CORRELATION BETWEEN BIOFILM OF DENTAL MICROBES AND ANTIBIOTIC RESISTANCE, *IN VITRO* STUDY

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ABSTRACT:

Microbial biofilms, complexes containing embedded bacterial cells in secreted extracellular polysaccharides, has raised significant issues in dentistry. They are providing ongoing nutrient supplementation since they can adhere strongly to teeth and their inherent resistance to conventional antibiotics and cleaning techniques. This study aimed to identify and characterize biofilm-forming dental microbes and evaluate statistics relating biofilm formation to antibiotic resistance under aerobic and anaerobic conditions. Ninety-six swab samples were collected from different sites of oral cavities, and 184 microbial isolates were isolated under aerobic and anaerobic conditions. All isolates were identified, and the ability of the isolates to form biofilms has been assessed using qualitative and quantitative methods. The isolates were then tested against a variety of antibiotics. Analyzing the relationship between antibiotic resistance and dental biofilm formation statistically, Minitab 19 and SPSS 25 using ANOVA one-way were used. The result revealed that 54.95% of the aerobic isolates can form biofilms with different degrees, while the other 45% haven't. Indeed, among isolates of anaerobic bacteria, 60.27% form biofilm while 39.72% haven't. *Enterococcus faecium, Pseudomonas. aeruginosa, Klebsiella pneumoniae, Streptococcus mutans, Staphylococcus aureus, and Lactobacillus rhamnousa* were found to be multidrug-resistant (MDR) strains that are the strongest biofilm formers. The present study's statistical analysis of aerobic isolates showed that biofilm formation is negatively correlated with susceptibility (P - value< 0.05) to Cefadroxil, Cefoxitin, and Piperacillin. Formation of biofilms and susceptibility to Cefadroxil, Cefoxitin, Piperacillin, Cefamandole, Aztreonam and Amoxicillin are also significantly negatively correlated (P- value <0.05) in the case of anaerobic isolates. Our findings can conclude that anaerobic conditions may be more favourable for microorganisms to disseminate the resistance genes via the biofilm matrix. Detection of such correlations in dental isolates is helpful in studying the behavior of this pathogen and may provide a new target for the treatment of MDR infections of oral cavity.

Key word: Dental bacteria, biofilm, correlation, antibiotic resistance, dental caries.

Introduction:

Bacterial biofilms are a collection of microorganisms in which the cells are surrounded by an extracellular polymeric substance (EPSs) (Ben-Zaken *et al.*, 2021 & Flemming, 2016). In contrast to planktonic bacteria, which move around freely in a bulk solution, this state is very different. Biofilms consist of multilayered cells that interact with each other. These biofilms can either be directly attached to the solid surface or be created in flocs where they are mobile and do not adhere to the surface (Funari and Shen, 2022), infections of the middle ear, ocular implants, native valve endocarditis, chronic lung infections in cystic fibrosis patients, and dental caries are associated strongly with biofilm formation (Pugazhendhi *et al.*, 2022; Raghavendran *et al*., 2020; Sonkusale & Tale, 2015). A complex microbiological process called biofilm formation involves a number of developmental stages, some of which are particular to the type of bacterium present, while others involve numerous species of microbes (Rather *et al*., 2021). The formation of biofilms during infection has been explained by three theories defense, colonization, and communal benefits (Jefferson, 2004). Modern molecular biology techniques have found that dental biofilm contains 1000 different bacteria, twice as many as can be grown *in vitro* (Saini *et al.*, 2011). There are four main stages of oral biofilm formation. Pellicle formation is the adhesion of salivary glycoproteins to a perfectly smooth tooth surface. The binding proteins found in the acquired pellicle are recognized by the pioneer bacteria in saliva during the initial adhesion and connect to them. Bacteria from different species co-aggregate and mature biofilm formations, pellicles, and connect to them during maturation. The co-aggregation of various bacterial species and development of fully developed biofilms. Dispersion: as bacteria spread from their surfaces, colonizing new areas (Heller *et al*., 2016).

The environment of warm, humid and neutral pH conditions in the mouth encourages bacteria to grow. Compared to isolated bacterial communities in different regions and subgingival areas, the microbial makeup of typical plaque biofilms is very different. Compared to free-floating bacteria, the explanation for this diversity is the existence of a diverse collection of genes (Souza *et al.*, 2016 & Valm, 2019). Dental biofilm includes bacterial strains of different types but is primarily made up of the *Streptococcus* species, including *Streptococcus mutans*, which plays a significant role in the development of dental caries. Bacteria like this produce acid and decrease the mouth pH when dietary sugars ferment when sugar is present *(*Aas *et al.*, 2008). The first step in the production of oral biofilm, the demineralization of the teeth (Chen *et al*., 2020). According to several studies, environmental biofilms can act as flashpoints for the spread of antibiotic resistance *(*Flores-Vargas *et al., 2021;* Nassar *et al., 2022* & Said *et al.*, 2021).

This research aimed to determine the statistical relationship between dental isolate biofilm development and antibiotic resistance. Also, it was designed to isolate different microbial isolates and assess the connection between isolates sites, the degree of biofilm, and antibiotic resistance from distinct oral cavity sites under aerobic and anaerobic circumstances.

Materials and Methods

Ethical approval:

The sampling process in this research was done at the College of Dentistry, Al-Azhar University, Cairo, Egypt. The College of Dentistry, Al-Azhar University Ethical Committee (Approval code 914/341), in addition to the Faculty of Science, Ain Shams Ethical Committee (Approval code ASU/SCI/MICR/2023/4/3) granted ethical approval to the research.

