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IDENTIFICATION OF ACTIVE COMPOUNDS FROM BACILLUS AMYLOLIQUEFACIENS JUSC-02 AGAINST BIOFILM FORMING ORAL PATHOGENS

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ABSTRACT
The prevalence of most oral infections arises due to the rising antibiotic resistance of oral bacteria against different kinds of antibiotics, which can be attributed to their biofilm forming ability. The most prevalent illnesses of the oral cavity, dental caries and periodontal disease, are caused by dental plaque. Numerous oral isolates such as Kocuria spp. (JUFC 01), Bacillus sp. (JUFC 10), and Neisseria sp. (JUFC 11) were isolated from Periodontitis, dental plaque, and dental caries individuals. These isolates displayed evident resistance to antibiotics such Trimethoprim, Vancomycin, Clindamycin, and Levofloxacin. The production of biofilm was one of these organisms' distinctive traits. As a result, these isolates' defense mechanism of biofilm formation can be linked to their antibiotic resistance. Secondary metabolites of various LABs were extracted to test their effectiveness against oral isolates because LABs are well-known for their wide range of anti-microbial, antibiofilm, and anti-adhesive characteristics. From pomegranate juice, *Bacillus amyloliquefaciens* (JUSC-02) was identified. To test for their inhibitory efficacy against biofilm generated by Kocuria spp, Bacillus spp, and Neisseria spp, the crude extract of this B. amyloliqu0efaciens was extracted. To investigate the inhibitory activity of crude extracts of LABs against the oral isolates and the biofilm formed by the isolates, a variety of procedures including Microtitre plate assay, MIC analysis, and antibiotic susceptibility tests were used. The fractions of CEs of LABs were to be separated using TLC method. Fluorescence microscopy and the TLC bioautography assay were used to confirm the separated fractions' inhibitory effects on the oral isolates. The bioactive fractions have been identified by FTIR and LC-MS analysis, indicating that the substance may be a phenolic component.

KEYWORDS: Oral isolates, Lactic acid bacteria, Antibiotic resistance, Anti-biofilm activity, bioactive compounds.

1. INTRODUCTION
An individual's oral health depends on the surface microflora of their teeth, gums, and oral cavity linings being in good health. It is made up of a wide range of species, including bacteria, fungus, and viruses. Streptococcus, Lactobacillus, Lactococcus, Enterococcus, Staphylococcus, and Corynebacterium species are common in the normal oral cavity (Wang et al., 2012). The oral infections contain Porphyromonas gingivalis, Fusobacterium nucleatum, Peptostreptococcus micros, Streptococcus intermedius, and Treponema denticola. Numerous systemic illnesses, like periodontitis, dental plaque, and dental caries, are brought on by these oral bacteria (Alghamdi, 2021). The most prevalent illnesses of the oral cavity, dental caries and periodontal disease, are caused by dental plaque. The first step of microbial colonization is the salivary pellicle protein adsorption by all available oral surfaces which is followed by microbial adhesion and growth. As microorganisms accumulate, they form structures known a\s biofilms. Dental plaque is a microbial biofilm made up of organisms that are closely attached to one another and to the solid substrate by way of an exopolymer matrix. Organisms such as Kocuria sp., Bacillus sp. and Neisseria sp. were isolated from individuals with oral infections such as dental plaques, caries and periodontal infections.

Kocuria sp. isolated from clinical specimens is typically oropharynx commensals. Kocuria sp are typically treated as laboratory contaminants and despite claims that they are a natura component of the flora in the mouth cavity and on human skin, their potential pathogenicity is reduced (Lim, Eun Seob, et al., 2017).

Bacillus sp. are responsible for oral infections such as thrush, Cavity, Periodontal disease, Syphilis, Gingivitis, Oral herpes, Herpangia. Infections caused by these

organisms were found to be severe or lethal. Gram-negative bacilli are abundant and diverse in the microbiota associated with periodontitis, and they are less resistant to β-lactam antibiotics (Espindola et al, 2022). For fundamental research on supragingival and subgingival biofilms, it has a lot of potential.

Neisseria sp. is a commensal bacterium of the oral cavity and respiratory tract of humans (Knapp and Rice, 1995). Infrequently, N.subflava can enter the submucosa and cause opportunistic infections, such as meningitis, septicemia and endocarditis (Pollock et al., 1988).

Most oral bacterial species are found exclusively within the mouth and do not typically have an environmental niche (Rachid et al., 2000). These bacteria would be immediately washed away by saliva, ingested, and destroyed within the stomach's acidic contents due to their planktonic development. The majority of these bacteria's natural lives are probably spent developing biofilms.

The term "biofilm" refers to a microbial community made up of clusters of bacterial cells that are attached to a surface and encased in a self-produced extracellular matrix (Sanchez et al., 2013). An essential component of virulence, biofilm offers the organisms a safe environment that allows them to survive. Many pathogenic and nosocomial bacteria have been associated with the increase in antibiotic resistance and chronic recurrent infections (Harika et al., 2020).

Kocuria sp., Bacillus sp. and Neisseria sp., form a biofilm as a protective mechanism. The biofilm structure provides several advantages to colonising species, such as protection against antimicrobials and host-defence, enhanced co-aggregation, and interaction properties (Flemming & Wingender, 2010). These protection mechanisms between microorganisms make biofilms challenging therapeutic targets (Socransky & Haffajee, 2002). Dental caries, periodontal disorders, implant-related infections, and oropharyngeal candidiasis are just a few examples of the many oral diseases that oral biofilms are the primary etiologic factor in (Yin et al., 2019).

