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Biofilm-Forming oral Microorganisms in Dental Caries: Isolation, Identification, and Nanoemulsion Intervention

Sofia. A*[1], Rimal Issac. R. S[2], Aneesh Nair[3], Sujina. M. G[4]

[1]Research Scholar, NICHE Kanyakumari

[2]Associate Professor (Dept. Of Nanotechnology) NICHE Kanyakumari

Deemed-To-Be-University, Accredited by Ministry of Education NAAC(Cycle2)

Email ID: rimalisaac@niuniv.com

[3]Principal Scientist NIMS-CGM &Associate Professor NICHE

[4]Scientific Officer NIMS-CGM NIMS Medicity

***Corresponding Author**

Sofia. A

[1]Research scholar, NICHE Kanyakumari

Email: sofia.dentalresearch@gmail.com

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ABSTRACT

Introduction: Oral biofilm, or dental plaque, is a complex microbial community composed of bacteria, fungi, and other microbes that form extracellular polymeric substances (EPS), leading to dental caries, periodontal disorders, and other oral health problems. Biofilms are resistant to antimicrobial agents and host immune responses, necessitating alternative treatment strategies. Herbal extracts, probiotics, quorum-sensing inhibitors, and biomaterials are being explored as biofilm inhibitors.

Objectives: This study aims to isolate and morphologically characterize microbial species from oral swabs and assess their biofilm-forming potential through a multidisciplinary approach involving colony morphology, VITEK® 2-based taxonomic identification, and biofilm evaluation using crystal violet and Congo red agar assays.

Methods: Swabs from eight patients with dental caries and plaque were collected and used for laboratory analysis. Bacteria were isolated through serial dilution and cultured on nutrient and Mitis salivarius agar (MSA) media. Anaerobic and aerobic bacteria were identified using Gram's staining and the VITEK® 2 automated system. The next step will involve nanoemulsion preparation to assess its antimicrobial and biofilm inhibition effects.

Results: The study identified ten bacterial isolates from oral swabs, including Alloiococcus otitis, Enterococcus faecium, Klebsiella pneumoniae, Kocuria kristinae, Gemella bergeri, and Candida albicans, with distinct morphological features and confirmed taxonomy via the VITEK® 2 system. Among these, Enterococcus faecium (OD 1.32) and Alloiococcus otitis (OD 0.91) demonstrated significant biofilm-forming ability as confirmed by both crystal violet assay and Congo red agar assay, highlighting their potential role in oral biofilm development. Identified microorganisms included Alloiococcus otitis, Enterococcus faecium, Klebsiella pneumoniae, Kocuria kristinae, Gemella bergeri, and Candida albicans. Biofilm formation was assessed using Congo red and crystal violet assays, revealing biofilm production in Enterococcus faecalis and Alloiococcus otitis.

Conclusions: This study concludes that among the six microbial species identified from oral swabs of eight patients with dental plaques, Alloiococcus otitis and Enterococcus faecalis demonstrated significant biofilm-forming ability as confirmed by crystal violet and Congo red agar assays, suggesting the need for future exploration into antibiofilm strategies.

KEYWORD: *Oral Biofilm, Periodontal Diseases, Nanoemulsions, Antimicrobial Efficacy, Biofilm Inhibition, Crystal Violet, Congo Red Agar, Extracellular Polymeric Substance.*

1. INTRODUCTION

Oral biofilm (dental plaque), a complex microbial community formed on teeth and oral surfaces, which consists of bacteria, fungi, and other microorganisms embedded in a matrix of polymers (Jakubovics et al., 2021; Costa et al., 2023). Controlling and eliminating oral biofilm is crucial for maintaining oral health. Theformation of oral biofilm begins with the attachment of bacteria to the tooth surface, multiplying and forming micro-colonies. As the biofilm matures, it becomes more complex, with different species of bacteria interacting and communicating with each other. Biofilms are difficult to control, causing dental caries, periodontal disorders, and other oral health issues (Ray, 2022).

The oral cavity is exposed to air, but the surface of teeth becomes anaerobic when it gets colonised (Khan & Sarmah, 2023). Their complex structure makes them highly resistant to antimicrobial agents and host immune responses, necessitating effective biofilm inhibition strategies. Once the biofilm develops, the proteolytic enzymes develop and cause damage to tissues (Ramírez-Larrota & Eckhard, 2022). If this is not properly treated, it will lead to more infections like aspiration, pneumonia, and COPD (Chronic Obstructive Pulmonary Disease) (Kolpen et al., 2022). Due to the limitations of conventional antibiotics and antiseptics, there is an increasing focus on herbal extracts, probiotics, quorum-sensing inhibitors, and biomaterials to disrupt biofilm formation. Treating biofilm became a challenge, so we came to the new study, which is free of side effects.

