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Research Article

Illuminating Biofilm Formation and Pathogen Persistence in Household Refrigerators: Evidence from Karachi, Pakistan

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Abstract

In the modern household, refrigerators play a central role in preserving food freshness. However, the microbial landscape within these appliances, particularly the formation of biofilms, remains a significant concern for food safety and hygiene. This study investigated biofilm formation in household refrigerator environments to understand the potential risks associated with microorganisms and their implications for food safety. The study was conducted by examining a total of 80 refrigerators of residents of Karachi, over a 15-day period. employing rigorous methodologies to assess optical density (OD), Colony Forming Units (CFU/cm^2), and Scanning Electron Microscopy (SEM) to uncover bacterial species' abilities to develop biofilms. Results revealed that 12 out of the 80 slides displayed positive biofilm formation based on both OD and CFU criteria. Strong biofilm producers encompassed *Acinetobacter, Listeria, P. aeruginosa, E. coli*, Bacillus, Salmonella, and others. Notably, *P. aeruginosa* was a common component across different biofilm categories. In vitro studies on individual isolates further confirmed biofilm-forming capabilities. *P. aeruginosa* isolates exhibited strong biofilm formation, while *E. coli* isolates displayed weak capabilities. *Enterococci* isolates generally formed weak to moderate biofilms. In summary, our study reveals the prevalence of biofilm formation in household refrigerators, with significant microbial risks posed by species like *P. aeruginosa*. These findings underscore the importance of stringent hygiene practices for ensuring food safety at home.

Keywords: Adhesion, Colony Forming Units, Dispersion, Multi-Species, Optical Density, Single Species.

1. Introduction

Modern refrigeration contributes to the relatively excellent quality of food service meals. Although they serve as a common storage environment for various food items but provide conditions that can support the growth and survival of pathogens as well (Ye et al, 2019). That's how the food can become contaminated and spoil even when refrigerated (Azad et al, 2019). The inoculum in refrigerators frequently gets its supply from the environment, raw materials,

improper handling practices, or inadequate cleaning procedures (Arun, 2018).

Pathogens introduced through contaminated food, improper handling practices, or inadequate cleaning procedures can multiply within the refrigerator and spread to different surfaces through cross-contamination (Okpala, Charles, and Ifeoma, 2019). These pathogens, encountering low temperatures, adopt a biofilm mode of life as a survival strategy, allowing them to persist and thrive in challenging conditions (Parrilli et al, 2021). The formation of biofilms in refrigerators presents a complex phenomenon involving the interaction of multiple bacterial species (. Treccani, 2023). Within these biofilm consortia, pathogens employ two distinct strategies: persistence and dispersal (Shree et al, 2023). While some pathogens remain within the biofilm structure, benefiting from protective matrices and cooperative interactions with other bacterial species, others detach from the biofilm and seek out new colonization sites within the refrigerator (Parrilli et al, 2021; Treccani, 2023). This dispersal strategy can lead to the colonization of additional surfaces, potentially spreading the infection to other food items or refrigerator compartments (Shree et al, 2023). The survival and persistence of pathogens within biofilm structures can be attributed to suboptimal conditions and stress experienced at low temperatures (DeFlorio et al, 2021). While low temperatures are generally unfavorable for the growth of most bacteria, the adoption of a biofilm lifestyle enables pathogens to withstand these conditions (Sharan et al, 2022). Moisture, space, and nutrient availability within the biofilm structure play crucial roles in promoting the resilience and persistence of bacteria (Alegbeleye et al, 2022). Furthermore, the presence of diverse bacterial species within multi-species biofilm consortia highlights the complexity of microbial interactions and dynamics within the refrigerator environment (Alegbeleye et al, 2022). By using the culture-isolation-identification approach, some researchers examined the microbial species present in household refrigerators and discovered that the predominant microbiota on the surface of these refrigerators included Bacillus, Staphylococcus, Kocuria, Pseudomonas, Cladosporium, Aspergillus, and Penicillium, Aeromonas, Listeria spp, Yersinia spp (Ye et al, 2019; Yang et al, 2013). Understanding biofilm formation mechanisms, pathogen prevalence, and interactions is crucial for targeted interventions and management (Shao et al, 2021). Therefore the study is designed to study biofilm formation in refrigerators so that by effectively controlling and mitigating biofilm formation, we can ensure food safety, minimize the spread of pathogens, and safeguard public health.