Samples collection:

Ninety-seven swab samples were taken from male and female patients with different ages in the College of Dentistry, Al-Azhar University. The samples were collected from posterior teeth 12(12.3%), interior teeth 22(22.6%), left premolar 30 (31%), right premolar 20(20.7%) and molars 13(13.4%). Then nutrient agar, mannitol salt agar and mitis salivarius agar plates were used to inoculate each swab and then incubated at 37°C under aerobic and anaerobic conditions for 48 h.

Isolation of biofilm-forming bacteria:

Firstly, a smear from each isolate was prepared directly and stained with Gram stain for initial identification. Then, on nutrient agar with 0.8 g/l of Congo red dye, the primary identified isolates were streaked and incubated for 48 h at 37°C. Biofilm formation was indicated by the development of dry, crystalline, black colonies, whereas red colonies were only produced by non-biofilm generating isolates (Mathur *et al*., 2006).

Selection of the most potent isolates:

The Microtiter Plate Method (MTP) was used for quantitative analysis.(Bedidi-Madani *et al.*, 1998 & Desouky *et al*., 2014) using tissue culture plates of 96 flat-bottomed wells. Each well was filled with 0.2 ml of 0.5 McFarland standard of a bacterial suspension in Tryptic soya broth (TSB) medium. After 48 h incubation at 37°C under aerobic and anaerobic condition, the contents were aspirated and phosphate-buffered saline was used to wash plates twice (PBS, pH: 7.2), fixed by methanol and dyed for five minutes with 0.1% crystal violet. After drying, the content of each well was suspended in 30% acetic acid and then read in ELISA reader (StatFax, USA) at 492 nm. Sterile TSB was used as a negative control. At least each of the experiments was repeated twice, and after then, the optical density (OD) measurements were averaged. A twice-grade scale was used to evaluate the strain biofilm producing ability by comparing it with OD of cut-off. The parameters from the previous study were used to interpret the biofilm production (Stepanović *et al*., 2007) as the following: ≤ ODc: Non, ≤ 2x ODc: Weak, 2x ODc < ~ ≤ 4x ODc: Moderate, > 4x ODc: Strong. *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 90028 were used for quality control as biofilm forming Gram-positive, Gram-negative and fungi strains, respectively.

Antibiotic susceptibility test:

The standardized Kirby-Bauer disc-diffusion method was performed on the basis of the Clinical Laboratories Standards Institute (CLSI) guidelines (2020). Antibiotic discs including Streptomycin 10µg (S), Rifamicin 30µg (RA), Cefadroxil 30 µg (CFR), Aztreonam 30µg (ATM), Chloramphenicol 30µg (CL), Cefamandole 30µg (MA), Cefoxitin 30µg (Fox), CLarithromycin 15µg (CLR), Piperacillin 100µg (PRL), Amoxicillin 25µg (AX) and Ofloxacin 5µg (FL) were placed using sterilized forceps, 0.5 McFarland standards of the selected isolates were used to inoculate the dried Muller Hilton agar plates. The plates were then incubated at 37°C for 24 h under aerobic condition for aerobic isolates and under anaerobic condition for anaerobic isolates. After incubation, the inhibition zone around each disc was measured. According to the diameters of inhibition zones and antibiotic discs producer guidelines and the recommended standards of CLSI, 2020, the isolates were categorized into three categories: sensitive, intermediate, and resistant. Bacteria that resist three or more groups of antibiotics were considered MDR strains. Each test was performed in triplicate (Kebede *et al*., 2021).

Identification of the selected isolates by the VITEK 2 system:

All the isolate were primarily identified by the traditional biochemical tests, then with the highest biofilm production poential biochemically were identified using the VITEK 2 system (6 isolates). The VITEK 2 technique is an automated microbial identification system that provides highly accurate results using colourimetric reagent cards. The reagent cards have 64 wells, each contains an individual test substrate of various biochemical tests for different species of bacteria. Various metabolic biochemical processes, such as acidification, alkalization, and enzyme hydrolysis, are measured by substrate utilization by the tested bacteria.

Statistical analysis:

Three practical replicates for each assay were obtained. The resultant values are the averages of three separate experiments. To examine variations between a sample and the corresponding control, data of the isolated strains antibiotic sensitivity against S, RA, CFR, FOX, ATM, CLR, AX, MA, OFL, PRL and CL in relation to sites of samples, (S) analysis of biofilm development in the oral cavity via Minitab 19 and SPSS 25 was performed. Descriptive statistics, including mean, standard deviation, standard error mean, minimum, maximum, median, first quartile, third quartile, and interquartile range have been calculated for all variables. Inferential statistics have been used to compare the results of different groups. All variables' parametric assumptions have been tested. Different comparisons were done using analysis of variance, one-way (ANOVA) under the fit general linear model. P-values were considered significant at $\alpha < 0.05$. Post hoc analyses of the interactions among all groups were done using the Tukey test for pairwise comparisons. Post hoc analyses are represented as letters where groups that share the same letters are non-significantly different, while different letters express significant differences among different groups. Pearson correlation was analyzed to all antibiotics to test the relationship between strong biofilm production, sites of oral isolation and antibiotic sensitivity. Multiple simple linear regressions have

been used to generate prediction equations of antibiotic sensitivity based on the source of affecting sensitivity results.

Results:

Sample collection and bacterial isolates:

Ninety-seven swab samples were collected from different sites inside the oral cavity of patients as the following posterior teeth 12(12.3%), interior teeth 22(22.6%), left premolar 30(31%), right premolar 20(20.7%) and molars 13 (13.4%). Under aerobic and anaerobic conditions, all samples were incubated on a nutrient agar plates medium. One hundred eighty four microbial isolates were isolated from patients. The primary isolates identification demonstrated how many isolates were grown under aerobic conditions, 111(60.32%) isolates included 23(20.7%) Gram-negative and 88(79%) Gram-positive isolates. these isolates 53(47.7%) cocci, 57(51%) rods and one isolate identified as candida. In comparison, the total number of isolates grown under anaerobic condition was 73 (39.67 %) isolates incluced 55(75.3%) Gram-positive and 18(16%) Gram-negative isolates. these anaerobic isolates included 36 (49%) rods and 37 (50.6%) cocci.