Nanoemulsions offer a powerful approach to biofilm inhibition due to their enhanced penetration, antimicrobial efficacy, and controlled release properties (Nabawy et al., 2022; Garcia et al., 2022). Nanoemulsions may include essential oils, antibiotics, antimicrobial peptides, or other oral pathogen-fighting chemicals. Their nanoscale size allows them to disrupt the EPS matrix, reaching deep within biofilms and effectively targeting oral pathogens responsible for dental caries and periodontal diseases. Samples collected from patients' swabs were first pre-inoculated. For isolating aerobic bacteria Serial dilution method was used (Aneja, 2009), and the Mitis salivarius agar method was used for isolating anaerobic bacteria. For the Identification of bacteria Gram staining method was used, and nine +cocci and one-rod microorganism were identified. Bacterial isolates were identified using the VITEK 2 automated system following the manufacturer's protocol. Based on the biochemical reaction patterns identified, the seven microorganisms are six bacteria and one fungus.

With the growing prevalence of antibiotic-resistant infections and chronic oral diseases linked to biofilm formation, it is imperative to continue this line of research. Although some work has been done to establish a foundation of knowledge regarding microbial communities in the mouth, there remain gaps in the full characterization of the biofilm-forming organisms, particularly those related to robust, automated systems that exceed the traditional biochemical or culture-based investigations. In addition, many studies focus chiefly on phenotypic characterization, causing these investigators to link morphological diversity and biofilm production only superficially. The need for more rapid and comprehensive microbial identification tools such as the VITEK® 2 system, validated biofilm detection methods like crystal violet and Congo red agar assays, becomes paramount for the advancement of clinical microbiology and research into oral health. Furthermore, the increasing social interest in understanding how microbial diversity enhances biofilm resilience has underlined the need to reconsider oral microbial profiling using new tools and methods. Microbial identification married to biofilm assessment holds promise for supporting the development of directed antimicrobial interventions and preventive strategies, particularly with vulnerable groups.

Many research works have been conducted to isolate and characterize biofilm-producing microbes from different oral niches, which form a solid basis upon which microbial ecology understanding in the mouth is constructed. Alghamdi (2022) explored the bacterial flora from healthy and unhealthy dental samples by isolating and identifying bacteria using biochemical tests for screening their ability to produce bacteriocin. A total of 120 swabs were collected from subjects attending dental clinics in Makkah City and incubated under nutrient broth. From these swabs, 15 bacterial strains were isolated, of which 8 were Gram-positive and 7 Gram-negative. Streptococcus pneumoniae (n = 26) was found to be the most dominant Gram-positive bacterium, while Escherichia coli (n = 26) was the leading Gram-negative species. The family Enterobacteriaceae showed the highest prevalence (19.36%), followed by Streptococcaceae (13.83%). Notably, the pathogen Klebsiella pneumoniae (n = 14), which is known to be cariogenic, was also identified. Bacteriocin production was particularly evident in Enterobacter species, which showed hostile antagonism against E. coli and K. pneumoniae. According to Viksne et al. (2023), they examined the biofilm formation and antibiogram profiles of these potential pathogens in the tonsillar crypts of healthy individuals: Staphylococcus aureus, K. pneumoniae, Pseudomonas aeruginosa, and Acinetobacter spp. Out of the patients sampled, 90 participants were considered because 40.7% of their samples contained some of the common oropharyngeal microbiota. S. aureus is the major pathogen (45%); K. pneumoniae is second (7.7%), then Acinetobacter spp. (5.5%) and P. aeruginosa (2.2%). A total of 51 strains are identified as biofilm producers, whereas 31 strains create moderate or strong biofilms.

In the same manner, Mohammed et al. (2022) researched the use of nanoparticles made by laser ablation concerning the inhibition of oral pathogens in biofilm formation. Patients of gingivitis and dental caries provided the samples, and it was found that the use of nanocomposites such as silver with polymer inhibited bacterial growth quite extensively. Other examples are graphene oxide-silver-polymer and zinc-polymer. The Ag + Zn + poly composite, however, did not exhibit activity against Sphingomonas paucimobilis, S. pneumoniae, Serratia plymuthica, and Staphylococcus hyicus. Treatment effects were also validated by an appreciable reduction in biofilm formation, as confirmed through a significant drop in optical density at 490 nm ($p \leq 0.05$) after treatment. Ali Hameed AL-Dabbagh et al. (2023) focused on the virulence factors identification from Candida species which were isolated from oral and vaginal swabs. A total of 58 samples were received,

out of which Candida isolates varied in their virulence enzyme expressions. Phospholipase, esterase, and proteinase were produced by 61.29%, 51.61%, and 83.87% of isolates, respectively. All strains except for Candida dubliniensis produced coagulase, while all the isolates showed hemolysin production and biofilm formation to various extents. Ngwu et al. (2022) studied the antibiogram profiles of oral Streptococci species forming biofilms in patients with dental caries. Of the total 450 swabs collected from dental plaque, carious samples, and saliva, streptococcus species were isolated predominantly from carious samples (37.1%). The age group of 21-35 years showed the highest prevalence (36%), with male patients more affected (40.2%) than female counterparts. However, no significant association was observed concerning the presence of Streptococcus and dental carries in female patients.