According to Kim et al. (2009), pathogenic bacteria can't form biofilms when exposed to biopolymers or EPS from LAB. Inhibition of bacterial biofilm formation by EPS did not occur directly. By weakening cell surface modifications or by reducing cell to cell noticeably, it prevents the initial attachment and autoaggregation of bacterial cells (Kanmani et al., 2011). This suggests that the EPS from S. phocae PI80 has a broad spectrum of antibiofilm activity against bacteria that form biofilms.

3. MATERIALS AND METHODS

3.1 Ethical Approval

The study was conducted at the Department of Microbiology from School of Sciences, Jain University, Bangalore. The studies involving human participants were reviewed and approved by the Internal Review Board of Ethical Clearance Committee (Refno. PHD/EC/SC/PSY/013-AUG2022). The participants provided their return informed consent to participate in this study.

3.2 Collection of samples

11 adult participants were chosen for the current study; swab selections with oral infections like dental plaques, caries, and periodontal infections were chosen. The samples were collected from Bangalore Dental College, Bengaluru. Any patient visiting the hospital with oral infections and falling within the age range of 25 to 55 met the study's eligibility requirements. Samples were obtained and delivered to the Department of Microbiology, School of Sciences, Jain (Deemed-to-be) University, in pre-sterilized sample collection containers containing 1 ml of transport media. For later usage, the samples were stored at 4°C.

3.3 Isolation of bacteria from oral swabs

According to the protocol given by Alghamdi S. (2022) with slight modifications, the samples were collected into sample containers, containing 1 ml of transport media and transferred to the laboratory, where it is enriched by incubating overnight at 37°C in nutrient broth. Enriched samples were serially diluted and spread plate on to the nutrient agar plates containing beef extract (0.3%), peptone (0.5%), and agar (1.5%). Pure bacterial cultures obtained were stored at 4°C for further investigation.

3.4 Isolation of Lactic Acid Bacteria

Isolation of Lactic acid bacteria (LABs) was done according to the protocol given by Sobrun et al., 2012. Sugar cane juice, mosambi and pomegranate juice were collected from a local vendor, of which 10 ml was incubated in test tubes in duplicate for 20 hours at 37°C. Aliquots of the incubated juice were serially diluted to a dilution factor of 106, and 0.1 ml was spread uniformly onto the MRS (De Man, Rogosa and Sharpe agar) agar plates amended with Calcium carbonate. Following that, the plates were incubated for 48 hours at 37 °C. Only bacterial colonies with a clear zone were individually selected, subcultured and stored at 4°C for further usage.

3.5 Screening of biofilm formation in oral pathogens

A modified version of the O'Toole et al 1998; described technique was used to examine biofilm formation. Obtained isolates were cultured aerobically in NB broth and incubated at 37°C in an orbital shaker for 24hrs. Biofilm producing ability of the pathogenic bacteria was screened using microtiter plate assay. In each well 50 microlitres of the overnight culture of test organisms, 150 microlitres of the sterile fresh medium. 200 microlitres of sterile medium was considered as negative control, incubated for 24 hours at 37°C under static conditions. The biofilm formation assay for the test organisms was performed using crystal violet assay

according to the protocol followed by Aman et al. (2021).

3.6 Biochemical characterization of bacteria from oral samples

The basic biochemical tests used to identify bacteria include gram staining, citrate utilization test, Oxidase, Catalase, Indole test (Onoriode & Oshomoh., 2018).

3.7 Biochemical characterization of lactic acid bacteria

LABs characterization was performed by culturing them aerobically overnight in MRS broth (HiMedia, India). According to Guesh et al. 2019 different tests such as Gram staining, KOH test, pH tolerance, catalase and oxidase production were performed. Cell morphology and colonial characteristics were observed for selected isolates.

3.8 Antibiotic susceptibility testing

The Kirby-Bauer diffusion test was used to examine the antibiotic susceptibility profiles of all oral isolates. Vancomycin (5mcg), clindamycin (10mcg), trimethoprim (10mcg) and levofloxacin (5mcg) were the antibiotic discs (HiMedia) utilized in this study. Based on the zone of inhibition widths suggested by the European Food Safety Authority (EFSA) the classification of organisms as "susceptible," "intermediate," or "resistant" was made (EFSA, 2012), according to Kaur et al., 2018.

3.9 Molecular identification and phylogenetic analysis of bacteria from oral sample

DNA was extracted from test organisms and amplified using a set of forward (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse (1492R, 5′-ACGGCTACCTTGTTACGC TT-3′) primers (Genie, Bangalore, India). The reaction mixture (25 µl) contained 1 µl of each primer, 1 µl of template DNA and 23 µl of one fold diluted master mix (Bangalore Genie, India). The thermocycling settings were as follows: 94 °C for 4 min of initial denaturation, 35 cycles of amplification lasting 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min, and 94 °C for 8 min of final polymerization. A Gen Elute gel elution kit was used to purify the final PCR amplicon after it had been eluted in 1.5% agarose gel.

3.10 Molecular identification and phylogenetic analysis of LABs

DNA was extracted from LABs and amplified using a set of forward (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse (1492R, 5′-ACGGCTACCTTGTTACGC TT-3′) primers (Genie, Bangalore, India). The reaction mixture (25 µl) contained 1 µl of each primer, 1 µl of template DNA and 23 µl of one fold diluted master mix (Bangalore Genie, India). The thermocycling settings were as follows: 94 °C for 4 min of initial denaturation, 35 cycles of amplification lasting 94 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min, and 94 °C for 8 min of final polymerization. A Gen Elute gel elution kit was used to purify the final PCR amplicon after it had been eluted in 1.5% agarose gel. Obtained amplicon was sequenced using Sanger's method and subjected to NCBI-BLAST analysis. The sequences were deposited in NCBI-GenBank for accession number (Aman et al., 2021).