Biofilm formation:

Among 111 aerobic isolates, 61 (54.95%) were able to form a biofilm with different degrees, while 50(45%) isolates weren't capable of forming biofilm. The biofilm forming aerobic strains were differentiated into 11(18%) weak biofilm formers, 28(45.9%) moderate biofilm formers and 22(39.3%) strong biofilm formers. Indeed, among 73 isolates of anaerobic bacteria, 44(60.27%) were able to form a biofilm, while 29(39.72%) couldn't form biofilm. The biofilm-forming anaerobic isolates included 21 (47.72%) formed strong biofilm, 8(15.9%) formed weak biofilm and 15(36.36%) formed moderate biofilm. Table 1 illustrates the total number of isolates with different biofilm formation degrees.

Table 1: Total microbial isolates with different biofilm categories.

Growth condition	Total No.	Biofilm forming isolates		Biofilm degree		Percentage of biofilm producers
Aerobic	111	+	61	Weak	11	54.95%
				Moderate	28	
				Strong	22	
		-	50	-		
Anaerobic	73	+	44	Weak	8	60.27%
				Moderate	15	
				Strong	21	
		-	29	-		
Total	184					

Antibiotic susceptibility pattern:

Antibiotic susceptibility was performed for strong biofilm-forming aerobic and anaerobic isolates. The isolated organisms showed resistance, with different patterns,

against various commercially available antibiotics. The results showed that 91.9% of aerobic isolates were sensitive to streptomycin and 90.9% were resistant to ofloxacin while 100% of anaerobic isolates were sensitive to Streptomycin and Clarithromycin and resistant to Cefoxitin. Tables 2 and 3 show the antibiotic susceptibility pattern for aerobic and anaerobic strong biofilm-producing bacterial isolates, respectively. The results showed 6 isolates multidrug resistant (27.27%) out of 22 aerobic isolates and 5 isolates multidrug resistant (23.8%) out of 21anaerobic isolates. Also the results revealed that isolates A48a, B70c, and C3 aerobic isolates and B72b, C8b and GTP anaerobic isolates are the most potent MDR isolates and biofilm producer .

Table 2: Antibiotic susceptibility for strong biofilm-forming aerobic isolates.

Sample code	S	RA	CFR	F0X	ATM	CLR	AX	MA	FL	PRL	CL
A6	S	I	R	S	R	I	S	R	I	S	S
A14	R	I	S	S	I	S	R	S	R	I	S
A19a	S	I	S	R	I	S	R	S	R	I	S
A19b	R	I	S	R	S	I	R	I	R	I	S
A25	S	R	I	R	I	I	S	I	R	R	S
A26	S	R	I	S	I	I	R	I	R	S	S
A36	S	I	R	S	R	R	I	S	R	R	S
A41	S	S	R	I	R	R	I	I	R	R	S
A42a	S	I	R	I	R	R	I	S	R	R	I
A48a	I	R	R	R	R	R	R	I	R	R	S
A48b	S	I	R	R	R	I	R	S	R	R	S
B61	S	S	R	R	R	I	I	S	R	R	S
B62	S	I	I	R	R	R	R	S	R	R	S
B63	S	R	R	S	R	R	R	R	S	I	S
B67	I	I	R	R	R	R	R	I	R	S	S
B70c	R	R	R	R	R	R	I	R	R	R	S
B71	I	R	R	R	R	R	I	R	R	R	S
B77	S	R	R	I	R	R	S	R	R	I	S
C2	S	R	R	R	I	R	R	R	R	R	I
C3	S	R	R	R	R	R	R	R	R	R	S
C8	S	R	R	R	R	R	R	R	R	I	S
C9	S	R	R	R	R	R	R	I	R	R	S
Resistance%	13.6	43	69.5	56.5	69.5	60.8	52	21.7	87	52	0

Table 3: Antibiotic susceptibility for strong biofilm-forming anaerobic isolates.

Sample code	S	RA	CFR	F0X	ATM	CLR	AX	MA	FL	PRL	CL
A9b	S	R	R	R	R	I	S	S	R	R	S
A24	S	R	R	R	R	R	S	R	R	R	S
A30	S	R	R	R	I	R	R	R	R	I	S
A31	S	R	I	R	I	R	R	R	S	R	S
A32	S	R	I	R	S	R	R	R	S	R	S
A34	S	R	R	R	R	R	S	R	R	I	S
A37	S	R	R	R	I	R	R	R	R	S	S
A41	I	R	R	R	S	R	R	S	R	I	I
A45	S	R	R	R	R	I	R	R	S	R	S
A46	S	I	R	R	S	R	R	R	R	I	I
B55	S	R	R	R	S	I	R	R	R	S	S
B66	I	R	R	R	I	R	R	I	R	R	S
B72b	S	R	R	R	R	R	R	R	R	I	I
B82	S	R	R	R	R	R	R	R	R	I	S
B83	S	R	R	R	R	R	R	R	R	S	S
C5	S	R	R	R	R	R	R	R	R	S	I
C6	S	R	R	R	R	S	R	R	R	S	S
C8b	I	R	R	R	R	R	R	R	R	R	S
C9	S	R	R	R	R	S	R	S	R	R	S
C10	S	R	R	R	R	I	R	S	R	R	S
GTP	I	R	R	R	R	R	R	R	R	R	I
Resistance%	0	95	90	100	62	71	85.7	76	85.7	47.6	0

Biochemical identification of the strongest biofilm-producing isolates:

The VITEK 2 technology was used to identify the most MDR bacterial isolates that are strong biofilm producers biochemically (Table 4). The six isolates A48a, B70c, C3, B72b, C8b, and GTP were identified as *Enterococcus faecium, Pseudomonas. aeruginosa, Klebsiella pneumoniae, Streptococcus mutans, Staphylococcus aureus,* and *Lactobacillus rhamnousa,* respectively.