Radunovic et al. 2022 studied the Candida species in tongue and subgingival samples from periodontal patients. Among the 163 subjects, Candida spp. was found in 23.3% of tongue samples and 21.5% of subgingival samples. The isolates were generally found susceptible to amphotericin B, whereas fluconazole resistance appeared in about 35% to 47% of the isolates site-dependent. Asghar et al. in 2023 characterized S. aureus strains isolated from the oral microbiota and investigated demographic associations. A total of 18 S. aureus strains were isolated and confirmed from 84 human oral cavity samples through the nuc gene. Their identification was particularly aided through the use of sheep blood agar and mannitol salt agar in conjunction with associating environmental and host-related factors. Chen et al. 2020 examined biofilms of extracted maxillary incisors for the existence of potentially cariogenic microbes. After determining acidogenicity and aciduricity, four strains- Streptococcus salivarius, Streptococcus anginosus, Leuconostoc mesenteroides, and Lactobacillus sakei- were selected based on the aforementioned criteria and confirmed through 16S rRNA gene sequencing. Those isolates showed the complex and varied cariogenic potentials of oral biofilm communities. Wu et al. in 2020 screened biofilm-producing bacteria from caries and identified P. aeruginosa DC-17 as a strong biofilm former. The strain produced a dense EPS matrix consisting of eDNA, proteins, and lipids, which played an important role in stabilizing the biofilm and conferring resistance. Omori et al. (2021) studied the impact of four different saliva sampling methods—spitting, chewing, swabbing, and rinsing—on oral microbiome analysis. There was no variation in microbial community compositions observed across the methods and time points concerning the genus level. Khalili et al. (2018) conducted a molecular study on bacteria responsible for dental caries among the public in Tehran. The dominant biofilm-producing microorganism in 133 samples was S. mutans. Others include S. mitis, S. sanguinis, S. gordonii, Lactobacillus gasseri, Jemela hemolysin, and Granulicatella adiacens. Molecular characterization was performed using 16S rRNA sequencing, whereas susceptibility to zoocin A and clove extract was evaluated, proving its potential alternative to traditional antimicrobials.

However, many of the existing works find some limitations in their full establishment of biofilm inhibition strategies, with many studies having delved into biofilm-producing microbes of the oral cavity. Most studies focused on establishing the infection levels of specific pathogens, S. aureus, K. pneumoniae, and P. aeruginosa, to other forms of biofilm disruption dimension, that is, non-antibiotic forms. More focus is found in nanoparticle and biochemical studies, which ever showed limited reference to the inhibition of biofilm formation by naturally derived inhibitors such as phytodrugs, probiotics, or quorum sensing inhibitors. In addition, some studies have focused only on phenotypic or biochemical identification, which cannot be compared to accuracy or efficiency by advanced automated systems. It will, therefore, be an advantage to address all these gaps with an introduction of novel intervention techniques in biofilm inhibition, such as plant extracts, probiotic application, quorum sensing inhibition, and biomaterials. Additionally, it involves an advanced, robust dual-layer microbial identification protocol using Gram staining and the VITEK® 2 automated system, thus ensuring precise classification and efficient identification of anaerobic and aerobic bacteria. By using swabs from patients with dental caries and plaque and culturing bacteria on both nutrient and Mitis salivarius agar media, the study offers a much more focused, practical approach to managing oral biofilm formation, thereby contributing a new and holistic perspective to the field.

2. OBJECTIVES

The ultimate aim of this study is to isolate and morphologically characterize the microbial species present in the oral swabs collected from different individuals and assess their biofilm-forming ability by standard laboratory techniques. The specific aims include: (1) providing visible differentiating characteristics of the microbial colonies like color, shape, margins, and texture; (2) the VITEK® 2 automated system to identify and accurately classify the taxa of the isolate; (3) evaluating the biofilm-producing potential of the isolates by crystal violet and Congo red agar assays. In a multidisciplinary approach combining morphology, biochemistry, and biofilm analyses, we aim to arrive at a finer resolution concerning the biofilm-producing microbial habitat of the oral cavity. The findings will help in the development of proper diagnostics and therapeutics in oral microbiology towards the clinical management of biofilm-associated oral infections.

3. METHODS

Ethical clearance

For the work, Ethical clearance was taken from the hospital for collecting oral swabs. There is a need to assure patients that identification will be kept confidential, and there will be no advantage or disadvantage to their assessment in which research is being undertaken. The collected data will be used only for research. The consent form was also collected from each patient before swabs were collected.

Sample area

The dental caries swabs were collected from the Nims dental hospital in NIMS Medicity from eight different patients (N=8) with dental plaques and caries visiting NIMS dental Hospital. Samples were collected from the infected area of the tooth using a sterile cotton swab, and the samples were used for further laboratory examination.

