRL), Abasyn University, Peshawar.

Isolation of Salivary Normal Flora

The normal flora was isolated from saliva of 2 healthy males and 2 healthy female subjects by using swab method, for which a sterile swab stick was rubbed over the surface of gums. For the collection of normal flora; subjects with completely healthy oral cavity were selected. Nutrient agar media was prepared and sterilized by autoclaving at 121 °C for 15 mins and then poured into the petri dishes to solidify. After solidification, the sample containing swabs were streaked on the surface of nutrient agar. The plates were then incubated at 37 °C for 24 hrs.

Isolation Of Pathogenic Bacteria From Periodontitis

In this study a total of 20 clinical samples were collected from the periodontitis patients through swab method. For sample collection, the sterile swab stick was taken and rubbed over the surface of the gums of periodontitis patients. Nutrient agar media was prepared. The media was sterilized by autoclaving at 121 °C for 15 mins and then poured into the petri dishes to solidify. After solidification, the sample containing swabs were streaked on the surface of nutrient agar. The plates were then incubated at 37 °C for 24 hrs.

Identification and Characterization of Bacterial Isolates

The following tests and procedures were carried out for the identification of bacteria.

Morphological and Cultural Characterization

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Cultural characteristics were used for the identification of microorganisms. Growth on nutrient agar plates was used to demonstrate well isolated colonies by observing following parameters. Size: (small, moderate and large). Pigmentation: (color of colony). Form: (circular, irregular).

Gram Staining

Gram staining is a method of staining use to distinguish and classify bacterial species into two large groups i.e., Gram positive and Gram negative (Gupta *et al.*, 2017). The Gram staining process included four basic steps; a sterile slide was taken and the bacterial colony was heat fixed. A primary stain (crystal violet) was applied for 60 seconds and then washed with running tap water. A mordent (Gram's iodine) was added for 30 seconds and then washed with running tap water. Rapidly depolarized with ethanol, acetone or a mixture of both for 5 seconds and then washed with running tap water. Counter staining was done with (safranine) for 60 seconds and then washed with running tap water. The slides were air dried and emulsion oil was applied on it for observation under microscope. Purple color showed gram positive bacteria while pink color resulted gram negative bacteria (Greenwood *et al.*, 2012).

Biochemical Tests

The isolated bacteria were characterized biochemically according to the Bergey's Manual of Determinative Bacteriology (9th Edition) by performing the following tests.

Triple Sugar Iron Test (TSI)

The top of a well-isolated colony was touched with a straight inoculation needle. The colony was then inoculated in TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant. The test tube was incubated at 37 °C in for 18 to 24 hours. Red slant, yellow butt indicated only glucose fermentation, yellow slant, yellow butt indicated triple sugar fermentation (glucose, lactose and sucrose), red slant, red but indicated no sugar fermentation, blackening of the medium indicated H_2S production by the microorganism (Carr, 2017).

Indole Test

Sterilized test tubes containing 4 ml of peptone water media was prepared. The tube was incubated aseptically by taking the growth from 18 to 24 hrs culture. The test tube was incubated at 37°C for 24 hours. A 0.5 ml of Kovac's reagent was added to the broth culture. The formation of a red ring indicates a positive indole result (KOMAL, 2019).

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Citrate Utilization Test (CUT)

A slant was prepared by autoclaving Simmons citrate agar at 121 °C. The bacteria were inoculated gently on the slant by slightly touching the tip of the needle to the colony, which was 18 – 24 hours old. In the citrate medium, organisms required more time to grow. Therefore, the media was incubated at 37 °C for 18 hours. The color change in the solution was checked and the development of blue color indicated the alkylation process which was interpreted as a positive result (MacWilliams, 2009).

Catalase Test

A glass slide and hydrogen peroxide was required. A drop of hydrogen peroxide was poured on top of slide. Using a sterilized inoculating loop, small quantity of bacteria was smeared onto the slide containing hydrogen peroxide. Bubbles produced, presented positive result for bacteria's ability to catalyze H_2O_2 (Reiner, 2010).

Oxidase Test

A strip of filter paper was soaked with a little freshly made 1% solution of the oxidase reagent (tetra- methyl- *p*- phenylenediamine- dihydrochloride). A speck of culture was rubbed on it with a platinum loop. The result was then observed immediately after rubbing the colonies on reagent. The colour change of the oxidase reagent to blue- purple within 10- 30 seconds indicated a positive oxidase test result (Shields and Cathcart, 2010).