To understand the phylogenetic relationship of the LABs from the current study with previously deposited sequences in NCBI-GenBank, MEGA-X software was used to build an unrooted phylogenetic tree using neighbour-joining and maintain it using 1000 bootstraps (Aman et al., 2021).

3.11 Extraction of Secondary Metabolites from Culture Filtrates of LABs

Obtained LABs were cultured in 500ml Erlenmeyer flask containing minimal MRS broth and incubated for 7 days under laboratory conditions. Cultures were centrifuged and the supernatant was extracted with ethyl acetate. The organic phase was concentrated using a rotary flash evaporator (Buchi) and the obtained extract was used as crude extract (CE) for further investigations (Aman et al., 2021).

3.12 Disc Diffusion Assay of CEs against oral pathogens

Protocol was followed according to Chen et al., (2012), where agar plates were inoculated with the three distinct bacteria stated, then sterile discs were impregnated with all four CEs (1 mg/ml) and loaded onto to the top of inoculated plates, incubated for 24 hours at 37°C. Distilled water was employed as negative control. The inhibitory zone was measured in mm.

3.13 Antibiofilm properties of LABs culture filtrates against oral pathogens

According to Aarti et al., (2018), biofilm inhibition activity of the purified metabolite extracts was examined using microtiter plate assay. Obtained pathogenic antibiotic resistant isolates were cultured in the nutrient broth added to the 96-wells microtiter plate and incubated it for 24 h. After 24 h of incubation, the content of the respective wells were removed. After that, 100 µl of methanol (99% v/v) was added into each well and kept undisturbed for 15 min. The methanol was discarded and the wells were allowed to dry. After air dry, 100 µl of crystal violet stain solution was added into the wells and held for 10– 15 min. The stain was discarded after the required incubation period and the wells were washed gently. The wells were allowed to dry and absorbance was read at 570 nm using ELISA reader.

3.14 MIC determination of CEs against oral pathogens

A modification of the technique by Shukla et al, (2017) was used to examine biofilm inhibition activity of the purified metabolite extracts, using microtiter plate assay. Obtained pathogenic antibiotic resistant isolates were

cultured aerobically in NB broth and incubated at 37°C in orbital shaker for 24hrs. Different dilutions of the extract were prepared with fresh broth (1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81 µg/ml). In each well 25 µl of the overnight culture of test organisms, 150 µl of the extracts of different dilutions were added. 25 µl of inoculated broth and 50 µl of acetone was considered as negative control, incubated for 24 hours at 37°C under static conditions. The biofilm inhibition assay for the test organisms by the virtue of extracts was performed using crystal violet assay according to the protocol followed by Aman et al. (2021).

3.15 Colour Analyses for biofilm estimation on harvested tooth sections

To reflect the levels of biofilm formation and antibiofilm properties, the thin tooth sections (2mm) in thickness were used. The tooth sections were placed in sterile eppendorf tubes and submerged in a media containing crude extract (1 mg/ml), inoculum and culture broth. Setup was incubated for 48 hrs at 37°C for the development of biofilm on tooth sections. Incubated tooth sections were separated and stained with 0.1% crystal violet (CV) for 30 minutes after the bacterial solutions had been withdrawn from the wells. Following the removal of the CV solution, pure water was poured into the wells to flush away any non-specially absorbed CV that had accumulated on the surfaces of the specimens. The water was then immediately removed. Three times this washing process was carried out. As a result, we were able to get stained specimens that reflect the levels of biofilm that were present (Kamimura et al., 2022).

3.16 Fluorescence Microscopy

The test organisms and the lyophilized LABs culture filtrates were used in the study. Each LAB was examined for its inhibitory effects on biofilm formation. Pathogenic isolates were inoculated in a 90 mm petri plate containing the lyophilized extract and nutrient broth. A sterile microscopic slide was immersed in the whole setup and incubated at 37 °C for 24hs. After incubation the slides were treated with isoamyl alcohol to fix the biofilm and treated with acridine orange and imaged under UV using fluorescence microscopy (Rajesh & Rai, 2014).

3.17 TLC analysis

TLC separations were performed using manufactured normal phase glass backed silica gel plates (Si-60, 0.2 mm thickness) and developed in a solvent system of ethyl acetate–hexane (3:1). Each chromatogram was loaded and developed in duplicate (with one plate intended for bioautography and the other for visualisation of separated active constituents). Duplicate plates were developed simultaneously in a saturated, twin trough developing tank to minimise variation in chromatographic conditions. Plates intended for visualising isolated compounds were observed using a UV illuminator. The protocol was according to Smith et al., (2007).

3.18 TLC Bioautography Assay

The developed chromatograms were carefully dried for the complete removal of the solvents. The bacterial inoculum was added to 50 ml of semi strength nutrient agar. 5–6 ml of this suspension was spread out equally across a pre eluted TLC strip (20 X 20 cm). The TLC plates were incubated overnight. After that, the plates were spotted through the UV illuminator to observe possible zones of inhibition. The technique was followed according to the protocol given by Balouiri et al., (2016).