Isolation of aerobic bacteria

For the isolation of bacteria, first, it should be pre-inoculated. These microorganisms were isolated from freshly collected swabs. Swabs containing different bacteria were separately inoculated into 1 mL of 0.1% buffered peptone water and incubated at 37℃ for 24 hours. The serial dilution method was used to isolate aerobic bacteria. 100 µl of peptone containing microorganisms was transferred to 1 ml of sterile distilled water in an Eppendorf tube. Dilutions from 10-1 to 10-5 were prepared using sterilized distilled water (Aneja, K. R.,2009). From the serially diluted sample, 0.1 ml of each sample was spread on a nutrient agar medium for aerobic bacterium. These plates were then incubated at 370C for 24-48 hours. After incubation, distinct colonies were found on the agar plate, and selected bacterial colonies were purified by repeated streaking (Prescott et al., 2005).

Isolation of anaerobic bacteria

Mitis salivarius agar (MSA) supplemented with 1% potassium tellurite was used for the isolation of anaerobic bacteria. 100 µl of peptone containing microorganisms was transferred to 1 ml of sterile distilled water in an Eppendorf tube. Dilutions from 10-1 to 10-5 were prepared using sterilized distilled water (Aneja, K. R.,2009). From the serially diluted sample, 0.1 ml of each sample was spread on an MSA medium for anaerobic bacterium. These plates were then incubated at 370C for 24-48 hours in anaerobic conditions. After incubation, distinct colonies were found on the agar plate, and selected bacterial colonies were purified by repeated streaking (Prescott et al., 2005).

Identification of bacteria

The Gram's reaction property and cell morphology of all ten bacterial isolates were examined using a standard staining procedure. For this, the Gram staining method is used. Gram staining was used for distinguishing both Gram's positive and negative bacteria. The pure culture of the selected colony was heat fixed on a clean glass slide. The primary stain crystal violet was poured over the slide and left to stand for 1 minute, then rinsed thoroughly with running tap water. The mordant Gram's iodine was pored over the slide and kept for 1 minute, then thoroughly rinsed with running tap water. This was followed by decolorization with 95% ethanol and rinsing with tap water. This slide was counterstained with safranin for 1 minute. Then, again rinsed with tap water and allowed to air dry. The dry slide was covered with immersion oil and viewed under a microscope (Cappuccino and Sherman,2005).

Identification of microbes

Bacterial isolates were identified using the VITEK® 2 automated system (bioMérieux, France) following the manufacturer's protocol. Pure colonies were loaded onto the VITEK® identification cards, incubated in the instrument, and results were automatically generated based on biochemical reaction patterns. The identified organisms are Alloiococcus otititis, Enterococcus faecium, Klebsiella pneumoniae, Kocuria kristnae, Gamella bergeri, and Candida albicans.

Screening Assays for Biofilm Formation

Screening of test organisms for biofilm formation is done by two methods: Crystal violet assay and Congo red agar assay. In the Crystal violet assay, a single colony of suspected oral biofilm-producing bacteria was inoculated on nutrient broth and incubated for 24 hrs at 37°C. After incubation, the cells were dumped out by discarding the broth, and the tubes were washed with 1X PBS two times. A freshly prepared 0.1% solution of Crystal Violet (CV) in water (3 ml) was poured onto the tubes and incubated at room temperature for 10-15 min with intermittent shaking. After incubation, the tubes were rinsed

with sterile water and dried overnight at room temperature. To each tube, 3ml of 30% acetic acid solution was added and incubated for 15 min for solubilizing the CV solution. The solubilized CV solution was transferred to fresh tubes, and the absorbance at 540 nm was measured using a UV-visible spectrophotometer using 30% acetic acid in water as the blank. Congo red agar assay was used to detect biofilm formation among the microbial isolates (Freeman et al., 1989). The medium was composed of Brain heart infusion broth 37g/l, Sucrose 50g/l, agar 10g/l, and Congo red 0.8 g/l. Congo red stain was prepared as a concentrated aqueous solution and autoclaved (1210C for 15 minutes) separately from other medium constituents and was then added when the agar had cooled to 55oC. Plates with Congo red agar medium were inoculated and incubated aerobically for 24 hours at 37 oC. The results were interpreted based on colony morphology.

4. RESULTS

A total of six distinct colonies were observed with different morphological characteristics from eight different swabs (Fig. 1).

