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ORIGINAL ARTICLE

Rhodococcus equi biofilm development in relation to external environmental variables

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ABSTRACT
Rhodococcus equi (R. equi) is a Gram-positive bacterium that causes infections in animals and immunocompromised humans. Biofilm formation by R. equi plays a significant role in its persistence and survival across various environments, including medical, industrial, and agricultural settings. This study aimed to assess biofilm development under different variable parameters like media compositions, pH, Temperature, presence of metals as well as effect of surface materials. The results revealed that biofilm formation is highly dependent on nutrient availability, with brain heart infusion (BHI) medium supporting the highest biofilm production, followed by Luria-Bertani (LB), tryptic soy broth (TSB), and minimal media (M9). Optimal biofilm growth was observed at neutral to slightly alkaline pH levels, with increased biofilm formation at 37°C. Glucose supplementation significantly enhanced biofilm production whereas higher salt concentrations inhibited biofilm growth. The study also highlighted the impact of metal ions, with non-inhibitory concentrations of iron and zinc playing critical roles in biofilm enhancement. Iron was particularly effective in the early stages of biofilm development, while zinc exhibited a delayed but notable influence. Biofilm formation on different surfaces revealed that stainless steel was the most conducive, followed by aluminum, while glass exhibited the lowest biofilm formation due to its smooth texture. These findings provide important insights into the biofilm-forming abilities of R. equi and may suggest strategies for mitigating biofilm-related challenges in medical devices and industrial settings.
Keywords- Rhodococcus equi, biofilm, heavy metals, Stainless steel, Aluminum

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INTRODUCTION

Rhodococcus equi (*R. equi*), formerly known as *Corynebacterium equi,* is a gram-positive bacterium which is an environmental pathogen that primarily a pathogen of foals but also recognized to cause opportunistic infections in humans. It is a facultative intracellular pathogen normally reside in soil and is reported to cause rare infections in immunocompromised individuals [1,2]. It causes equine rhodococcosis, primarily affecting foals aged between three weeks to six months [3]. This bacterium is prevalent globally and commonly found in diverse environments including horse-breeding farms [4]. The presence of virulence-associated plasmid is a crucial factor in pathogenicity of *R. equi*. There are different types of virulence plasmids in *R. equi*, often associated with specific animal hosts. For example, the pVAPA plasmid is found in equine isolates, while pVAPB is found in porcine isolates, and pVAPN in bovine isolates [5,6]. These plasmids have evolved to adapt to their respective hosts, showing variations in their genetic makeup. The most studied strain of *R. equi* (103+) contains pVAPA plasmid, which typically ranges between 85 to 90 kb and carries genes that encode virulence-associated proteins, such as VapA [7,8]. These proteins are essential for survival and replication of bacterium within macrophages [9,10,11]. Other important genes present on these plasmids include conjugation related genes. *vap*A containing

plasmid is shown to be conjugative and are able to get transferred to another related actinomycetes [12]. Both of these characteristics are important for biofilm formation by microorganisms.

R. equi has also been reported to form biofilms, which help them to cause virulence, persistence and resistance against antibiotics [13,14]. Biofilms are the structured communities of bacteria embedded in a self- produced extracellular polymeric substance (EPS). EPS provides a protective covering around the bacteria that enhance their survival rate and resistance towards external stressors.

The biofilm formation is a complex phenomenon and involves different stages, starting with initial attachment to the surface, followed by production of EPS for irreversible attachment and maturation [15]. Last stage is the dispersion and start of new cycle of biofilm formation. The role of EPS is not only to provide the structural stability but it also protects the bacteria from harsh environment such as desiccation, antimicrobial agents and the host immune system. EPS components vary with the organism and have role in the structural and functional stability of biofilm. Composition of EPS can influence the biofilm's physical properties and its resistance to antimicrobial treatments. EPS matrix also facilitates communication between bacterial cells through quorum sensing, a process that regulates gene expression and coordinates biofilm development [16].

Along with protecting bacteria from host immune system, biofilm also functions as a physical barrier for antibiotics. It alters the microenvironment including gradient of oxygen, nutrient and waste inside the biofilm, creating conditions for bacteria to enter dormant stage or slow growing stage on which the antibacterial agents don't work. Some bacterial cells within biofilm employ efflux pump that continuously expels the antibiotics out of the cells thus reducing the effective intracellular antibiotics concentration. Bacterial cell within biofilm also transfers antibiotics resistance capability to others cells in close proximity through horizontal gene transfer thus spreading protection against antibiotics. Thus, ultimately bacterial biofilm increases the antibiotics tolerance capacity upto thousand-fold than its planktonic form. The clinical implications of *R. equi* biofilm formation are immense, particularly in the context of antibiotic resistance in medical setup. Biofilms can act as reservoirs for antibiotic-resistant bacteria, making infections difficult to eradicate with conventional antibiotic therapies. This is particularly concerning as new antibiotics are not getting discovered readily. In veterinary settings, like *R. equi* infections in foals, it can lead to significant economic losses due to the high costs associated with treatment and prevention. Moreover, in human healthcare settings, infections caused by biofilm-forming bacteria are associated with increased morbidity and mortality [17-20].