2. Methods & Materials

2.1 Sample Collection and Biofilm Development

This study investigated 80 household refrigerators of various models and sizes. For this, a clean slide was precisely placed on each refrigerator floor after thorough cleaning and disinfection to eliminate existing biofilm. The refrigerators were kept at 4-4.5^{o}C with 40-60% humidity for two weeks to facilitate biofilm development. The refrigerators' doors were opened minimally to prevent disturbances to biofilm development. After two weeks of incubation, slides were aseptically collected from the refrigerator floor using sterile forceps to avoid surface contact. The slides

were individually placed in sterile Petri dishes for examination. Gram staining and microscopy were then conducted to visualize bacterial adhesion.

2.2 Colony-Forming Unit (CFU) Count by sonication method:

To quantify the viable bacteria adhered to the slides, a sonication method was employed. The slides were submerged in a Phosphate Buffer Saline (PBS) solution (PH=7.4), and ultrasonication was applied to dislodge the adhered bacteria. Serial dilutions of the resulting suspension were plated on appropriate agar media. After incubation, the number of bacterial colonies was counted, and CFU/cm² was determined.

2.3 Segregation of Individual Isolates from Biofilm Consortia

2.3.1 Serial Dilution and Plating Method:

Biofilm-positive slides were placed in a phosphate buffer and vortexed to disperse the cells. The buffer was serially diluted using sterile water or saline solution to decrease the population density. Aliquots of the diluted suspension were plated onto Tryptone Soy agar plates using pour plate techniques. After incubation, well-separated colonies were transferred to separate plates for further isolation (Marmion et al, 2022).

2.3.2 Selective Isolation of bacteria by using selective and differential media:

Well-separated colonies from dilution and plating were inoculated onto specific media tailored for each organism: Cetrimide agar for Pseudomonas aeruginosa, Eosin Methylene Blue agar for Escherichia coli, Baird-Parker agar with egg yolk tellurite for Staphylococcus aureus, Listeria selective agar for Listeria, Brilliant Green agar, Hektoen Enteric agar, and Xylose Lysine Deoxycholate agar for Salmonella, Salmonella-Shigella agar for Shigella, M-enterococci agar for Enterococci, Mackonkey agar for Acinetobacter, Chromogenic coliform agar for Coliforms and Fecal Coliforms, TCBS agar for Vibrio species, and MYP agar. Incubation was 24 hours for isolation and purification, with a 15-day duration for biofilm formation.

2.4 Quantification of Biofilm Biomass by Using Crystal Violet Staining and Optical Density (OD)

Biofilm thickness was evaluated using crystal violet staining and OD measurement. Biofilm-positive slides were stained with crystal violet dye, followed by rinsing and drying. The stained biofilm was visualized under a microscope. For OD measurement, biofilm-positive slides were dispersed in phosphate buffer, and the cell suspension was mixed well. The OD of the suspension was measured using a spectrophotometer, providing a quantitative assessment of biofilm density (Desai and Linda, 2019).

2.5 Scanning Electron Microscopy (SEM)

Slides for SEM analysis were fixed with 2.5% glutaraldehyde, rinsed with phosphate buffer, and dehydrated through ethanol washes (30%, 50%, 70%, 90%, and 100%). Biofilm-positive slides were sectioned, washed with distilled water, and negatively stained with 2% uranyl acetate. Ethanol dehydration (50%, 75%, 95%) preceded platinum coating using an auto-fine coater. Images were acquired with a JSM IT 100 JEOL Electron Microscope (5-20 KV) in high vacuum conditions using a Secondary Electron Detector.