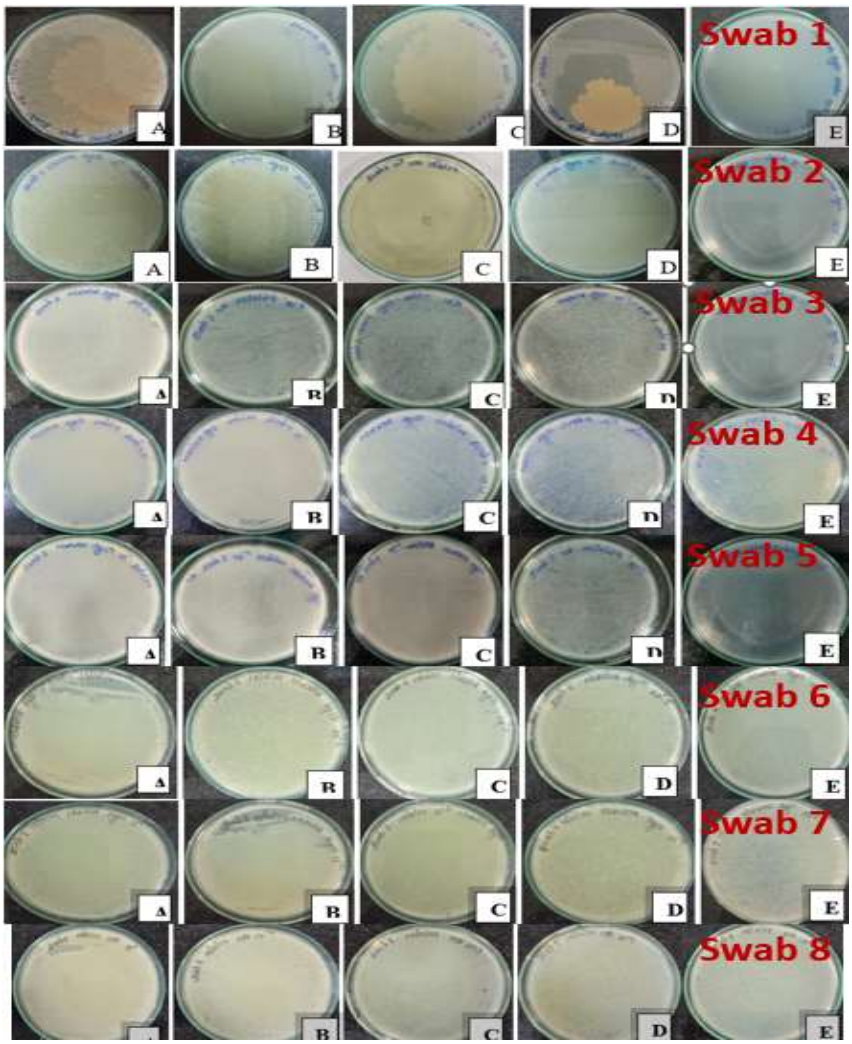

Fig. 1: Isolation of microorganism from dental caries (swab 1 to 8) "A:10^{-1}, B:10^{-2}, C:10^{-3}, D:10^{-4}, E:10^{-5} dilutions"

The pure cultures of these isolates were further tested for macroscopic observations. Morphological characteristics such as colony colour, shape, margins, elevation, and opacity were carefully observed to characterize the bacteria into 6 distinct groups. Six isolates (S1A, S2B, S3B, S5A, S6A, and S8A) were creamy white, yellow, and light green with irregular shapes and spreading, smooth textures. They were again subcultured into a nutrient agar (Table 1 & Fig 2).

Table 1: Morphological characteristics of different isolates

Isolates	Colour	Shape	Texture	Margin	Elevation
S1A	Off white	Irregular	Rough	Entire	Flat
S2B	Yellow	Irregular	Smooth	Entire	Flat
S3B	Light green	spreading	Rough	Entire	Raised
S5A	Cream	Irregular	Rough	Entire	Flat
S6A	Light green	Irregular	Smooth	Entire	Flat
S8A	Cream	Irregular	Smooth	Entire	Flat

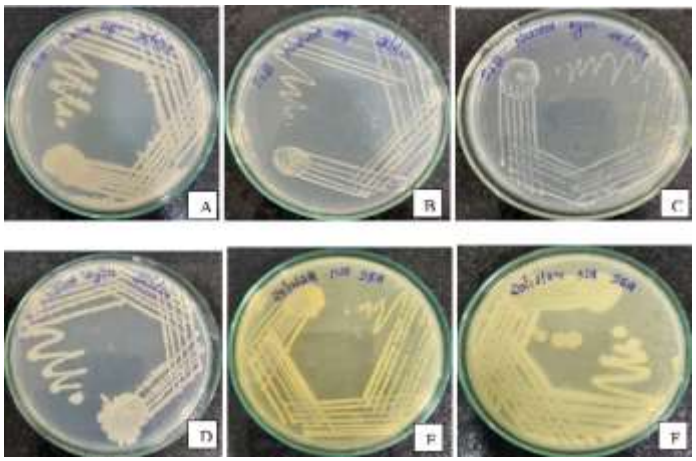

Fig. 2: Different bacterial isolates A: S1A, B: S2B, C: S3B, D: S5A, E: S6A, F: S8A.