Biofilms have a significant impact on several industries, often leading to substantial economic costs and operational challenges [21]. One important problem is related to biofilms on medical devices and implants, which can cause persistent infections with antibiotic resistance. Important medical devices affected by biofilm include catheters, prosthetic heart valves, pacemakers, orthopedic implants, and contact lenses etc. [22]. These biofilms can lead to device-related secondary infections, which are often difficult to treat. This can not only increase complications in patient treatment but also lead to increased healthcare costs due to prolonged hospital stays, additional treatments, and device replacements [23,24].

So, it becomes very important to study the biofilm formation of pathogenic bacteria like *R. equi* for better understanding the process and to plan effective treatment regimen. Understanding the conditions that influence biofilm formation by *R. equi* and associated aspects are essential for developing strategies to control and treat infections caused by this pathogen.

Present study is designed to evaluate biofilm formation by *Rhodococcus equi* under variable physical conditions as this will have significant implications for both veterinary and human medicine. By understanding the environmental factors that influence biofilm formation researchers can develop targeted strategies to prevent and treat biofilm-associated infections as well as design biofilm-resistant medical devices. The integration of advanced molecular techniques and innovative therapeutic approaches holds promise for overcoming the challenges posed by biofilm-forming pathogens and improving outcomes for infected individuals.

MATERIAL AND METHODS

Bacterial strain, media and growth condition

Rhodococcus equi (MTCC no. 2558) was obtained from Microbial type culture collection and gene bank (MTCC) Chandigarh, India. According to the need the *R. equi* has been grown and cultured in Luria Bertani media (LB) broth or agar (HiMedia) or brain heart infusion (BHI) broth or agar (HiMedia) at 37°C and supplemented with appropriate antibiotics when necessary. In certain experiments, bacteria were grown in minimal M9 medium (HiMedia) with supplements as described by Miller, et al., [25].

Microscopical study of biofilm

To study biofilm formation and its structure at different time intervals, glass surface was chosen. Sterile glass slide was placed inside 50 ml collection tubes containing 20 ml LB broth with bacterial culture. The tubes were incubated at 37°C in static condition. Glass slides were removed at every alternate days, up to day 16 and rinsed with saline to remove any un-adhered bacteria. The biofilm formed on slide was stained with crystal violet and observed under compound microscope at 100x magnification.

Scanning electron microscopy (SEM) is a powerful tool for analyzing biofilms, offering high-resolution images that reveal their three-dimensional structure. The SEM analysis was conducted using the Carl Zeiss UHR FESM Model Gemini SEM 500kmat (IIT Bhilai, Chhattisgarh). In order to study the planktonic form of *R. equi* under SEM, bacteria were grown in LB broth medium overnight and one to two drop of culture was used to prepare a thin smear over a cleaned glass coverslip. Smear was air dried for few minutes before further processing for SEM analysis. For growing biofilm on a glass coverslip, bacterial culture was grown in glass tube along with inserted coverslip and was incubated at 37°C. After incubation the coverslips were removed and air dried. Coverslips (planktonic and biofilm) were processed for the SEM analysis as described below. Coverslips were dipped into 2% glutaraldehyde for 30 minutes followed by washing with phosphate buffer saline (PBS). The coverslips were then dipped into a series of acetone solutions (30%, 50%, 70%, 90% and 100%) for dehydration of sample. Before SEM visualization the coverslips were coated with the gold using gold sputter coater (Quoram Sputter Coater Q150R S Plus). The coated samples were then mounted on the SEM sample holder for visualization of micrograph on the screen.

Biofilm formation assay

For quantitative estimation of biofilm formation, test tube method (TM) given by [26] was used with some modifications. Briefly, 2 ml of LB broth were inoculated in test tubes with o/n grown culture (in 1:100 ratio). The uninoculated broth was taken as control. After 1 h of shaking, the tubes were incubated at 37°C without shaking (static condition). Some tubes were removed on alternate days up to 16 days, for evaluation of the biofilm formation. Media was decanted and tubes were allowed to air dry for 10-15 min. After that tubes were washed with saline for 2-3 times and air dried again for 20 min before adding 2ml of 0.1% Crystal violet solution. After 15 min incubation, crystal violet was discarded and tubes were rinsed with distil water until the water ran clear. Tubes were left for 30- 60 min to air dry. The resultant biofilm was dissolved in 2ml of 95% ethanol and resultant solution was studied spectrophotometrically (Systronics UV-Vis double beam spectrophotometer) at 570 nm. Biofilms attached to surface, absorbs the crystal violet stain in direct proportions to the number of bacteria present. So, higher the biofilm formation more will be crystal violet absorption. This will be visualized by higher absorbance at 570nm. This method was used to estimate biofilm formation in different experimental conditions.

Effect of varying physical conditions on biofilm formation

The test tube method, as described earlier, was used to check biofilm formation under different physical conditions. To study the effect of media composition on biofilm formation, bacteria were grown in brain heart infusion (BHI), Luria Bertani (LB), Tryptic soya broth (TSB) and Minimal media (M9), separately and biofilm formed on test tubes was compared. pH is another factor which is known to affect the biofilm formation capability of the organism. *R. equi* was grown in LB media with different pH (6, 7, 8, 9 & 10) and Biofilm formation was studied. To see the effect of temperature on biofilm formation, bacteria were grown at different temperatures (22°C, 27°C and 37°C) before comparing the amount of biofilm formed. Other factors affecting biofilm formation include salt concentration, glucose concentration as well as the presence of different metallic salts in the media. In one experiment different concentrations of NaCl (0.5%, 1%, 1.5% and 2%) was added in LB media, in addition to normal salt concentration and effect was studied. Similarly additional Glucose (0.25% and 0.5%) was added in media to see how it affects the biofilm formation by bacteria. Bacterial growth and biofilm formation was also checked in presence of 0.1mM concentration of different metals salt ($AgNO_3$, $CuSO_4$, $FeCl_3$, $MnSO_4$ and $ZnSO_4$).