Analysis of statistics:

Results of one-way ANOVA for different biofilm degrees among dental isolates from several sites show that non-significant differences were noticed in the number of isolates forming biofilms among sample sites (posterior teeth, anterior teeth, left premolar, right premolar and molars). However, some patterns were discovered in the case of premolar samples, for example, isolates were weaker biofilm forming than those collected from other sites (Fig 1). One-way ANOVA results for comparing different antibiotic sensitivity among different isolates in anaerobic and aerobic isolates from various sites were illustrated in Fig. 2 and Fig. 3.

Table 4: Morphological, and biochemical identification of the most potent isolates by VITEK 2 technique.

Test	A48a	B70c	C3	B72b	C8b	GTP
Gram stain	+	-	-	+	+	+
Cell shape	Cocci	Bacilli	Bacilli	Cocci	Cocci	Bacilli
Coagulase	-	-	-	-	+	+
Catalase	-	+	+	-	+	-
D-Amygdalin	+	-		-	-	+
Ala-phe-pro- Aryl amidase	-	-	-	+	-	+
Leucine Aryl amidase	-	-	-	-	-	+
Alanine Aryl amidase	-	+	-	+	-	+
D-Ribose	+	-	-	-	+	+
Novobiocin Resistance	+	-	+	-	+	+
D- Raffinose	-	-	+	+	-	-
Optochin resistance	+	-	+	+	+	+
Phosphatidylinositol Phospholipase C	-	+	+	-	-	-
Cyclodextrin	+	-	+	-	-	-
L-Prolin Aryl amidase	-	+	-	-	-	+
Tyrosine Aryl amidase	-	-	-	+	-	+
L-Lactate Alkalinization	-	-	-	-	+	-
6.5% NaCl growth	+	+	+	-	-	+
Resistance to O/129	-	+	+	-	+	
D-Xylose	-	-	+	-	-	-
L- Aspartate Aryl amidase	-	-	+	-	-	-
(Beta- Glucuronidase)	-	-	-	+	-	-
D-Sorbitol	-	-	+	-	-	-
Lactose	+	-	+	-	-	+
D-manitol	+	-	+	+	-	+
Salicin	+	-	+	+	-	+
Argnine Dihydrolase	+	+	-	+	+	-
Beta galactopyranosidase	-	-	+	-	-	+
Alpha- galactocidase	-	-	-	+	-	+
N- Acetyle-D- Glucosamine	+	-	-	+	+	+
D-Mannose	+	-	+	-	-	+
Sucrose	-	-	-	+	+	+
Urease	-	-	+	-	-	-
Alpha-Mannosidase	-	-	+	-	-	-
Beta-galactosidase	-	-	+	-	-	-
L-Pyrrolidonyl-Arylamidase	+	-	-	-	+	+
Polymixin B resistance	+	-	-	+	+	+
D-maltose	+	--	+	-	+	+
Methyl-B-D-Glucopyranoside	+	-	+	+	-	-
D-Trehalose	+	+	+	+	+	+
Alpha-glucosidase	+	-	-	+	+	+
Beta Glucuronidase	+	-	+	-	-	+
Phosphatase	-	-	+	-	+	+
D-Galactose	+	+	+	+	+	+
Bacitracin Resistance	-	-	-	-	-	+
Pullulan	-	+	+	-	-	+
Arginine Dihydrolase2	+	+	-	+	-	+
ID 99%	*E. faecium*	*P. aeruginosa*	*K. pneumoniae*	*S. mutans*	*S. aureus*	*L. rhamnousa*

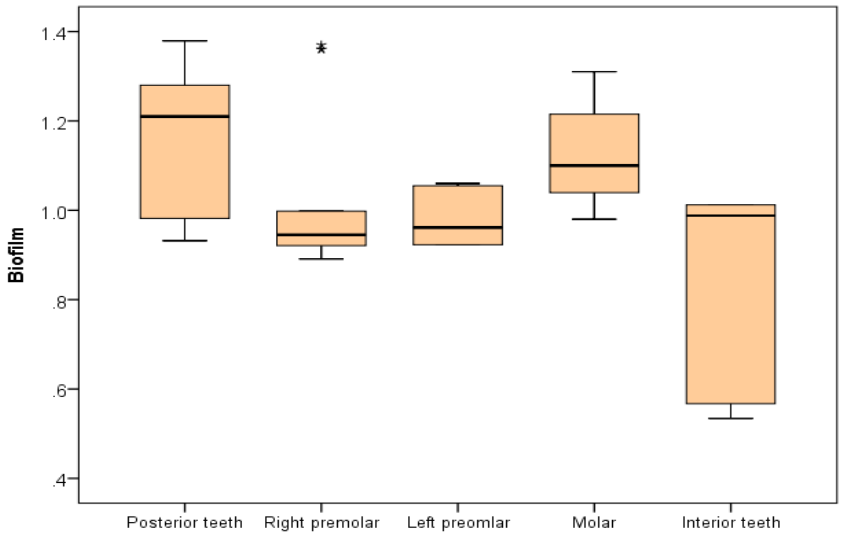

Fig. 1: Results of one way ANOVA comparing the sites of the oral cavity and the isolates with the strongest biofilm formation.