Identification of microorganisms

All ten bacterial isolates were identified by using VITEK and identified the culture. VITEK is an automated system for identifying the cultured sample. This manufacturer's protocol is used. A pure colony was loaded in the machine and inoculated. Identification cards are present in a 10-well cassette holder. For different organisms, different cards are present. Having a standard acceptable range. The purity of culture is checked by the McF range, which varies for different organisms. After 3 hours, the result were obtained.

A total of four distinct colonies were observed from eight different patients' swabs in Mitis salivarius agar. They were again subcultured into MSA plates until the pure cultures were observed. This study gives insight into the importance of the biofilm formation. Results were summarized in Fig 3 & Table 2.

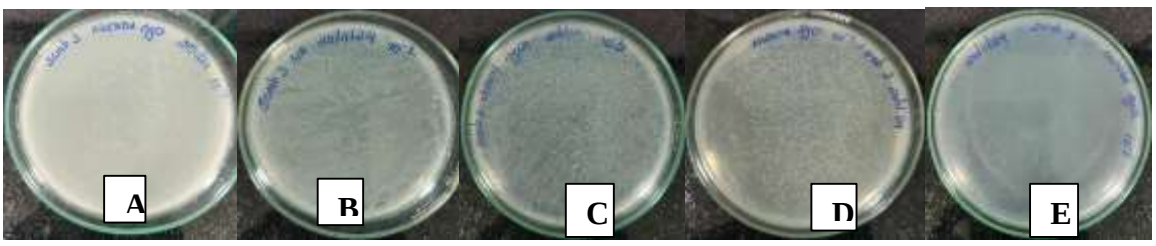

Fig.3: Isolation of anaerobic microorganisms from dental caries (swab 1-6) A:10-2, B:10-3 dilutions

Table 2: Morphological characteristics of different isolates

Isolates	Colour	Shape	Texture	Margin	Elevation
S1	Bluish white	Irregular	Smooth	Entire	Flat
S2	Bluish white	Irregular	Smooth	Entire	Flat
S6	Bluish white	spreading	Smooth	Entire	Raised
S8	Bluish white	Irregular	Smooth	Entire	Flat

Fig. 4: Different bacterial isolates A: S1, B: S2, C: S6, and D: S8

Table 3 Gram's Staining

Isolates	Gram's staining
S1A	+ cocci
S2B	+ cocci
S3B	+ cocci
S5A	+ cocci
S6A	- rod
S8A	+ cocci
S1	+ cocci
S2	+ cocci
S6	+ cocci
S7	+ cocci

The microbial isolates revealing the presence of *Alloiococcus otitis, Enterococcus faecium, Klebsiella pneumoniae, Kocuria kristinae, Gemella bergeri,* and *Candida albicans* (Table 4).

Table 4: Identification of microorganisms in oral swabs by Vitek method

Isolates	Microorganisms
S1A	*Alloiococcus otitis*
S2B	Unidentified
S3B	*Enterococcus faecium*
S5A	*Alloiococcus otitis*
S6A	*Klebsiella pneumoniae*
S8A	Unidentified
S1	*Kocuria kristinae*
S2	*Gemella bergeri*
S6	Unidentified
S7	*Candida albicans*

Crystal Violet Assay

Isolated colonies were checked for biofilm formation using crystal violet assay. The cultured sample is inoculated for 72hrs in 3ml Broth. Biofilm is formed in the test tube. Then, discard the broth. To this, 3ml Crystal violet solution was added and incubated at normal temperature. Again, drain out and wash with PBS solution two times. Add Glacial Acetic Acid and incubate for 15 minutes. Then, the solution is transferred to fresh tubes. For taking OD (Optical Density) values, 1 ml solution is transferred to a Quiet tube and placed in the calorimeter. Absorbance at 540nm was recorded and tabulated as follows: Bacterial colonies Enterococcus faecalis and Alloiococcus otitis showed absorbance values of 1.32 and 0.91 at 540 nm, indicating a biofilm production value above 1 gives the confirmation of biofilm.

Fig.5: Crystal violet assay: Tube 1: Control; Tube 2: *Klebsiella* pneumoniae; Tube 3: *Alloiococcus otitis;* Tube 4: *Kocuria kristinae;* Tube 5: *Gemella bergeri;* Tube 6: *Enterococcus faecium*

Congo Red Agar Assay

The Congo Red Agar assay was used to detect biofilm formation among the microbial isolates. Place the culture in prepared Congored agar medium(10gm agar and 1 pinch of Congored powder mixed in distilled water and media prepared). Streaking is done and inoculated for 24 hours. Brownish-black colonies in dry crystalline form were observed in Congo red agar plate inoculated with Enterococcus Faecalis. Strong biofilm producers exhibited black, dry, and crystalline colonies, indicating the presence of an extracellular polysaccharide matrix.

The morphology of the colonies in the Congo agar plate was observed and shown in Fig. 6.