Biofilm formation on different surfaces

Nature of the surface where biofilm is growing, greatly affect the quality and amount of biofilm formation. Three different surfaces (Aluminum, Glass and Stainless steel), which has more chance to be present in hospital conditions and are used mostly in medical devices, are used in this study. A strip of 5 cm x 1 cm (Length x breadth) were prepared of each material and were immersed in test tubes with bacterial culture. Test tubes were incubated for different time intervals and biofilm formation was estimated as described earlier. All assays were performed in triplicate and repeated three times.

Statistical analysis

The statistical analysis was conducted using the most recent version of GraphPad Prism software developed by GraphPad Software, San Diego, California, USA. The data underwent one-way analysis of

variance (ANOVA), and significant differences between means were identified using posthoc Tukey tests with a significance level of $P<0.05$. The outcomes are illustrated in graphs, with distinct letters denoting significant variation. The values represent the mean of three replicates with standard deviation. The graphs were created using GraphPad Prism software, California, USA, 2024.

RESULTS

Microscopical study of *R. equi* biofilm

Biofilm formation by *R. equi* at the liquid-air interface on the glass slide was analyzed to observe the pattern of development over different time (days). The slides were half immersed in media with bacterial culture and incubated for various time periods and biofilm formation was observed under a compound microscope at every 4-days interval up to 16 days. The initial observation was made on the first day of the incubation, by preparing a smear of the bacterial culture on the slide. Fig. 1A shows the microscopic image of planktonic bacterial cells, where each cell is individually seen and not attached to each other. On day 4, there is an increase in bacterial growth and crowding, with visible group formations in some areas (Fig. 1B). This stage represents the initial attachment phase of biofilm formation. Fig. 1C-E illustrate the maturation stages of the biofilm. In Fig. 1C, the early maturation stage is depicted, where the number of bacterial cells increases and EPS (extracellular polymeric substances) production begins.

Fig. 1D and 1E represent the late maturation stages of biofilm formation. In these images, a thick layer of bacterial cells is observed, heavily covered by EPS. At this stage, maximum production of EPS was observed and the biofilm is so dense that the bacterial cells are completely enveloped in the thick EPS layer.

SEM analysis of *R. equi* biofilm

The scanning electron microscopy (SEM) micrograph of planktonic *Rhodococcus equi* reveal that the R. equi cells are rod-shaped and do not exhibit structural appendages such as pili or fimbriae, which are commonly associated with bacterial attachment and interaction (Fig 2). The dimensions of these planktonic cells vary, with lengths ranging from approximately 1.7 µm to 2.7 µm and widths around 500 nm, as detailed in Figure 2C. The cells are free-floating, without the extracellular matrix or polymeric substances that typically facilitate cell aggregation and biofilm formation. The images are captured at varying magnifications (2k X, 5k X, and 20k X), providing a comprehensive view of the cellular morphology and spatial organization of *R. equi* in its planktonic state.

Images 3A through 3E offer detailed views of bacterial biofilm structures under different magnifications. Image 3A depicts a mature and dense biofilm of *R. equi*, featuring a robust, multilayered architecture. This panel reveals a complex and thick biofilm structure, where layers of bacterial cells are closely packed together, forming a substantial, well-developed biofilm. On the other hand, image 3B represents a different area with less mature stage of biofilm formation. Here, the biofilm appears as a thinner, single-layered structure with visible gaps between the bacterial cells, indicating an earlier or less dense stage of biofilm development.

The figure 3C underscores the presence of an extracellular matrix (ECM) surrounding the bacterial cells. This ECM is crucial for biofilm integrity, as it provides structural support and facilitates the interconnectedness of individual cells within the biofilm. The extracellular polymeric substances (EPS) that make up a significant component of the ECM are highlighted with arrows in the figure 3D and 3E, emphasizing their role in the biofilm's architecture. Additionally, individual bacterial cell is marked with a notch arrow (Fig 3E), distinguishing them from the surrounding ECM and EPS. The comprehensive view provided in the figure helps illustrate the progression of biofilm maturation and the structural complexity of the biofilm matrix.

Biofilm formation at different physical condition

Biofilm formation by *R. equi* has been studied at different parameter such as different media, pH, temperature, additional glucose and salinity to see their effect on biofilm formation.

To see the effect of media composition on biofilm formation, different complete (LB, BHI and TSB) as well as a minimal media (M9) were used to grow the bacterium. Results show that the highest amount of biofilm formation has been observed in case of brain heart infusion (BHI) media followed by Luria Bertani (LB) media, and tryptic soy broth (TSB) media and lowest or almost no biofilm formation were observed in case of minimal (M9) media (fig. 4A).

While studying the biofilm formation at different pH levels, biofilm formation showed an increasing pattern up to the 8 days, after which it became stagnant. At pH 7, biofilm formation was high from the very beginning. In contrast, biofilm formation was relatively slow at other pH levels. Interestingly, at pH 10 the biofilm formation began at a much slower pace compared to the others but continued to increase

steadily until the day 16. The highest absorbance, 1.102, was recorded on day 16 at pH 10 (fig. 4B). In case of temperature variations, the highest biofilm formation was seen in case of physiological temperature (37°C) while in other 22°C and 27°C temperature the biofilm formation was very low (fig. 4C).