3.19 Column Chromatography

Using column chromatography (CC), the crude extract was fractionated and separated using pre-activated silica gel packed in a glass column that measured 40 cm by 2 cm in length and width, according to Aman and Rai V (2016). Ethyl acetate and hexane were used as the mobile phase for column chromatography in a 7:3 ratio. Each sample of crude extract that was passed through the column yielded approximately 26 fractions. The extracts were centrifuged at 15000 rpm for 3 minutes after being suspended in ethyl acetate. The supernatant was air dried, and each fraction was given a distinct name and stored in Eppendorf tubes.

3.20 FTIR analysis

The ethanol precipitated and dried EPS samples were used for Fourier transform infrared (FTIR) spectroscopy. Three litres of 100% cold ethanol were used to precipitate EPS, which was then left to sit on the ice for two hours. The precipitates were dried in an oven for an entire night at 50 °C after being centrifuged at 17500 g for 20 minutes at 4 °C (Aman & Rai, 2016).

3.21 LC-MS analysis

The bioactive fraction fractions which exhibited antibiofilm properties were further processed for identification of bioactive compounds by LCMS/MS analysis. The detection was performed through direct injection mode with Electrospray Ionization (ESI) probe, at positive-mode. Separated fraction was diluted to 100 folds, and 10 µl of the sample was injected to the C-18 column. Chromatographic separation was obtained using methanol (100%) at the flow rate of 1 mL/min; mass of molecular ions was detected in SQD by setting electrospray ionisation (positive mode). MS conditions were source temperature (150 oC), desolvation temperature (300 oC), nitrogen flow (550 L/min), cone voltage (80 V); MALDI-TOF MS was used in the reflectron mode of detection (Aman et al., 2021).

4. RESULTS

4.1 Isolation of bacteria from oral swabs

The samples were collected into sample containers, containing 1 ml of transport media and transferred to the laboratory, where they were enriched by incubating overnight at 37°C in nutrient broth. Enriched samples

were serially diluted and spread on the nutrient agar plates containing beef extract (0.3%), peptone (0.5%), and agar (1.5%). Obtained isolates were coded according to the source of isolation and morphological characteristics for internal reference.

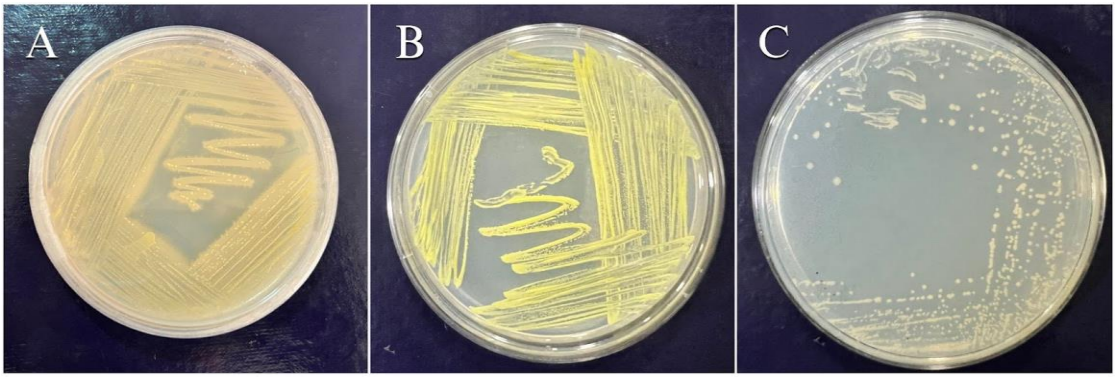


Fig 1: Representation of isolates exhibiting different cultural characteristics -(A) JUFC-08, (B) JUFC-09, (C) JUFC-11.

4.2 Isolation of LABs

LABs were isolated from pomegranate and sugarcane juice from local vendors. Isolates were selected based on their capacity to break calcium carbonate, which was amended in the MRS agar media (Fig-4). The isolates were purified further using quadrant streaking and maintained on agar slants for further usage.

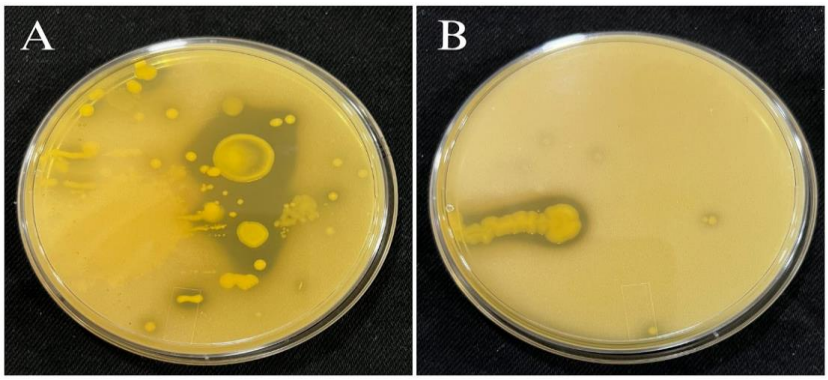


Fig-2: LABs on calcium carbonate amended MRSA media exhibiting the zone of clearance(A) Isolate from pomegranate juice (JUPG-03), (B)Isolate from sugarcane juice (JUSC-02).

4.3 Antibiotic susceptibility testing of bacterial isolates using disc diffusion method

Results revealed that the isolate JUFC-01 was completely resistant to trimethoprim, followed by clindamycin and vancomycin, JUFC-10 was resistant to trimethoprim, followed by clindamycin and vancomycin and JUFC-11 was resistant to trimethoprim, followed by clindamycin and vancomycin.