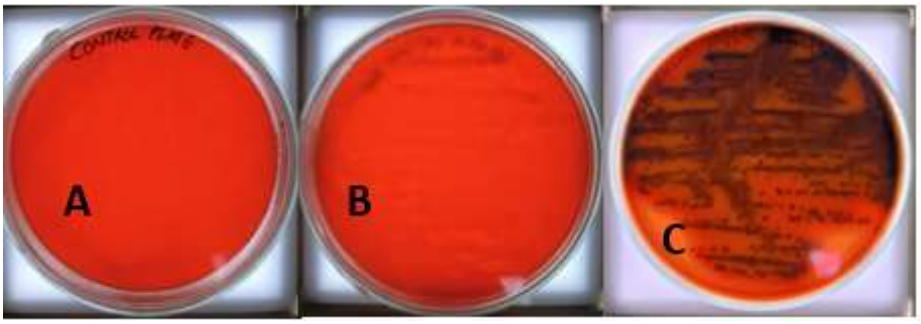

Fig 6: Congo Red Agar Assay: (A) Control plate; (B) *Alloiococcus otitis; (C) Enterococcus Faecalis*

This study helps to understand the oral mucosa, which also gives insight into the biofilm formation of microorganisms. Crystal violet assay and Congored agar assay used for getting the biofilm producing microbes. Thus, two organisms, Aloi coccus otitis with OD value 0.91 and Enterococcus faecium with OD value 1.32 which help to understand biofilm formation in the microbes. The OD value of above 1 will be the best biofilm formation microbes in the study.

Table 5: OD VALUE

SI.NO	Sample	OD at 540 nm
1.	Control	0.00
2.	*Klebsiella pneumoniae*	0.47
3.	***Alloiococcus otitis***	**0.91**
4.	*Kocuria kristinae*	0.53
5.	*Gemella bergeri*	0.62
6.	***Enterococcus faecium***	**1.32**

5. DISCUSSION

This study on the Isolation of Biofilm-Producing Microbes from Oral Swabs includes substantial advances to the knowledge of microbial communities inhabiting the oral cavity as well as their potential to produce biofilms, complex, matrix-enclosed microbial communities that provide resistance against environmental stressors and antimicrobial agents. The study provides an extensive insight into the diversity of biofilm-forming bacteria isolated from 8 patients using careful application of the full range of modern microbiological techniques including serial dilution, Gram staining, VITEK 2 automated identification,

Crystal Violet assay, and Congo Red Agar assay. Its significance has been amplified by identifying such important biofilm-forming pathogens like Enterococcus faecalis, Alloiococcus otitis, Klebsiella pneumoniae, Kocuria kristinae, Gemella bergeri, and Candida albicans, which are now known and again be an important part of the literature as clinically relevant mdlroahn and systems in which they are involved.

The dissection of biology and application of VITEK 2 for microbial identification in the present study have given a new paradigm for any conventional biochemical methods, identifying microorganisms in a relatively faster, automated, and standardized way with more precision. Many earlier papers that were published, like Alghamdi (2022), have to rely on biochemical profiling for describing microbial flora from the oral niches. In contrast, this study throws light on the advanced methodology through the VITEK system, in which it intends to automize the effective categorization of aerobic and anaerobic bacteria. This corresponds to best practices in modern clinical microbiology. In addition, classical morphological observation and Gram staining configuration improve the dual-layer validation that heightens the credibility of the process involved in the microbial identification.

Unlike Alghamdi's work, which reported a predominance of Streptococcus pneumoniae and Enterobacteriaceae family members as determined by biochemical screening, this investigation includes a wider variety of bacteria, which includes Klebsiella pneumoniae, another pathogen isolated from Alghamdi's samples and one that is known for its intense biofilm formation. Finding this organism in both studies underscores its relevance as an oral pathogen that keeps coming back to haunt us, also showing its adaptability for many niches in the oral cavity. The current detection of Enterococcus faecalis is interestingly congruent with Viksne et al.'s (2023) observations regarding biofilm formation in the oropharyngeal microbiota, identifying several biofilm-producing species, including Staphylococcus aureus and K. pneumoniae. In Viksne's research, nearly 51 isolates demonstrated strong biofilm formation, which further complements the assumption of biofilm phenotypes being prominent amongst common oral and pharyngeal microbes. Indeed, the presence of E. faecalis with characteristics for an opportunistic pathogen and high resistiveness further adds weight to the evidence gathering regarding its importance in chronic oral infection, especially in endodontic failures and periodontal disease. Unexpectedly found is Alloiococcus otitis, albeit rarely reported from oral niches, considered an otitis-related aggressive organism. Its identification in the present study may insinuate an otherwise less appreciated presence in the oral cavity deserving attention.