Biofilm formation is directly dependent on the growth of bacteria, that's why experiment was designed to see the effect of additional glucose in the media. The effect of added glucose on biofilm formation was investigated using LB media with 0.25% and 0.5% of additional glucose. It was found that biofilm formation was higher with the higher amount (0.5%) of added glucose compared to lower amount (0.25%) (fig. 4D). Results signify that may be additional glucose is used for better growth of bacteria and in turn, better formation of biofilms. Another possibility is that additional glucose is used to generate high amount of extracellular polysaccharides [27]. To further assess the effect of stress conditions, additional salt (NaCl) was added in LB broth (0.5%, 1%, 1.5% and 2%). The results revealed that biofilm formation is negatively affected by increasing salt concentrations. The pattern of biofilm formation at different salt concentrations was as follows: 2% < 1.5% < 1% < 0.5% < 0% (no additional salt) (fig. 4E).

3.3 Effect of heavy metals on biofilm formation

Although metals play an important role in many physiological processes in bacteria, but when present in more than required amount they are toxic for the cell and hamper the regular processes in the bacteria. Initially, bacteria were grown in presence of lower concentration (0.1mM) to see the growth pattern. Those metal salts where good growth was seen at subinhibitory concentrations (0.1mM), were used to see the effect of metals on biofilm formation. Bacteria grown without metal was taken as control. The biofilm formation assay was done up to 16 days, with spectrophotometric analysis at 570 nm. The results, shown in fig. 5, indicates that iron promoted the high amount of biofilm formation initially upto 10^{th} day. While, in presence of copper, bacteria showed the peak biofilm formation at 14^{th} days. However, after 14 days, a slight decrease in biofilm formation by *R. equi* was observed in presence of copper. In case of zinc salts, biofilm formation was significantly higher from 10^{th} – 14^{th} day in comparison to control. However, at 16^{th} day biofilm formation in presence of zinc salt was the same, while it increases in control (no metal) tubes. Initially upto day 10, Iron was the metal to promote maximum amount of biofilm, but on later days (10^{th}, 12^{th} and 14^{th}) biofilm formation by copper metal were higher than most of the metals and shown its peak at day 14^{th}. Surprisingly at day 16^{th}, silver metal shows significant biofilm formation that is higher than all the metals, only less than control.

Biofilm formation on different surfaces

Nature of surface plays very important role in biofilm formation, as it promotes or declines the initial bacterial attachment. Biofilm formation was studied on various surfaces (glass, aluminum, and stainless steel) to assess bacterial adhesion over time and resultant biofilm formation. Comparative analysis shows that lowest biofilm was seen in case of glass from first day itself among all surfaces used. The biofilm formation in case of glass varies from 0.046 to 0.9 on different days. Maximum biofilm formation among all surfaces was observed on stainless steel at 14^{th} day (OD_{570nm}- 1.135). Aluminum also promotes high biofilm formation, just like stainless steel surface. Initially, biofilm formation was better on aluminum surface than steel surface, but later on growth were comparable with little more on stainless steel (Fig. 6).

DISCUSSION

Biofilm formation by *Rhodococcus equi* (*R. equi*) is a critical factor in its survival and persistence in various environments, including medical, industrial, and agricultural settings. Our study focused on evaluating biofilm formation under different physical conditions, as well as on various surfaces, and provided insights into how different parameters like pH, temperature, extra carbon source, salinity, and the presence of metals impact biofilm development.

Scanning Electron Microscopy is one of the best techniques to study the structure of biofilm [28]. Our analysis showed the colony morphology of *R. equi* in planktonic stage as well as all the characteristics of biofilm. Colony was rod shaped with size ranging between 1.7µ to 2.7µ. Biofilm contains lot of extra cellular material. It is seen that cells are embedded in ECM as expected. These results are similar to other findings reported for bacterial biofilms [29].

Biofilm formation by *R. equi* is highly dependent on the growth medium, with the highest biofilm formation observed in brain heart infusion (BHI) medium, followed by Luria-Bertani (LB), tryptic soy broth (TSB), and minimal media (M9). This finding is consistent with previous studies that emphasize the role of nutrient-rich media in enhancing biofilm formation [30]. In many studies BHI and TSB medium has been used for the different bacterial biofilm formation [30,31]. Although both BHI (Brain Heart Infusion) and TSB (Tryptic Soy Broth) media are good for biofilm growth, but sometimes BHI performs better, while other times TSB is more effective, depending on the specific bacteria [30-32]. In case of *R. equi* BHI