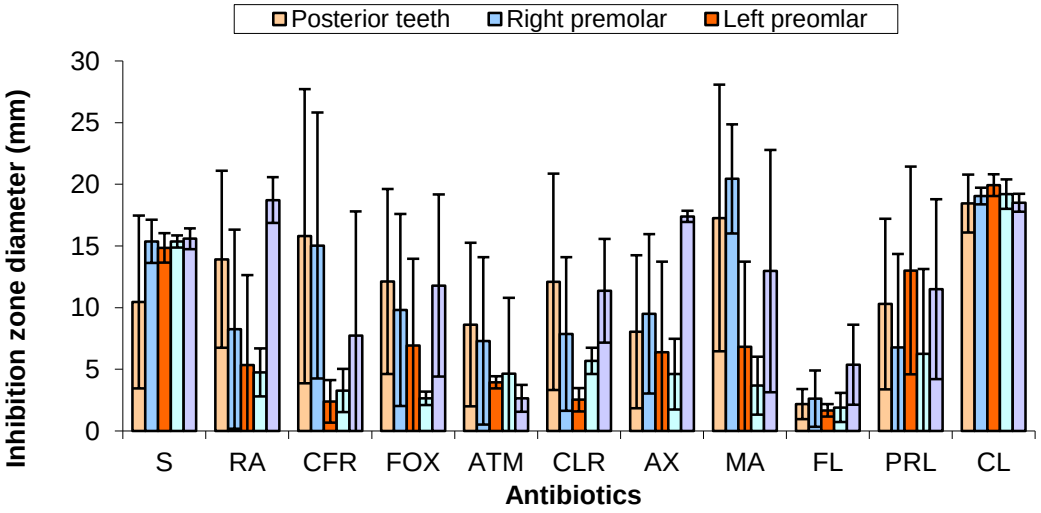

Fig. 2: Results of a one-way comparison of antibiotic sensitivity among different sites of oral cavity in case aerobic condition. S: Streptomycin; RA: Rifampicin; CFR: Cefadroxil; FOX: Cefoxitin; ATM; Aztreonam; CLR: Clarithromycin; AX: Amoxicillin; MA: Cefamandole; OFL: Ofloxacin; PRL: Piperacillin; CL: Chloramphenicol.

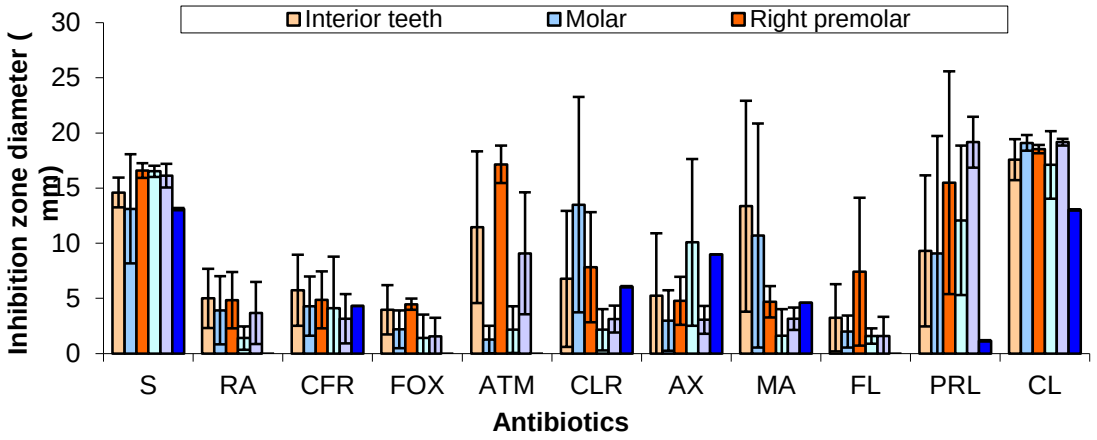

Fig. 3: Results of a one-way ANOVA comparison of antibiotic sensitivity at several regions in the oral cavity in case of isolates grown under anaerobic conditions. S: Streptomycin; RA: Rifampicin; CFR: Cefadroxil; FOX: Cefoxitin; ATM; Aztreonam; CLR: Clarithromycin; AX: Amoxicillin; MA: Cefamandole; OFL: Ofloxacin; PRL: Piperacillin; CL: Chloramphenicol.

Correlation between biofilm and antimicrobial resistance of the isolates:

Data from statistical analysis demonstrated a reasonable fit for different models with R > 91% and adjusted R > 90%. In addition, normal residual probability plots showed a linear attitude for all analyses. As a correlation of the obtained results, the bacteria under examination were evaluated for the capability of biofilm formation and antibiotic resistance; biofilm formation is negatively correlated with the use of Streptomycin, Cefadroxil, Cefoxitin, Ofloxacin and Piperacillin (P value < 0.05). The development of biofilms did not, however, exhibit a statistically significant relationship with Rifamycin, Aztreonam, Clarithromycon, Amoxicillin, Cefamandole, and Chloramphenicol in case aerobic isolates, while anaerobic isolates exhibit a negative relation between biofilm formation and Rifampicin, Cefadroxil, Cefoxitin, Aztreonam, Amoxicillin, Cefamandole, Piperacillin and Chloramphenicol (P value < 0.05) but Streptomycin, Ofloxacin, and Clarithromycin had no statistically significant relationship with biofilm capacity.

An illustration of Pearson correlation between various antibiotic susceptibilities and biofilm formation was shown in Tables 5 and 6. The result exhibited, among aerobic isolates, a negative relationship between formation of biofilm and susceptibility to the antibiotics Streptomycin, Rifampicin, Cefadroxil, Cefoxitin, Clarithromycin, Amoxacillin, Cefamandole, Ofloxacin and Piperacillin (r = ranged from - 0.474 to - 0.119, P < 0.05), with high significance to Streptomycin and Cefoxitin (P < 0.01). While a non-significant positive relationship was found between formation of biofilm and the susceptibility to Aztreonam and Chloramphenicol (r = ranged from 0.120 to 0.061 P > 0.05 respectively). On the other hand, among anaerobic isolates, a negative relation between the formation of biofilm and antibiotic susceptibilities to Streptomycin, Rifampicin, Cefadroxil, Cefoxitin, Clarithromycin, Amoxicillin, Cefamandole, Ofloxacin and Piperacillin, (r = ranged from - 0.585 to - 0.025, P -value < 0.05).