Table 1: Antibiotic susceptibility testing of oral isolates with different antibiotics.

Antibiotics	Zone of inhibition in mm*										
	JUFC-01	JUFC-02	JUFC-03	JUFC-04	JUFC-05	JUFC-06	JUFC-07	JUFC-08	JUFC-09	JUFC-10	JUFC-11
Vancomycin (5mcg)	10	17	20	16	17	17	18	21	18	10	8
Clindamycin (10mcg)	7	17	18	18	18	19	20	20	20	6	6
Trimethoprim (10mcg)	0	19	21	20	18	19	20	20	19	0	0
Levofloxacin (5mcg)	21	20	20	20	18	21	20	20	19	20	20

*Antimicrobial susceptibility pattern according to the National Committee for Clinical Laboratory Standards (NCCLS, 2000).

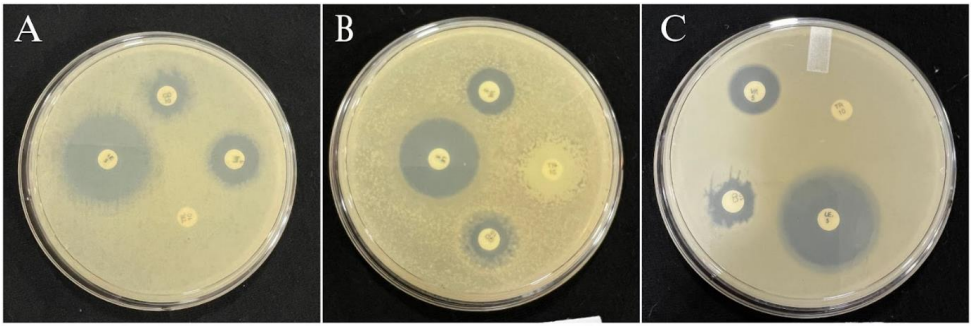


Fig 3: Isolates (A) JUFC 01, (B) JUFC 10 and (C) JUFC 11 exhibiting antibiotic resistance against different antibiotics

4.4 Screening of biofilm formation in oral pathogens

The obtained isolates were checked for biofilm production ability using a microtiter plate assay. It was performed using a crystal violet assay, and the OD reading was recorded at 570 nm.

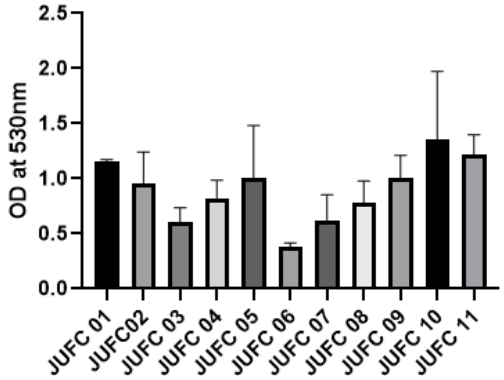


Fig. 4: 11 isolates form the oral samples exhibited varied biofilm forming ability among them JUFC-10 exhibited maximum biofilm forming ability, whereas JUFC-06 exhibited least biofilm forming ability.

4.5 Biochemical characterization of biofilm forming bacteria isolates

Basic biochemical tests such as Gram's staining, Oxidase and catalase tests were performed for identification of 6 different isolates (JUFC 01, JUFC 02, JUFC 05, JUFC 09, JUFC 10 and JUFC 11) collected from oral swabs.

Table 2: Biochemical characterization of 6 isolates from Oral samples.

Biochemical test	JUFC 01	JUFC 02	JUFC 05	JUFC 09	JUFC 10	JUFC 11
Gram staining	+	+	-	-	+	-
Indole test	-	-	-	-	-	-
Citrate	+	+	-	+	-	+
Oxidase	-	-	-	-	+	-
Catalase	+	+	+	+	+	+

4.6 Screening of antimicrobial properties of crude extracts using disc diffusion method

Results revealed that the isolate JUFC-01 was susceptible to the crude extracts of JUPG-03 and JUSC-2; JUFC-10 was susceptible to the crude extracts of JUPG-03 and JUSC-2; JUFC-11 was susceptible to the crude extracts of JUPG-03 and JUSC-2.

Table 3: Antibiotic susceptibility testing of LABs crude extract against biofilm forming bacterial isolates.

LABs CE	Zone of inhibition in mm					
	JUFC-01	JUFC-02	JUFC-05	JUFC-09	JUFC-10	JUFC-11
JUPG-03	**18**	12	13	13	**18**	**18**
JUSC-01	7	6	0	0	0	0
JUSC-02	**20**	12	11	8	**18**	**18**
JUMB-05	6	8	9	9	9	9

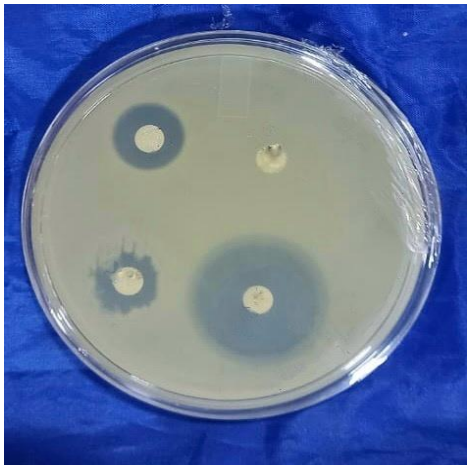


Fig 7: Representation of bacterial isolates loaded with discs, with added crude extracts (1 mg/ml) of JUPG 03, JUSC 02, JUSC 01 and JUMB 05 and a disc with positive and a negative control.