media better support biofilm formation. BHI media which is a nutrient rich media as it contains infusion of calf brains and beef hearts, which provide a complex mixture of amino acids, peptides, minerals, essential nutrients and growth factors that promote bacterial adhesion and biofilm development. In addition, BHI media contain 2% glucose which serves as carbon source and known to facilitate biofilm formation [33]. In contrast, the minimal media like M9 lack sufficient nutrients, leading to limited growth which may not be sufficient for biofilm formation, as observed in our study. The biofilm formation pattern on different pH by *R. equi* indicates its preference for neutral to slightly alkaline environments. Biofilm growth was high at pH 7 from the start, while at pH 10, it increased steadily until day 16. This suggests that *R. equi* can adapt to alkaline conditions by getting incorporated into biofilm matrix, potentially aiding its survival in high-pH environments like certain soils. Our study suggests ability of *R. equi* to form biofilm optimally at 37°C which reflects its pathogenic potential in humans and animals, as this temperature mimics the conditions within host organisms. This may also be the reason for biofilm formation on medical devices attached to the human patients in organ transplant cases. Biofilm formation, at 22°C and 27°C is significantly lower in comparison to 37°C. This result is in line with the findings of [34], who reported that biofilm formation by *Listeria monocytogenes* on various surface vary with the temperature. It was reported that as the temperature increased (from 4°C, 12°C, 22°C to 37°C), there was a corresponding increase in biofilm formation. The presence of additional glucose in the LB media results in increased biofilm formation. Higher the added glucose (0.25% vs 0.5%), higher the biofilm formation. This is consistent with earlier studies that highlight the role of glucose as a carbon source in promoting biofilm formation by providing energy for extracellular polymeric substance (EPS) production [35]. Glucose also induces biofilm-related genes, leading to the expression of factors important for attachment, maturation, and maintenance of biofilms [36]. In experiments to see the effect of salinity on biofilm formation, we found that increasing NaCl concentrations had a negative impact, with the highest biofilm formation observed in the absence of additional salt. Notably, the LB medium used in our experiments already contains 1% NaCl, indicating that the actual highest biofilm production occurred at this baseline concentration. As salinity increased beyond this point, biofilm formation progressively decreased. Salinity is known to influence osmotic pressure, which in turn affects bacterial metabolism and biofilm architecture. Elevated salt concentrations impose osmotic stress, disrupting cell communication and impairing the production of extracellular polymeric substances (EPS), essential for biofilm integrity. Similar observations were made by Jahid et al. (2015) [37], who reported a significant reduction in biofilm formation at higher salt concentrations, while lower NaCl levels promoted increased biofilm production in *Aeromonas hydrophila*. These findings parallel with our results, indicating that *R. equi* biofilm is similarly sensitive to salinity, with biofilm formation diminishing as salt concentration rises. This finding may be useful in patient's treatment plan who are susceptible to opportunistic infection by *R. equi*. All the devices used can be rinsed with saline water before use for those patients.

Metal ions, especially iron and zinc, influenced biofilm formation by *R. equi*, with iron showing the highest biofilm production initially and zinc peaking at 14th day. Metal ions play a critical role in biofilm formation by modulating bacterial metabolism and the production of biofilm matrix components such as EPS. Iron, in particular, is essential for bacterial growth and biofilm development, as it serves as a cofactor for various enzymes involved in metabolic pathways. On the other hand, some studies highlight the critical role of zinc in facilitating surface protein interactions that promote aggregation and biofilm formation in *Staphylococcus aureus*, a major human pathogen [38, 39]. However, other studies showed contrasting findings that elevated zinc concentrations can inhibit biofilm formation in the Gram-positive bacterium *Streptococcus suis* [40]. The role of iron in biofilm formation has been also described by Ojha et al., 2007 [41] supporting our study. Biofilm formation on different surfaces namely aluminum, glass and stainless steel were also studied in present work and after comparison it was found that stainless steel was the most conducive surface for biofilm development. In contrast, glass showed the lowest biofilm formation. The glass surfaces are smooth compared to stainless steel and aluminum making it a little difficult for bacterial attachment. However, Stainless steel surfaces often feature cracks and crevices that are capable of trapping bacteria [42]. Whereas aluminum surfaces possess even larger crevices and a sponge-like structure. This surface topography enables the release of bacteria that have become trapped within it. The findings may help to identify materials that resist biofilm formation, aiding in the selection of suitable surfaces for medical, industrial, and environmental applications to improve hygiene and durability.

Overall, present study explored the different aspects of biofilm formation by an opportunistic bacterium, *R. equi*, and findings may be utilized for better management of the disease caused by the bacterium.