Table 5: Pearson correlation among different antibiotics and formation of biofilm in aerobic isolates.

S: Streptomycin; RA: Rifampicin; CFR: Cefadroxil; FOX: Cefoxitin; ATM; Aztreonam; CLR: Clarithromycin; AX: Amoxicillin; MA: Cefamandole; OFL: Ofloxacin; PR: Piperacillin; CL: Chloramphenicol

	Biofilm		**S**		**RA**		**CFR**		**FOX**		**ATM**		**CLR**		**AX**		**MA**		**FL**		**PRL**		**CL**	
	R	**P-value**	**r**	**P-value**	**R**	**P-value**	**r**	**P-value**	**R**	**P-value**	**r**	**P-value**	**r**	**P-value**	**R**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**
Biofilm	–	–	**-0.474****	**0.000**	-0.224	0.068	**-0.303***	**0.013**	**-0.332****	**0.006**	0.120	0.335	-0.119	0.336	-0.211	0.086	-0.168	0.175	**-0.267***	**0.029**	**-0.245***	**0.046**	0.061	0.622
S	**-0.474****	**0.0001**	–	–	0.033	0.791	0.160	0.196	0.120	0.334	**-0.267***	**0.029**	0.023	0.851	0.039	0.754	**0.256***	**0.036**	**0.305***	**0.012**	-0.028	0.822	**-0.428****	**0.000**
RA	-0.224	0.068	0.033	0.791	–	–	**0.385****	**0.001**	**0.360****	**0.003**	-0.139	0.263	**0.518****	**0.000**	0.106	0.394	**0.530****	**0.000**	0.183	0.139	**0.342****	**0.005**	**-0.376****	**0.002**
CFR	**-0.303***	**0.013**	0.160	0.196	**0.385****	**0.001**	–	–	**0.469****	**0.000**	**0.400****	**0.001**	**0.553****	**0.000**	-0.059	0.638	**0.473****	**0.000**	**0.349****	**0.004**	**0.308***	**0.011**	0.050	0.689
FOX	**-0.332****	**0.006**	0.120	0.334	**0.360****	**0.003**	**0.469****	**0.000**	–	–	0.022	0.860	**0.333****	**0.006**	0.085	0.493	**0.303***	**0.013**	**0.617****	**0.000**	**0.437****	**0.000**	-0.103	0.408
ATM	0.120	0.335	**-0.267***	**0.029**	-0.139	0.263	**0.400****	**0.001**	0.022	0.860	–	–	**0.450****	**0.000**	-0.076	0.543	-0.055	0.656	0.081	0.515	**0.293***	**0.016**	**0.324****	**0.007**
CLR	-0.119	0.336	0.023	0.851	**0.518****	**0.000**	**0.553****	**0.000**	**0.333****	**0.006**	**0.450****	**0.000**	–	–	0.197	0.111	0.175	0.157	**0.347****	**0.004**	**0.528****	**0.000**	-0.039	0.754
AX	-0.211	0.086	0.039	0.754	0.106	0.394	-0.059	0.638	0.085	0.493	-0.076	0.543	0.197	0.111	–	–	-0.167	0.177	**0.347****	**0.004**	-0.094	0.447	0.076	0.541
MA	-0.168	0.175	**0.256***	**0.036**	**0.530****	**0.000**	**0.473****	**0.000**	**0.303***	**0.013**	-0.055	0.656	0.175	0.157	-0.167	0.177	–	–	0.102	0.412	0.067	0.587	**-0.354****	**0.003**
FL	**-0.267***	**0.029**	**0.305***	**0.012**	0.183	0.139	**0.349****	**0.004**	**0.617****	**0.000**	0.081	0.515	**0.347****	**0.004**	**0.347****	**0.004**	0.102	0.412	–	–	**0.496****	**0.000**	-0.149	0.228
PRL	**-0.245***	**0.046**	-0.028	0.822	**0.342****	**0.005**	**0.308***	**0.011**	**0.437****	**0.000**	**0.293***	**0.016**	**0.528****	**0.000**	-0.094	0.447	0.067	0.587	**0.496****	**0.000**	–	–	0.006	0.960
CL	0.061	0.622	**-0.428****	**0.000**	**-0.376****	**0.002**	0.050	0.689	-0.103	0.408	**0.324****	**0.007**	-0.039	0.754	0.076	0.541	**-0.354****	**0.003**	-0.149	0.228	0.006	0.960	–	–

Table 6: Pearson correlation among different antibiotics and formation of biofilm in anaerobic isolates.