2.6 Statistical Analysis

Statistical analysis, utilizing SPSS software Version 24, employed a qualitative approach to explore the correlation between CFU/cm² and OD in biofilm formation by a mixed

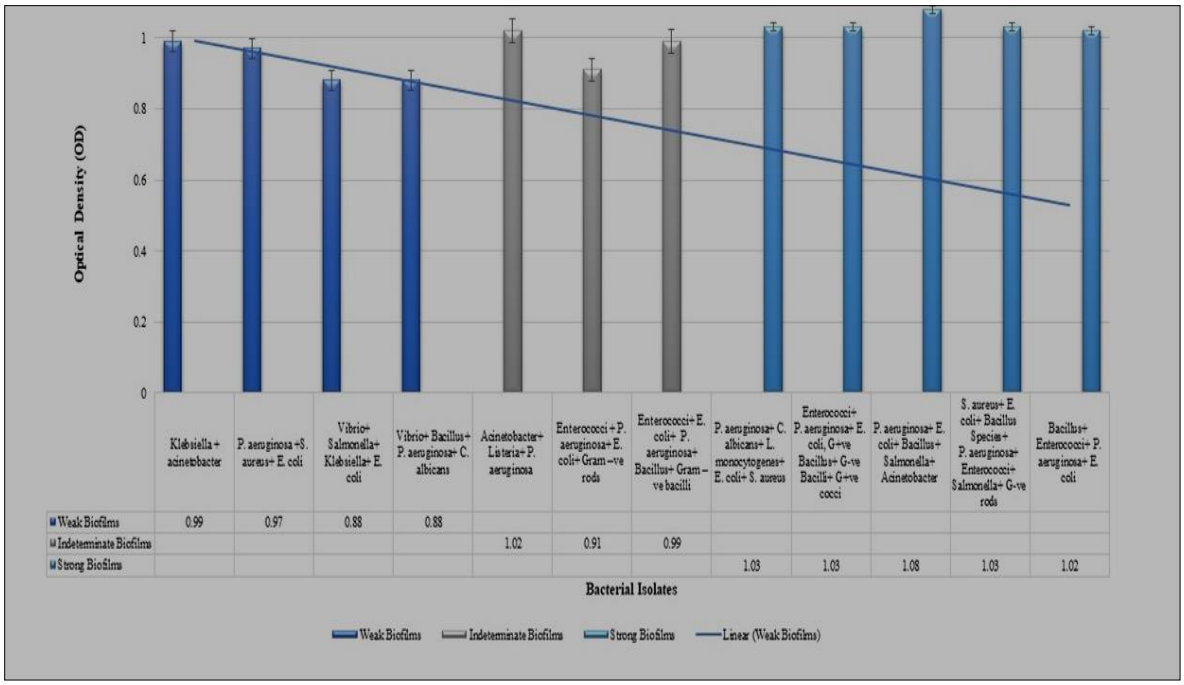

Figure 1: Categorization of biofilm producers into weak, intermediate, and strong groups based on Optical Density (OD). Weak producers (OD ≤ 0.9) exhibit limited biomass, while intermediate producers (OD > 0.9 to 1.0) show moderate maturity. Strong producers (OD > 1.0) indicate well-established biofilms.

bacterial culture. The Pearson correlation coefficient (r) gauged linear correlation, providing a numerical value between 0 and 1. Additionally, a t-test compared the means of two groups, assessing the impact of procedures or differences between groups. A significance level (P-value <0.05) determined variable significance.

3. Results

3.1 Biofilm Development

The findings showed that among the 80 collected slides, merely 12 exhibited evidence of biofilm formation, as confirmed by both the OD measurements and the total CFU/cm2 counts. It's noteworthy that the refrigerators were consistently maintained within a controlled temperature range of 4 to 4.5 degrees Celsius throughout the study. Furthermore, the humidity levels inside the refrigerators were maintained between 40% and 60%.

3.2 Exclusion and Inclusion Criteria for Slide Selection

Slides with CFU counts below 1000 CFU/cm² and OD under 0.6 were omitted to eliminate underdeveloped biofilms. Slides with an OD exceeding 0.8 were excluded if CFU counts were low (<1000 CFU/cm²) to prevent optical artifacts. Conversely, slides with high CFU counts (>10,000 CFU/cm²) and low OD (<0.5) were excluded to avoid overestimating biofilm density. Scanning electron micrographs aided in excluding debris-laden slides to ensure accurate biofilm structure assessment.