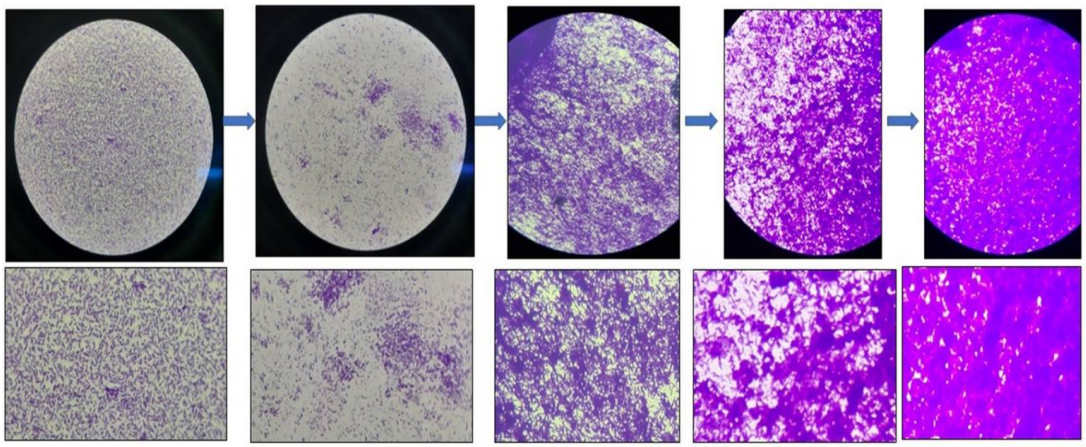

Fig 1. Microscopic image of Biofilm formed on glass slide surface at different days taken at 100x magnification (A) *R. equi* at planktonic stage, image taken on first day of bacterial inoculation (B) Image taken after 4 days of incubation. It shows accumulation of bacterial cell forming a group resemble with attachment stage of biofilm formation. (C) Image taken after 8days, showing increase in bacterial density as well as start of EPS production, representing early stage of Biofilm maturation. (D) Image taken after 12days. This shows increase in bacterial cell density covered with high amount of EPS, representing maturation stage (E) Image taken after16days. It shows maximum increase in bacterial cell density covered fully with high amount of EPS, representing fully matured stage.

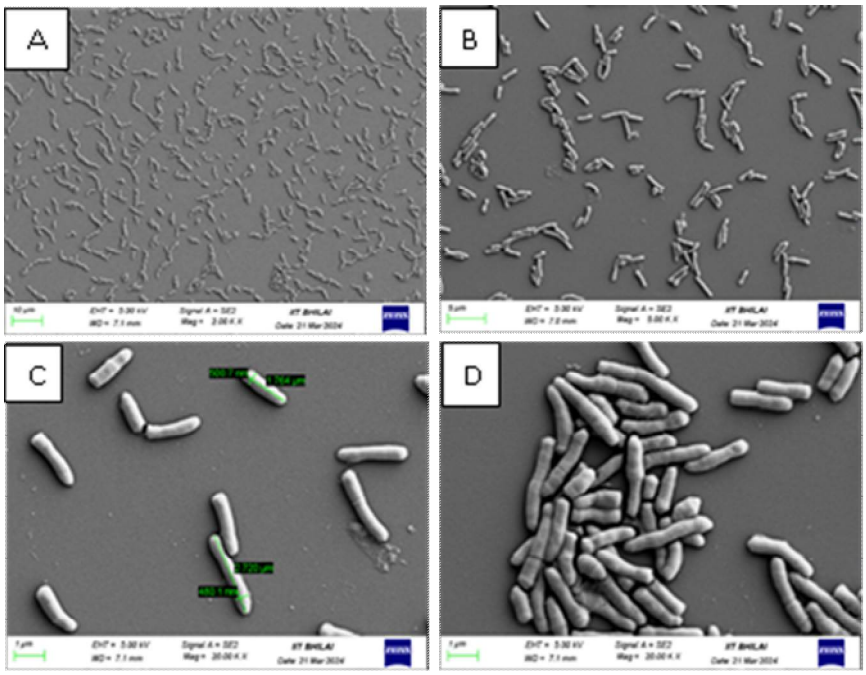

Fig. 2. SEM images of planktonic *Rhodococcus equi* cells at magnifications of 2K, 5K, and 20K. Panels A and B show dispersed rod-shaped cells without any appendages. Panel C & D shows details cell dimensions (~1.7–2.7 µm in length, ~500 nm in width) and clustered cells that remain unconnected respectively.

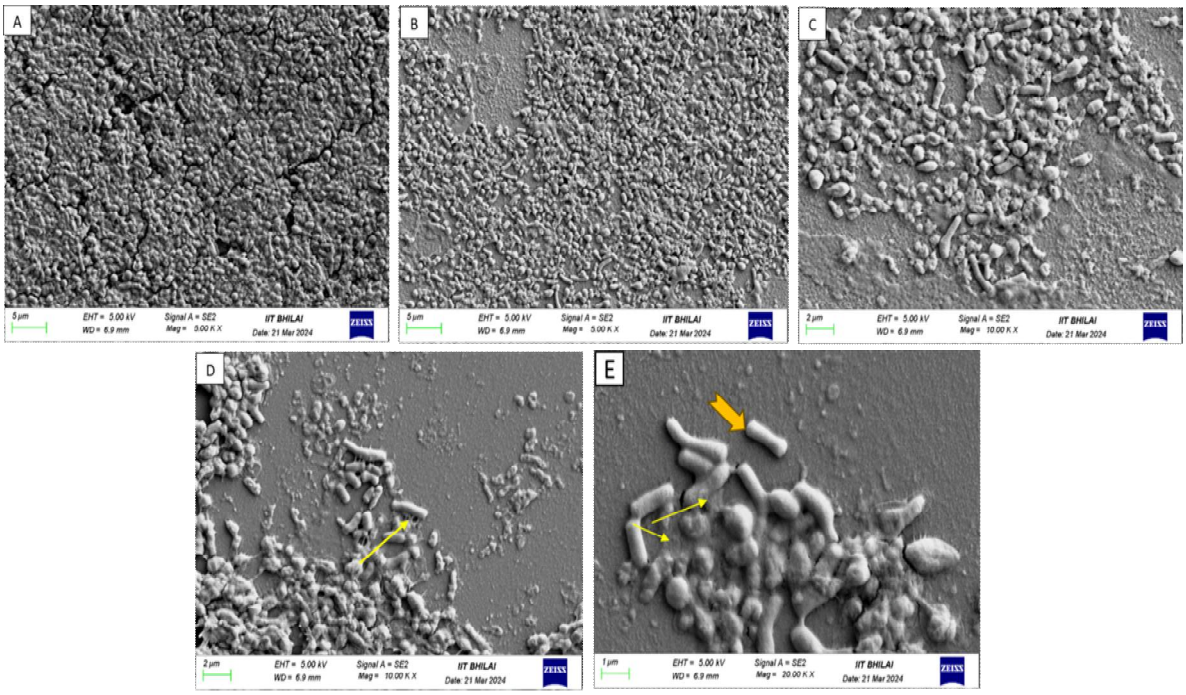

Fig 3. SEM image of biofilm structure of *R. equi*. Image A. shows robust biofilm structure, image B depicts less mature biofilm with few layer of biofilm structure. Image C,D,E shows magnified structure of biofilm indicating ECM with an arrow and bacterial with notch arrow.