	Biofilm		**S**		**RA**		**CFR**		**FOX**		**ATM**		**CLR**		**AX**		**MA**		**FL**		**PRL**		**CL**	
	R	**P-value**	**r**	**P-value**	**R**	**P-value**	**R**	**P-value**	**R**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**	**r**	**P-value**
Biofilm	–	–	-0.205	0.107	**-.577****	**0.000**	**-.327****	**0.009**	**-.384****	**0.002**	**-.319***	**0.011**	-0.106	0.408	**-.585****	**0.000**	**-.264***	**0.037**	-0.025	0.847	**-.385****	**0.002**	**-.331****	**0.008**
S	-0.205	0.107	–	–	-0.135	0.293	**-.264***	**0.037**	-0.043	0.736	0.003	0.981	0.061	0.635	0.070	0.585	-0.044	0.730	0.233	0.066	0.181	0.156	0.174	0.172
RA	**-.577****	**0.0001**	-0.135	0.293	–	–	**.631****	**0.000**	**.553****	**0.000**	**.497****	**0.000**	0.152	0.234	0.188	0.139	0.226	0.075	**.385****	**0.002**	**.293***	**0.020**	0.194	0.128
CFR	**-.327****	**0.009**	**-.264***	**0.037**	**.631****	**0.000**	–	–	**.490****	**0.000**	**.340****	**0.006**	0.204	0.109	**.286***	**0.023**	0.126	0.326	0.073	0.567	-0.011	0.931	0.218	0.085
FOX	**-.384****	**0.002**	-0.043	0.736	**.553****	**0.000**	**.490****	**0.000**	–	–	**.424****	**0.001**	**.306***	**0.015**	**.429****	**0.000**	**.486****	**0.000**	**.445****	**0.000**	0.059	0.644	0.044	0.732
ATM	**-.319***	**0.011**	0.003	0.981	**.497****	**0.000**	**.340****	**0.006**	**.424****	**0.001**	–	–	-0.237	0.062	0.038	0.766	0.198	0.120	**.314***	**0.012**	**.380****	**0.002**	0.033	0.799
CLR	-0.106	0.408	0.061	0.635	0.152	0.234	0.204	0.109	**.306***	**0.015**	-0.237	0.062	–	–	0.192	0.132	**.566****	**0.000**	-0.013	0.921	-0.216	0.088	0.130	0.310
AX	**-.585****	**0.0001**	0.070	0.585	0.188	0.139	**.286***	**0.023**	**.429****	**0.000**	0.038	0.766	0.192	0.132	–	–	**.257***	**0.042**	-0.033	0.794	0.158	0.217	0.073	0.569
MA	**-.264***	**0.037**	-0.044	0.730	0.226	0.075	0.126	0.326	**.486****	**0.000**	0.198	0.120	**.566****	**0.000**	**.257***	**0.042**	–	–	0.047	0.716	-0.197	0.121	-0.002	0.985
FL	-0.025	0.847	0.233	0.066	**.385****	**0.002**	0.073	0.567	**.445****	**0.000**	**.314***	**0.012**	-0.013	0.921	-0.033	0.794	0.047	0.716	–	–	0.127	0.323	0.208	0.101
PRL	**-.385****	**0.002**	0.181	0.156	**.293***	**0.020**	-0.011	0.931	0.059	0.644	**.380****	**0.002**	-0.216	0.088	0.158	0.217	-0.197	0.121	0.127	0.323	–	–	-0.017	0.893
CL	**-.331****	**0.008**	0.174	0.172	0.194	0.128	0.218	0.085	0.044	0.732	0.033	0.799	0.130	0.310	0.073	0.569	-0.002	0.985	0.208	0.101	-0.017	0.893	–	–

S: Streptomycin; RA: Rifampicin; CFR: Cefadroxil; FOX: Cefoxitin; ATM: Aztreonam; CLR: Clarithromycin; AX: Amoxicillin; MA: Cefamandole; OFL: Ofloxacin; PRL: Piperacillin; CL: Chloramphenicol.

Discussion:

Oral microflora contains more than 700 distinct bacterial species *(*Huang *et al*., 2011). They zcolonize the hard palate, carious lesions, periodontal disorders, teeth, tongue, and oral mucosa (Ptasiewicz *et al*., 2022). It has been demonstrated that the microbiota in the oral cavity is not distributed randomly. Depending on the ecosystem in the area, a majority of species favour some places over others (Huang *et al*., 2011 & Relucenti *et al*., 2021). In the current investigation, bacteria from various sites in the oral cavity were isolated, identified, and then tested for their capacity to form biofilms. The results showed that the bacteria that were isolated and that produced the strongest biofilm varied depending on where in the oral cavity they were found: more in the posterior teeth than in the molar, more in the internal teeth, more in the left molar than in the right premolar. According to the current study, dental caries bacteria form biofilms. Dental caries is habitat to a variety of microbial flora and these communities showed dynamic resistance against various tested antibiotics. Furthermore, because of the presence of biofilm and extracellular polysaccharides, the selected bacterial strain resisted unfavorable climatic conditions (Wu *et al*., 2020). Due to bacteria capacity to form biofilms, antibiotic sensitivity also differed. The effectiveness of the antibiotics for particular bacterial strains is considered while choosing specific medications. (Fair and Tor, 2014). Dental plaque is an aggregation of bacteria where one by-product acts as a nutrient for the other. High percentages of bacterial strains are developing resistance to different antibiotics. Antibiotic resistance in the oral cavity microflora may be caused by several bacterial species, depending on the prevalence of antibiotic-resistant bacteria (Fair and Tor, 2014). The current study results demonstrated that most dental isolates, which related to different species, exhibited Cefoxitin resistance. The bacteria develop β-lactamase due to the fact that existence of fundamental processes, an inhibitory barrier to bacteria cell wall, and an inability to bind protein to penicillin *(*Kapoor *et al*., 2017). The results of the present study statistical analysis revealed, in the case of aerobic isolates, a significant negative correlation (P -value < 0.05) between the formation of biofilm and the susceptibility to Cefadroxil, Cefoxitin, and Piperacillin while in case of anaerobic isolates there is also a significant negative correlation (P < 0.05) between the biofilm formation and the susceptibility to Cefadroxil, Cefoxitin, Piperacillin, Cefamandole, Aztreonam and Amoxicillin. All these antibiotics have the same mode of action for inhibiting bacterial cell wall. In contrast, the percentage of significant and non-significant correlations differs between anaerobic and aerobic isolates, where results revealed that the formation of biofilm and susceptibility to antibiotics showed a non-significant correlation in three antibiotics for anaerobic isolates and six antibiotics for aerobic isolates. Microbial biofilms may provide a favourable environment for transferring resistance genes between the implanted microbes (Auer *et al*., 2022). Our findings might indicate the significance of biofilm matrix in the transmission of resistance genes among the oral cavity bacterial population, also the possibility that anaerobic conditions might be more conducive to the success of this process. So, we can conclude that in addition to adding a new perspective that anaerobic conditions may be more favorable for the microorganisms to disseminate the resistance genes via the biofilm matrix. This work may introduce a confirmation to the earlier findings regarding relationship between microbial biofilm, from clinical and environmental origin, and antibiotic resistance. An innovative strategy to combat MDR infections by inactivation of biofilm activity using different compounds