4.7 Molecular identification and phylogenetic analysis of bacteria from oral sample

Isolates from oral samples were identified by amplifying genomic DNA followed by sequencing of 16S rRNA gene. The isolates were identified by BLAST analysis of obtained 16S rRNA sequence and deposited in the GenBank database under accession number OQ927096 (*Kocuria sp* JUFC 01), OQ927095 (*Neisseria sp* JUFC 11) and OQ931032 (*Bacillus sp*JUFC 10). Further understanding of the relationship of identified isolates with previously deposited different species of bacteria belonging to diverse isolation sources was done by generating Neighbor-Joining phylogenetic trees. Phylogenetic tree was constructed using MEGA X software followed by 1000 boot straps.

4.8 Molecular identification and phylogenetic analysis of LABs

LABs isolated from fruit samples were identified by amplifying genomic DNA followed by sequencing of 16S rRNA gene. The isolates were identified by BLAST analysis of obtained 16S rRNA sequence and deposited in the GenBank database under accession number *Bacillus amyloliquefaciens* JUSC-2 (OQ940385). Further understanding of the relationship of identified isolates with previously deposited different species of bacteria belonging to diverse isolation sources was done by generating Neighbor-Joining phylogenetic trees. Phylogenetic tree was constructed using MEGA X software followed by 1000 boot straps.

4.9 Biochemical characterization of LABs

Biochemical characterization of LABs was performed by Gram staining, KOH tests, pH tolerance, catalase and oxidase production. Cell morphology and colonial characteristics were observed for all 4 isolates.

Table 4: Biochemical characterization of LABs indicating JUPG 03 from pomegranate juice, JUSC 01 and JUSC 02 from sugarcane juice, and JUMB 05 from Mosambi samples collected from local vendors.

Sl	Name of the test	JUPG 03	JUSC 01	JUSC 02	JUMB 05
1.	Gram staining	+	-	+	+
2.	KOH test	+	+	+	+
3.	Catalase	+	+	+	+

4.10 pH tolerance test for LABs

pH tolerance of LABs, isolated from pomegranate (JUPG 03), sugarcane (JUSC 01, JUSC 02) and mosambi juice (JUMB 05) was analysed at ranges of pH 2 and pH 3. The strain's capacity to flourish at lower pH levels (2.0 and 3.0) was examined at 3 hrs and 6 hrs duration.

Table 5: Representation of pH tolerance of LABs for pH 2 and 3 at duration of 3 hours and 6 hours, indicating JUPG 03 - Pomegranate juice, JUSC 01 and 02 - sugarcane juice and JUMB 05- Mosambi juice.

Organism	pH 2		pH 3	
	3hrs	6hrs	3hrs	6hrs
JUPG 03	Positive	Positive	Positive	Positive
JUSC 01	Positive	Negative	Positive	Positive
JUSC 02	Positive	Positive	Positive	Positive
JUMB 05	Positive	Negative	Positive	Negative

4.11 Screening of antibiofilm properties of crude extracts by crystal violet assay

CEs (1 mg/ml) of 4 LAB isolates JUPG 03, JUSC 02, JUSC 01 and JUMB 05 were tested for their for biofilm inhibition properties against 6 different oral pathogens and results revealed that biofilms of Isolates JUFC 01, JUFC 10 and JUFC11 were inhibited efficiently by the CEs of JUPG 03 and JUSC02.

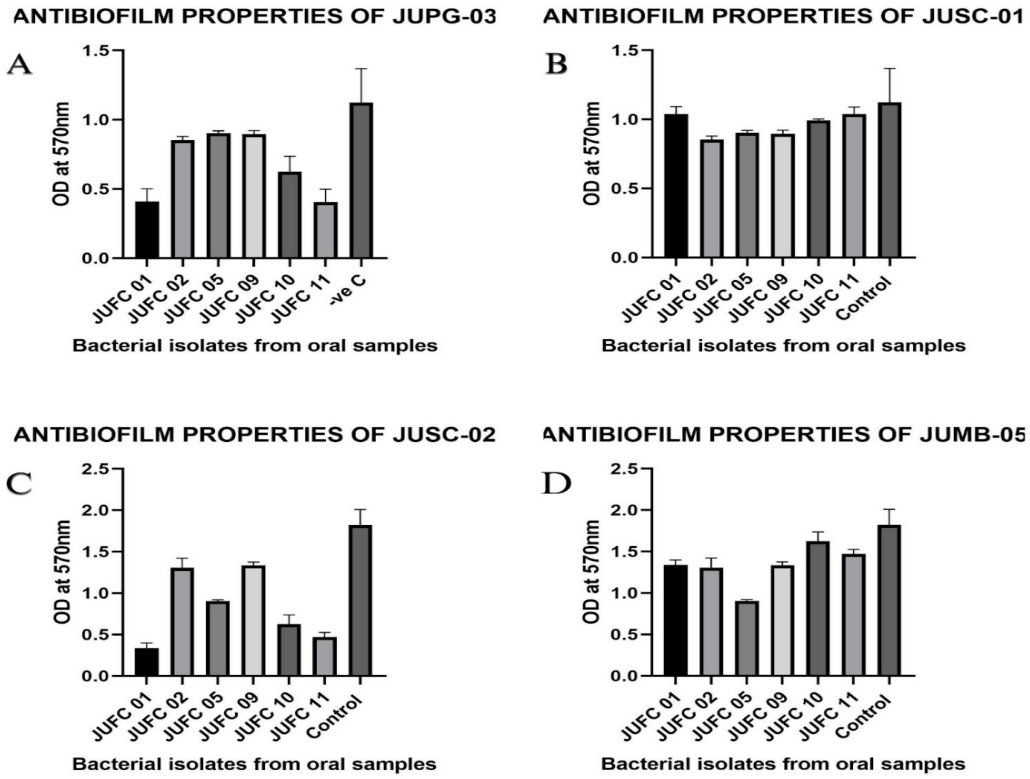


Fig. 10: Antibiofilm properties of crude extracts of LABs JUPG 03, JUSC 01, JUSC-02 and JUMB-05 against bacterial isolates from oral samples.