The detection of Candida albicans echoes with the findings of Ali Hameed Al-Dabbagh et al. (2023), wherein these authors reported diversified virulence factors among Candida species isolated from oral and vaginal swab samples mainly relating to the enzymatic production and strong biofilm formation. Like their findings, the present research also highlights the general plasticity of C. albicans as a biofilm producer, which also contributes to its pathogenicity and resistance to antifungal treatment. Biofilm formation by Candida has been extensively reported, especially in immunocompromised patients, wherein its presence in polymicrobial biofilms further complicates treatment regimens. The assessment of biofilms from the isolates was also confirmed by the Crystal Violet and Congo Red assays applied for this investigation. The value of optical density (OD) meters measurement is used to quantify the biofilm density, while the latter assay qualitatively states the presence of an extracellular polysaccharide matrix, which is typical for strong biofilm producers. These methods conform to what Wu et al. (2020) used in the assessment of the biofilm formation ability of P. aeruginosa from caries samples. Their work brought attention to the role of the extracellular polymeric substance (EPS) matrix, the same feature confirmed in this study through the presence of black, dry, and crystalline colonies on Congo Red Agar from E. faecalis isolates.

In addition, the present findings are in agreement with the observations made by Mohammed et al. (2022). Nanoparticle administration was tested for its biofilm inhibition properties. Our study did not investigate inhibition; however, the clear documentation of biofilm formation by various microbes in this study provides basic data on which such therapies can be further evaluated and tested. In the study by Mohammed et al., there was a significant reduction in OD readings due to the treatment by nanoparticles, which only reinforces that OD measurements are indeed a good indicator of biofilm biomass. Ecologically, this study lends credence to the suggestive ever-present oral microbial diversity and polymicrobial biofilm structures. As narrated by Ngwu et al. (2022) in their extensive enumeration of oral Streptococcus species, biofilm formation is anything but copybook, as these organisms interact with each other in a myriad of ways. While six different biofilm-forming organisms characterize this current investigation, the question of their further co-operative or competitive interactions in actual settings is raised in the turn of oral microbial consortia.

In this study, the detection of Kocuria kristinae and Gemella bergeri would add an aspect of less studied oral residents to biofilm research. These organisms, although not been targets in significant studies of biofilms, indeed widen the spectrum of biofilm-producing microbes in the oral cavity. Future studies ought to unravel their roles in coaggregation, succession between these microbes, or opportunistic infections. The combination of both nutrient agar and Mitis Salivarius agar for culturing aerobic and anaerobic bacteria is another methodological strength. That comprehensive cover enables an all-around impression of the aerobic-anaerobic interface of the oral microbiome: a critical issue, as Radunovic and others (2022)

demonstrated in their independent research since the concept of anatomical site-dependent variation in presence and antifungal resistance came out. The inclusion of both types of media provides maximal recovery of microbes while simulating in vivo oral ecological conditions, particularly in regions like the gingival sulcus or deep carious lesions where oxygen gradients vary.

With the majority of mentioned studies (e.g., Khalili et al., 2018; Omori et al., 2021) addressing the identification and characterization of oral microbiota based on the use of 16S rRNA or different salivary sampling methods, the current study contributes uniquely through the integration of morphological, biochemical, and automated identification techniques. All these methodologies together rectify some of the shortcomings of earlier studies regarding phenotypic identification and strongly endorse the requirement for high-throughput and automated microbial identification and susceptibility profiling. The limitation acknowledged, however, presents an encompassing gap in literature, including this one, and that is the scant coverage of biofilm inhibition measures. Following biofilm identifications and confirmations are very relevant thoughts; the next frontier in research should be centered on studying ways to disrupt or inhibit these biofilms. From Mohammed et al. (2022) to Khalili et al. (2018), more studies have explored biofilm inhibition using nanoparticles and plant-based agents, while a more thorough exploration of probiotics, quorum-sensing interference, and natural biofilm disruptors remains lacking.

The study was aimed at finding out which microbes are producing biofilm. For the work, Ethical clearance was taken from the hospital for collecting oral swabs. There is a need to assure patients that identification will be kept confidential and, the collected data will be used only for research. A consent form was also collected from each patient before the swabs were collected. The dental caries swabs were collected from the Nims dental hospital in NIMS Medicity from eight different patients (N=8) with dental plaques and identified 6 organisms from 8 patients swab that was collected. Aerobic and anaerobic bacteria were identified using the serial dilution method and Mitis salivarius agar plate for anerobic bacteria. The Gram staining method was done to isolate bacteria. One is -rod bacteria, and the rest of them are +cocci identified. Six organisms were identified by the Vitek automated system using the manufacturer's protocol. By doing the biofilm assays Identified Biofilm formation in two organisms: Alloicoccus otitis & Enterococcus faecalis. For this Crystal violet assay and Congored agar assay are used results of the study show two organisms are showing biofilm formation. Explore in future studies may explore the Antibiofilm assay, Nanoemulsion, Sequencing, CMS & Molecular Docking.

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Conflict of Interest Statement

The authors declare no conflict of interest with regard to the publication of this paper.

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