(Yousef *et al*., 2022) may be provided by the detection of such correlations. This is vital for researching how oral infections behave. (Ben-Zaken *et al.*, 2021; Chen *et al.*, 2020 & Flemming, 2016). This could be useful for researchers looking at new therapeutic compounds as antibiotic alternatives to focusing on anti-biofilm agent to combating the MDR epidemic (Abd Elkarim *et al*., 2020; Soliman *et al.*, 2022; Soliman *et al*., 2023 & Yousef *et al.*, 2022).

Declarations:

Ethical Approval

The sampling process in this study was carried out at the College of Dentistry Al-Azhar University, Cairo, Egypt. The College of Dentistry, Al-Azhar University's Ethical Committee (Approval code 914/341), in addition to Faculty of Science, Ain Shams's Ethical Committee (Approval code ASU/SCI/MICR/2023/4/3) granted ethical approval to the research.

Consent to Participate

Authors understand that their participation is voluntary.

Consent to Publish

Authors gave their consent for the article to be published in the Journal.

Authors Contributions

Asmaa Alhalafwy: Methodology, data recording, writing the manuscript, **HebatAllah Ibrahim Abdelazeim Youssef**: Methodology, writing the manuscript, data analysis, supervision, data reviewing, **Hassan M. Gebreel**: supervision, data reviewing. **Mohammed Abu-Elghait**: Conceptualization, Methodology, supervision, data analysis reviewing, submitting the manuscript to the journal,

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العلاقة بين الأغشية الحيوية لميكروبات الأسنان ومقاومة المضادات الحيوية: دراسة مخبرية

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الاغشيه الحيوية الميكروبية عبارة عن تجمع يحتوي على خلايا بكتيرية محاطة بطبقه خارجية من السكريات وهذه الخاصية في ميكروبات الأسنان أثارت مشكلات كبيرة لما لها من قدرة على الالتصاق بقوة بالأسنان والذي يؤدي إلى مقاومتها القوية للمضادات الحيوية التقليدية وكذلك مقاومة طرق التنظيف المختلفة. تهدف هذه الدراسة إلى تحديد وتوصيف ميكروبات الأسنان المكونة للأغشية الحيوية لتقييم الإحصائيات المتعلقة بتكوين الأغشية الحيوية ومدى القدرة على مقاومة المضادات الحيوية في ظل الظروف الهوائية واللاهوائية فقد تم جمع المسحات من التجويف الفمي للمرضي باختلاف اعمارهم ثم عزل البكتريا في وسط غدائي مناسب و تم وضعها تحت اختبارات كيفية وكمية لتحديد البكتريا ذات القدره علي تكوين الأغشية الحيوية وكذلك تم اختبار العزلات ضد مجموعة متنوعة من المضادات الحيوية وبعد ذلك تم تحليل العلاقة الإحصائية بين مقاومه البكتريا للمضادات الحيويه المختلفه و قدرتها علي تكوين الاغشية الحيوية عن طريق استخدام برنامج Minitab 19 و SPSS 25 باستخدام ANOVA أحادي الاتجاه. واظهرت النتيجة أن ٥٤.٩٥% من العزلات الهوائية يمكنها تكوين أغشية حيوية بدرجات مختلفة بينما الـ ٤٥% الأخرى لا تستطيع و من بين عزلات البكتيريا اللاهوائية، ٦٠.٢٧% تشكل الأغشية الحيوية بينما ٣٩.٧٢% لا تستطيع تكوين الاغشية الحيوية .وبعد كل التجارب تم اختيار أقوى الكائنات انتاجا للاغشيه الحيوية و أقدرهم على مقاومة المضاادات الحيوية المختلفه وهم انتيروكوكس فاشيوم و سيدوموناس اوروجينوزا، ستربتوكوكس ميوتنس، ستافلوكوكس اوريس ، لاكتوبسيلس رومنويساز. ومن خلال هذه الدراسة وجدنا في حالة العزلات الهوائية تكوين الأغشية الحيوية يرتبط سلبا قيمة ($P < 0.05$) مع القابلية لكل من السيفادروكسيل ، السيفوكسيتين، والبيبيراسيللين. ومن ناحية أخرى أظهرت الدراسة أن تكوين الأغشية الحيوية والقابلية للسيفادروكسيل، سيفوكسيتين. بيبيراسيلين، سيفاماندول، أزتريونام وأموكسيسيلين هي أيضًا مرتبطة بشكل سلبي بشكل كبير لقيمة ($P < 0.05$) في حالة العزلات اللاهوائية. تعد هذه الدراسة مهمة لتحديد الأسباب التي من خلالها تستطيع بكتيريا الفم والاسنان مقاومة المضادات الحيوية ومنها الأغشة الحيوية وبالتالي تمكن الباحثين فيما بعد في استكشاف مواد لمكافحة هذه الأسباب.

الكلمات المفتاحيه: بكتريا الاسنان، الأغشية الحيوية، العلاقة، مقاومه المضاد الحيوي، تسوس الأسنان.