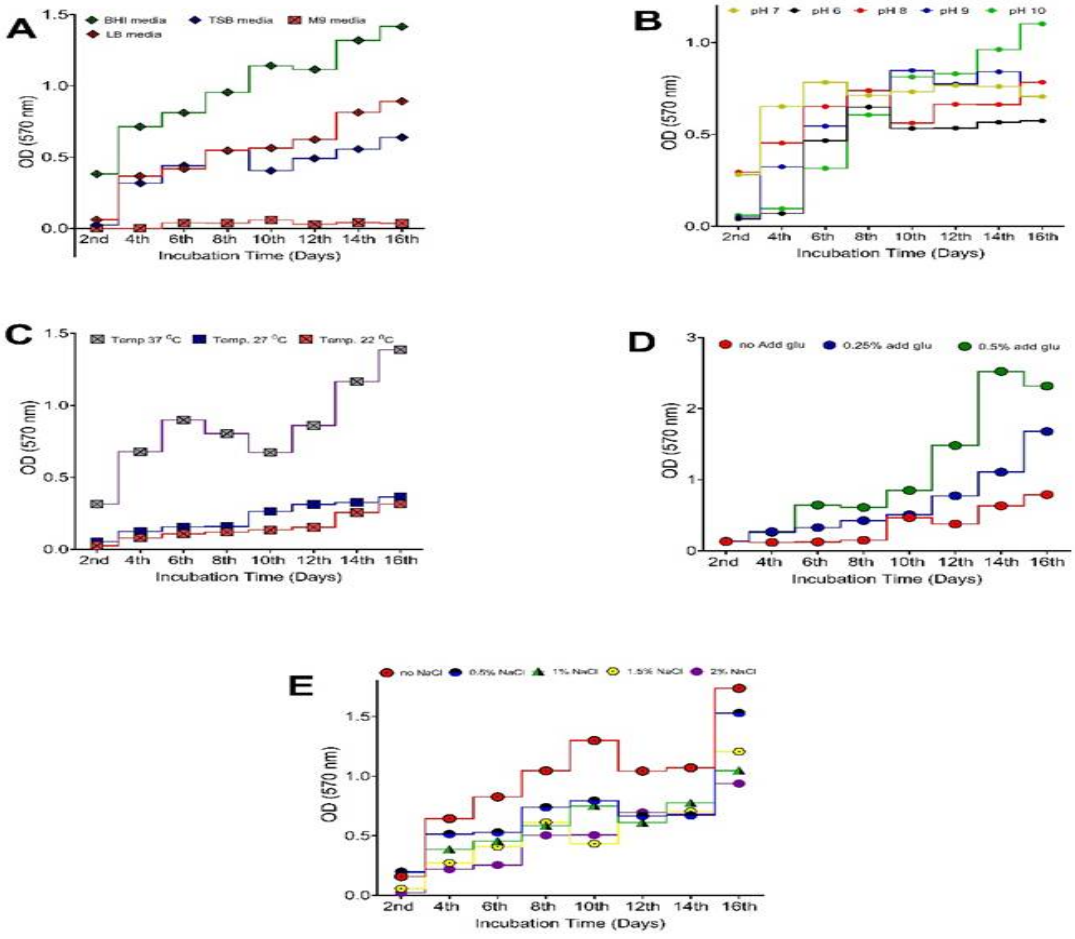

Fig 4. Distribution of biofilm formation pattern for different physical parameters (A) media (B) pH (C) temperature (D) additional Glucose & (E) additional salt. Data were collected till day 16.