Urease Test

The surface of a urea agar slant was streaked with a portion of a well-isolated colony from an overnight broth culture. The slant was incubated at 37 °C for 24 hours. The colour change of the urea agar from yellow to pink indicated the presence of urease enzyme and a urease positive result (Majumdar and Singh, 2014)

Coagulase Test

A drop of rabbit blood plasma was poured onto a glass slide. With a wire loop a portion of the isolated colony was emulsified in the drop to make a thick suspension. The clumping of the organisms was observed within 10 seconds which indicated a positive coagulase test (Brown *et al.*, 2005).

Extraction of Bacterial Bioactive Secondary Metabolites

Nutrient broth media was used to extract secondary metabolites from bacteria isolated from oral normal flora. The flasks were incubated at 37 °C with constant shaking at 120

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rpm for 3 days. After incubation, the broth media was centrifuged at 10,000 rpm for 10 min at 4 °C to collect the cell-free supernatant, Ethyl acetate was added at 1:1 (v/v) to the cell-free supernatant and the media, which was shaken vigorously for a few min. The ethyl acetate mixture was poured into separating funnels and allowed to stand until organic and aqueous phases were separated, and the organic phase was collected. The organic phase was evaporated to dryness using a rotary evaporator to yield ethyl acetate extracts (Majumdar and Singh, 2014).

Antimicrobial Activity of Normal Flora

For determination of antibacterial activity, Muller Hinton Agar (MHA) media was prepared and autoclave at 121 ℃ for 15 mins. After autoclaving, the media was allowed to cool down at room temperature and poured in the plates. Then bacterial lawn was prepared on the surface of MHA by using sterile cotton. With the help of sterile borer wells were formed at equal distance of 20 mm. The stock solution of the normal flora was prepared with 3 mg of the extracts dissolved with DMSO less than 1 % respectively. In each well 100 µl of extract (normal flora) from the stock solution was poured respectively. For positive control (Amoxicillin 30 micro grams) was used and for negative control DMSO was used. Next, the plates were incubated at 37 ˚C for 24 hours. After incubation time, cleared zones around wells were observed and result was noted in mm by using scale (Majumdar and Singh, 2014).

RESULTS

Isolation and identification of Salivary normal flora

The normal flora was isolated from saliva of two healthy males and two healthy female subjects. Two species were detected from the four samples. *Lactobacillus spp.* was identified as normal flora from 2 male samples and *Streptococcus spp.* were identified as normal flora from 2 female samples as shown in Table 1.

Table 4.1. Oral Normal Flora Identification

Isolated From	Identified As
Healthy Male Saliva	*Lactobacillus spp.*
Healthy Female Saliva	*Streptococcus spp.*

Gram staining and different biochemical tests were used for the identification of normal flora samples. The results are shown in the following Table 2.

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TABLE 2: BIOCHEMICAL TEST RESULTS OF *LACTOBACILLUS SPP.* AND *STREPTOCOCCUS SPP.*

S.No.	Species	Gram stain	Catalase	Oxidase	Coagulase	TSI	Urease	Indole	CUT
1	*Lactobacillus spp.*	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
2	*Streptococcus spp.*	+ve	-ve	-ve	-ve	A/A	+ve	-ve	+ve

Key words: +ve= positive, -ve= negative, A/A= yellow slant/yellow butt, TSI= Triple Sugar Iron test, CUT= Citrate Utilization Test.

Isolation and Identification of Bacteria from Periodontitis Samples

In this study, a total of 20 samples from the periodontitis patients were collected and were isolated through swab method. Out of the 20 samples, 5 showed positive results among the positive samples, three types of bacteria were then identified on the basis of Gram staining and different biochemical tests. The results are shown in Table 3.

Table 3: Identification of Bacteria from Periodontitis Samples

S no.	Species	Gram stain	Catalase	Oxidase	Coagulase	TSI	Urease	Indole	CUT
1	*Streptococcus spp.*	+ve	-ve	-ve	-ve	A/A,	-ve	-ve	+ve
2	*Actinomyces spp.*	+ve	-ve	-ve	+ve	A/A	-ve	-ve	-ve
3	*Escherichia coli*	-ve	+ve	-ve	-ve	A/A	-ve	+ve	-ve

Key words: +ve= positive, -ve= negative, A/A= yellow slant/yellow butt, TSI= Triple Sugar Iron test, CUT= Citrate Utilization Test

Antimicrobial Activity of Normal Flora

The antibacterial activity of salivary normal flora was performed by using agar well diffusion method. The maximum zone of inhibition of salivary normal flora was observed against *Escherichia coli* followed by *Actinomyces spp.* and lastly *Streptococcus spp.* Among normal flora *Streptococcus spp.* showed more zone of inhibition followed by *Lactobacillus spp.* The results of the zone of inhibition of the secondary metabolites extracted from salivary

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normal flora in comparison to the +ve and -ve control are depicted in the following Table 4.