4.12 MIC determination of CEs against Oral pathogens: To examine the biofilm inhibition activity of CEs JUPG 03 and JUSC 02 using microtiter plate assay, for the test organisms JUFC 01, JUFC 02, JUFC 05, JUFC 09, JUFC 10 and JUFC 11. Varying concentrations of CEs as 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.25 µg/ml, 15.62 µg/ml, 7.81 µg/ml were added to the wells. Each well consists of 25 µl of the overnight culture of test organisms, 150 µl of the extracts of different dilutions were added. 25 µl of inoculated broth and 50 µl of acetone was considered as negative control, incubated for 24 hours at 37°C under static conditions. OD readings were then recorded at 570 nm.

4.13 Colour analysis for biofilm estimation on harvested tooth sections: To measure the levels of biofilm formation and antibiofilm properties, the thin tooth sections with 2mm thickness were subjected to Colour analysis. The results revealed that the dental sections treated with the crude extracts of JUSC-02 and JUPG-03 inhibited the biofilm formation, which can be visualised by the staining ability of dental discs.

4.14 TLC analysis: Separation of fractions from CEs was done by thin layer chromatography (TLC) and yielded TLC fractions were coded according to their Rf values.

Table 6: Rf values of TLC plates for CEs of JUSC-02

CE - LAB	Compound	Rf TLC
JUSC-02	F1	0.32
	F2	0.43
	F3	0.55

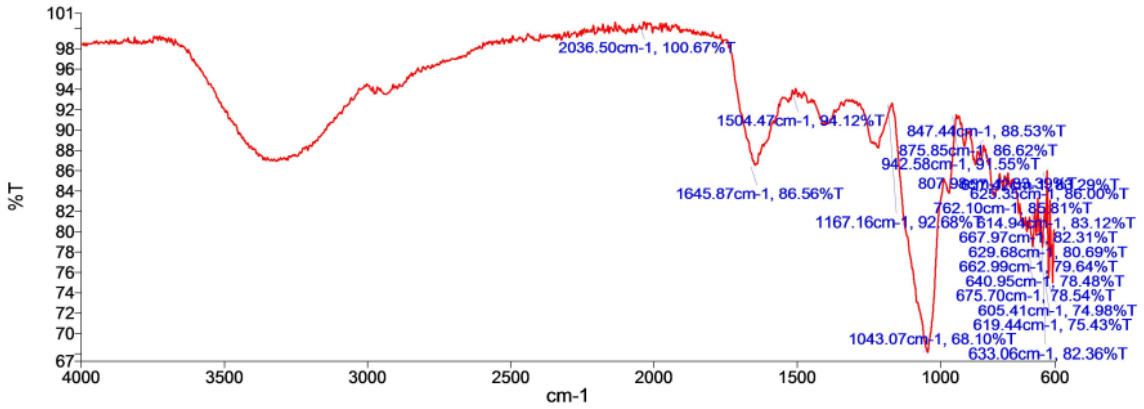
4.15 TLC Bioautography assay: Separated CEs fractions were checked for inhibition activity against the isolated oral samples, using TLC bioautography assay. Zone of inhibition was observed around the separated fraction on the TLC plate confirming the inhibitory activity of LAB.

4.16 Fluorescence Microscopy

The test organisms and the lyophilized LABs culture filtrates were used in the study. The LAB was examined for its inhibitory effects on biofilm formation.

4.17 FTIR

FTIR Analysis of JUSC-02/F2 fraction revealed different functional groups as shown in graph.