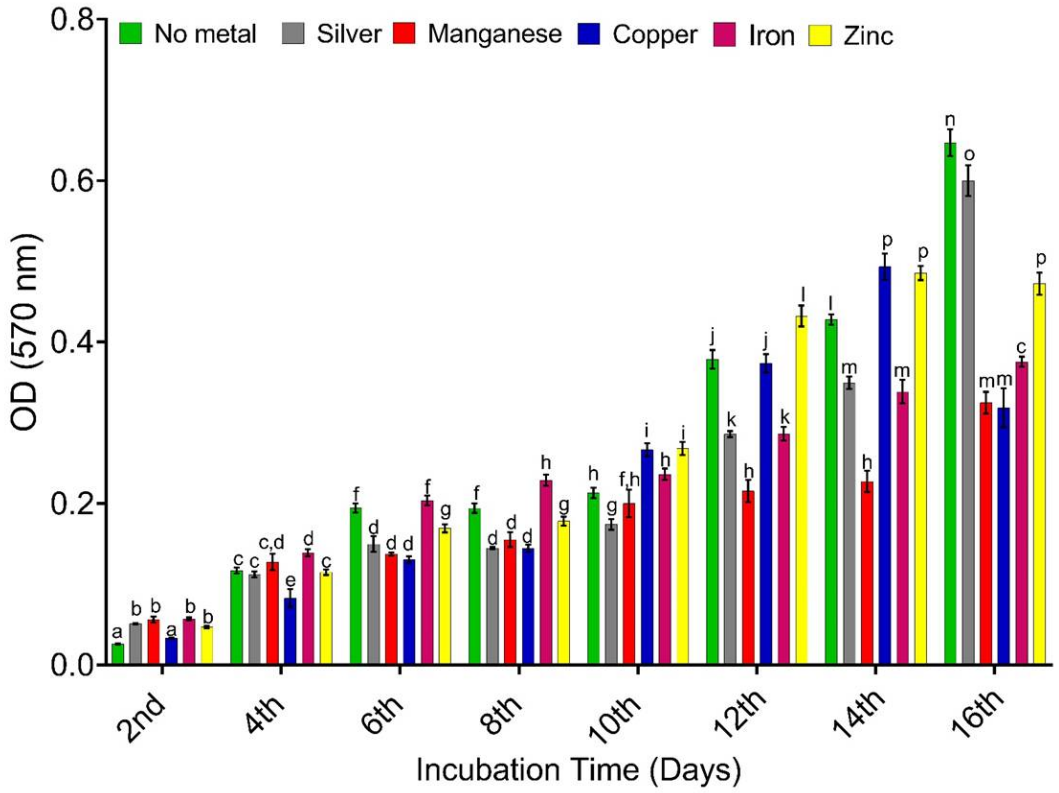

Fig 5. Biofilm formation over 16 days in presence of metal salts (zinc, iron, copper, manganese, silver) and a control (no metal). Significance was calculated by ANOVA, post hoc Tukey test at p≤0.05. Different letters show significant variation.

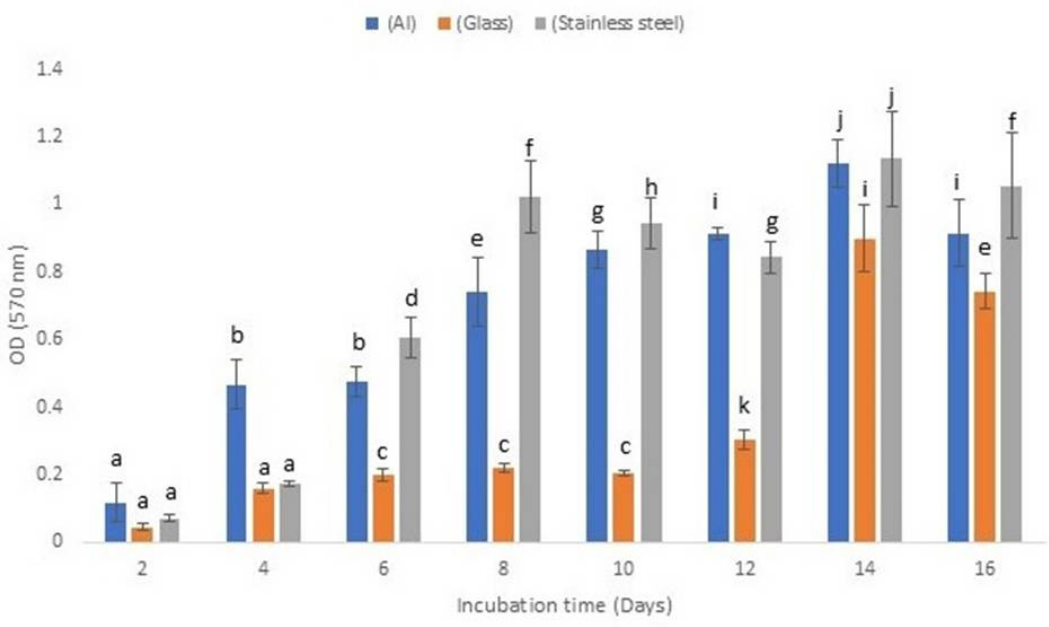

Fig 6: Comparative analysis of biofilm formation on three different surfaces (Aluminum, Glass and stainless steel) over time. Significance was calculated by ANOVA, post hoc Tukey test at p≤0.05. Different letters show significant variation.

CONCLUSION

This study demonstrates that biofilm formation by *Rhodococcus equi* is significantly affected by environmental conditions such as growth medium, temperature, pH, salinity, and the presence of metal

ions. The bacterium forms the strongest biofilm in nutrient-rich Brain Heart Infusion (BHI) medium, with optimal growth observed at physiological temperature (37°C), reflecting its potential pathogenicity in humans and animals. Biofilm formation was better in slightly alkaline conditions, while increasing salinity negatively impacted biofilm development. Among metals, iron promoted biofilm formation in the early stages, while zinc supported biofilm growth at later stages. Additionally, stainless steel proved to be the most conducive surface for biofilm development compared to aluminum and glass. These findings provide valuable insights into *R. equi* biofilm formation, offering potential strategies for managing infections and addressing biofilm persistence in medical, industrial, and agricultural environments.

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AUTHOR CONTRIBUTIONS
VNT conceptualize the idea for the manuscript. PB and SS performed the experiments. PB, SS and VNT write the original manuscript, reviewed and edited the draft. All authors read and finalize the manuscript.

CONFLICT OF INTEREST
The Authors has no conflicts of interest.

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