Table 4: Antimicrobial Activity of Secondary Metabolites extracted from Salivary Normal Flora

S. No	Species isolated from periodontitis	Zone of inhibition of secondary metabolites of *Lactobacillus spp.* (mm)	Zone of inhibition of secondary metabolites *Streptococcus spp.* (mm)	+ve control Amoxicillin (mm)	-ve control DMSO (mm)
1.	*Streptococcus spp.*	12	15	30	0
2.	*Actinomyces spp.*	18	20	32	0
3.	*Escherichia coli*	23	24	33	0

DISCUSSION

In this study antimicrobial activity of salivary normal flora was determined against bacteria isolated from periodontitis samples. This research is a contribution to biotechnological advancements and is capable to solve clinical and therapeutic problems related to periodontitis. The use of salivary normal flora to solve oral health issues such as periodontitis can be revolutionary in the progress of new medicinal and therapeutic sciences as it proves how significant body's own normal flora is when it comes to prevention and treatment and sometimes even reversal of diseases caused by pathogenic bacteria. In the present time, periodontitis can be a very common problem among all age groups. Different treatments and even surgical procedures are available for severe cases of periodontitis and related oral health issues.

However, in today's world due to an increase in industrialization a lot of chemicals have been oozed out in our environment, polluting it up to the brim. This caused a direct impact on our immunity. This the main reason why commercially available medications (which can be harsh due to their composition) for diseases like periodontitis have a tendency to cause adverse effects on people with lowered immunity. This research aimed

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to investigate the antimicrobial effect of salivary normal flora on periodontitis so that it can be utilized in the synthesis of medicines with minimal harmful chemicals and zero side effects for the treatment of periodontitis. Another important potential of this study is that it can help mitigate growing problem of antibiotic resistance. As the concept of the beneficial effects of normal flora as therapeutic agent is already known but not much commonly applied in our medicinal field (as studied by Rosier *et al.*, (2018), we can say that there is potential for salivary normal flora to work as an inhibiting medication for periodontal bacteria excluding the risk to be resisted but still further study in this area may be required.

In this research, *Streptococcus spp.* and *Lactobacillus spp.* were detected as normal flora. Similar investigation by Gao *et al.*, (2018), which suggested that the Human Oral Cavity is dominated by *Streptococcus spp.* Another study conducted by Majumdar and Singh (2014) reported the presence of several species of normal flora i.e., *Streptococcus spp.* and *Lactobacillus spp.* In this study, the periodontitis samples were collected from dental plaque and saliva on gums, similar to the way samples were collected by Aas *et al.*, (2016) in this study, from periodontitis samples *Streptococcus spp.*, *Escherichia coli* and *Actinomyces spp.* were identified, which mostly reside in the oral cavity naturally. Which was consistent with the study of Aas *et al.* (2005) and Zaura *et al.* (2017) which reported the presence of *Streptococcus spp.*, *Escherichia coli* and *Actinomyces spp.* in the human oral cavity. Another notable point is the difference of species of normal flora isolated from healthy male and female saliva. This key point relates to the study conducted by Zaura *et al.*, (2017) which suggested the gender- specific differences in the salivary microbiome.

In this study, *Streptococcus spp.* showed the most zone of inhibition against *Escherichia coli*, *Actinomyces spp.* and a little against *Streptococcus spp.* followed by the zone of inhibition formed by *Lactobacillus spp.* The effectiveness of *Escherichia coli* is consistent with the research of Carpenter (2020). Thus, this study proves that salivary normal flora can inhibit further spread of periodontitis caused by *Escherichia coli* and *Actinomyces spp.* The findings of this research can be further expanded by conducting further research that whether it is possible for salivary normal flora to reverse the effects of periodontitis or not. Further research can also be conducted on how salivary normal flora is significant in overcoming the problem of antibiotic resistance. Another gap that can be filled in this

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research by figuring out the right type of salivary normal flora that can work against periodontitis pathogen, *Streptococcus spp.*

CONCLUSIONS

In this study, two different bacterial species of *Streptococcus* were identified, one as a pathogenic from periodontitis and other as normal flora. Other than these, *Actinomyces* and *Escherichia coli* were identified from periodontitis. *Lactobacillus* was also identified as normal flora. This research has proved that salivary normal flora (*Lactobacillus* isolated from healthy male saliva and *Streptococcus* isolated from healthy female saliva) are effective and have antimicrobial properties to work against periodontitis caused by *Escherichia coli* and *Actinomyces*. Hence, this research proves that normal flora is affective in contributing to future biotechnological advancements in medicine.

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