4.18 LC-MS analysis

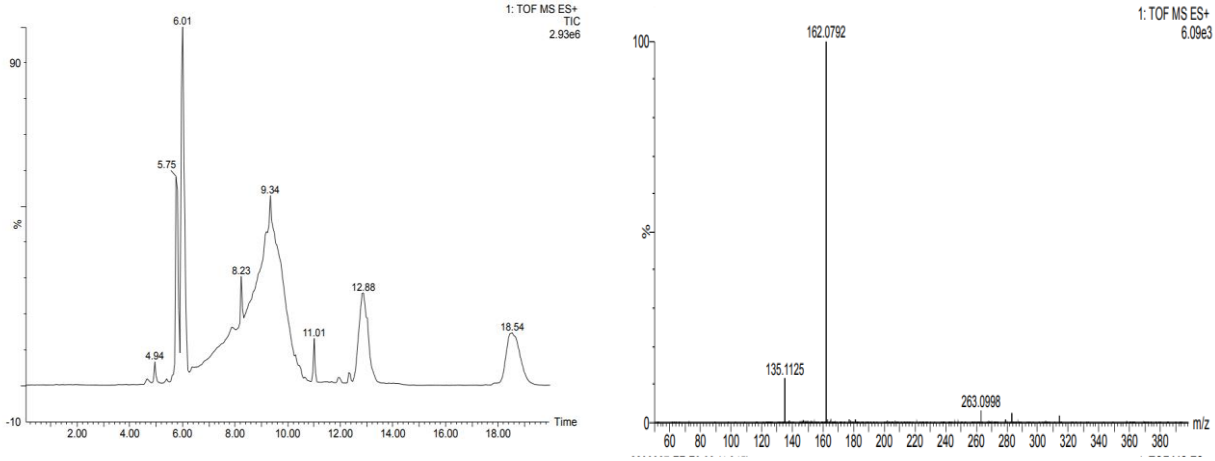


Fig 11: LCMS analysis of JUSC-02/F2 exhibited the molecular weight at 162.0792 which putatively indicates the presence of phenolic compounds.

5. DISCUSSION AND SUMMARY

Individuals with healthy native micro flora on the surface of their gums, teeth, and oral cavity linings have good oral health. It is made up of a wide range of species, including bacteria, fungus, and viruses. Numerous systemic illnesses, such as periodontitis, dental plaque, and dental caries, brought on by these oral bacteria (Alghamdi, 2021). Oral infections are characterised by the presence of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Bacteroides forsythus, Fusobacterium nucleatum, Campylobacter rectus, Peptostreptococcus micros, Streptococcus intermedius, Treponema denticola, and Eikenella corrodens.

Kocuria sp, Neisseria sp and Bacillus sp were isolated from oral swabs. Biochemcial characteristics were analyzed for these isolates. It was also observed that these oral isolates had an antibiotic resistance and biofilm formation capacity. A variable antibiotic resistance rate was observed among biofilm producing bacteria. Kocuria sp. (JUFC 01), Bacillus sp. (JUFC 10), and Neisseria sp. (JUFC 11) form a biofilm as a protective mechanism, and the biofilm structure offers several benefits to colonising species, such as protection against antimicrobials and host-defence, enhanced co-aggregation, and interaction properties (Flemming & Wingender, 2010). These defence mechanisms between microorganisms make biofilms challenging therapeutic targets (Socransky & Haffaje, 2002).

Antibiotic resistance of oral isolates was analyzed by checking it against different antibiotics as Vancomycin, Clindamycin, Trimethoprim and Levofloxacin. JUFC 01, JUFC 10 and JUFC 11 showed complete resistance to trimethoprim followed by Clindamycin and Vancomycin. The oral isolates showed varied biofilm forming ability amongst which JUFC 10 exhibited the maximum biofilm forming ability followed by JUFC 11 and JUFC 01. This was analyzed through Microtitre plate Assay.

Sugarcane juice from a local vendor was collected for the isolation of LABs. Lactic acid bacteria are most frequently found in environments rich in carbohydrates, amino acids, vitamins, or fatty acids because of their

complicated nutritional requirements for growth (Hutkins R, 2019). So, fruit juices serve as an excellent substrate for proliferation of LAB. One of the prominent bacteria isolated from Sugarcane juice was Bacillus amyloliquefaciens. Bacteria called B. amyloliquefaciens have great potential uses as novel probiotic supplements. It can create effective, non-pathogenic antibacterial chemicals. B. amyloliquefaciens B-1895 is capable of producing a wide range of proteolytic enzymes as well as other antibacterial substances (Algburi et al., 2016).

Secondary metabolites were extracted from the LABs to check their antibiofilm activity on the oral isolates. Disc diffusion Assay, Microtitre plate Assay were employed to check their biofilm inhibition capacity. Further, MIC determination of CEs was used to examine and confirm the minimum concentration required to inhibit the biofilm formed by the oral isolates.

To confirm the inhibitory effects of LABs on biofilm formation, Fluorescence Microscopy was performed. The fractions of crude extracts were separated using TLC analysis. To confirm whether the separated fragment has an inhibitory effect on the oral isolate, TLC bioautography assay was employed. With the help of obtained zones of inhibition, the inhibitory action of LAB was confirmed.

FTIR analysis was done to identify particular compounds such as Alkynes, amides, alkenes, chlorides etc. The bioactive fractions which exhibited antibiofilm properties were further processed for identification of bioactive compounds by LC-MS analysis. It was putatively found that the compound might be a phenolic compound in the culture filtrates of LAB.

These isolates showed visible resistance towards antibiotics such as Vancomycin, Clindamycin, Trimethoprim and Levofloxacin. Another major characteristic exhibited by these organisms was the biofilm formation. Hence, the antibiotic resistance can be attributed to the protective mechanism of biofilm formation by these isolates. As LABs are known for their diverse anti-microbial, antibiofilm and anti-adhesive properties, secondary metabolites of different LABs were extracted to check their activity on oral isolates. Bacillus amyloliquefaciens (JUSC-02) was isolated from the sugarcane fruit juice. The crude extract of this LAB was extracted to check for their inhibitory action against biofilm produced by Kocuria spp, Bacillus sp and Neisseria sp. Different techniques as Microtitre plate assay, MIC analysis; antibiotic susceptibility tests were examined to check the inhibitory activity of crude extracts of LABs against the oral isolates and the biofilm produced by the isolates. TLC technique was to used to separate the fractions of CEs of LABs. TLC bioautography assay, fluorescence microscopy were employed to confirm the inhibitory action of the separated fractions against the oral isolates. Through FTIR and LC-MS analyses, the bioactive fractions were putatively found that the compound might be phenolic compound.

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