3.3 Quantification of Biofilm Formation by Colony Forming Unit and Optical Density

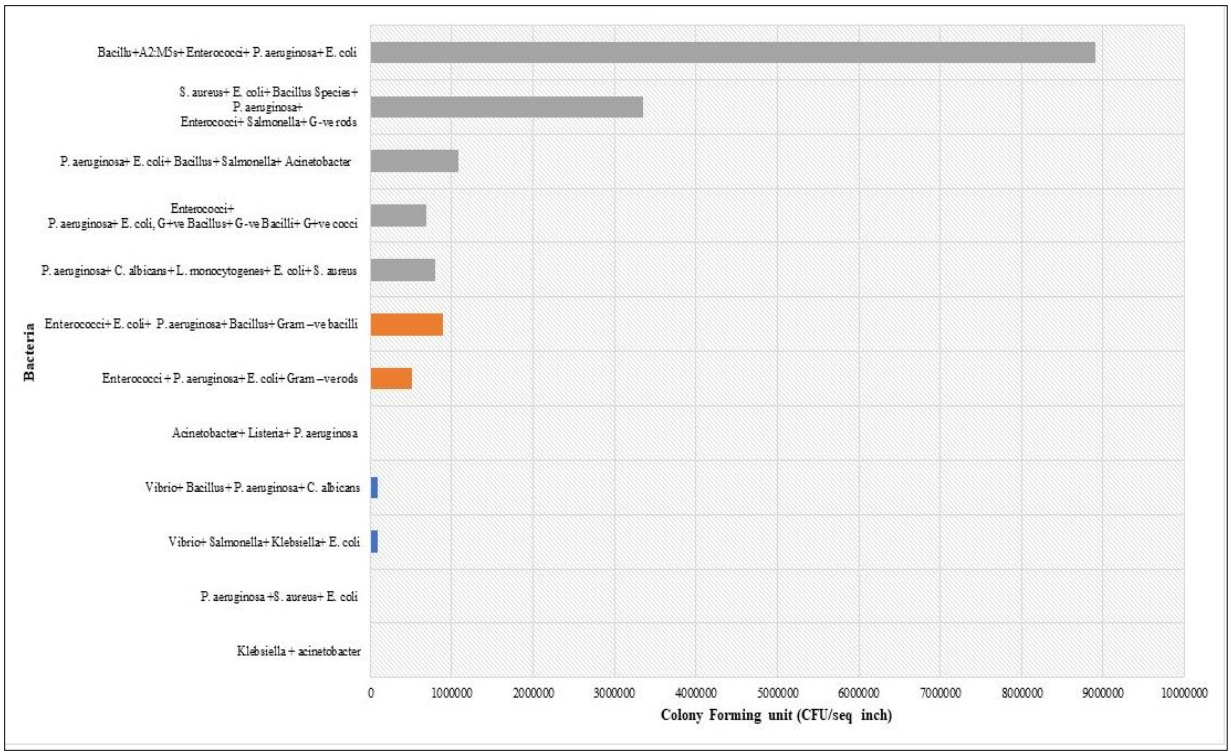

Figure 2: Comparative Analysis of Total Colony Forming Units (CFU) in Multispecies Biofilms

Among the 80 examined refrigerators, 51 (approximately 63.75% of the total) displayed bacterial counts below 100 CFU/cm² and showed no signs of biofilm. For 11 refrigerators (about 13.75% of the sample), slides exhibited bacterial counts below 1000 CFU/cm² and OD values below 0.6. In contrast, slides from 6 refrigerators (7.5%) were heavily debris-laden and consequently excluded from the study. This exclusion aimed to maintain the focus on well-defined biofilm samples, ensuring research reliability. Out of the 80 refrigerators scrutinized, only 12 slides (15% of the sample) met the criteria for total bacterial count and OD within the specified range (Figure 1). These slides were thus deemed suitable for further analysis and were included in the study.

3.4 Investigation and Characterization of Biofilm

In our study, we employed three criteria to investigate and characterize biofilms: OD, total bacterial count (CFU), and a combination of OD and CFU/cm² (Figures 1, 2, and 3).

The study categorized biofilm producers based on biofilm OD and total bacterial count. Weak producers (OD ≤ 0.9, CFU/cm² ≤ 100,000) included two consortia with OD=0.88: one comprising Vibrio, Salmonella, Klebsiella, and E. coli, and the other with Vibrio, Bacillus, P. aeruginosa, and C. albicans. Moderate producers (OD > 0.9 to 1.0) had four groups, and strong producers (OD > 1.0) comprised three groups. Notable strong biofilm producers included Acinetobacter, Listeria, and P. aeruginosa (OD=1.02, 15,520 CFU/cm²) and S. aureus, E.

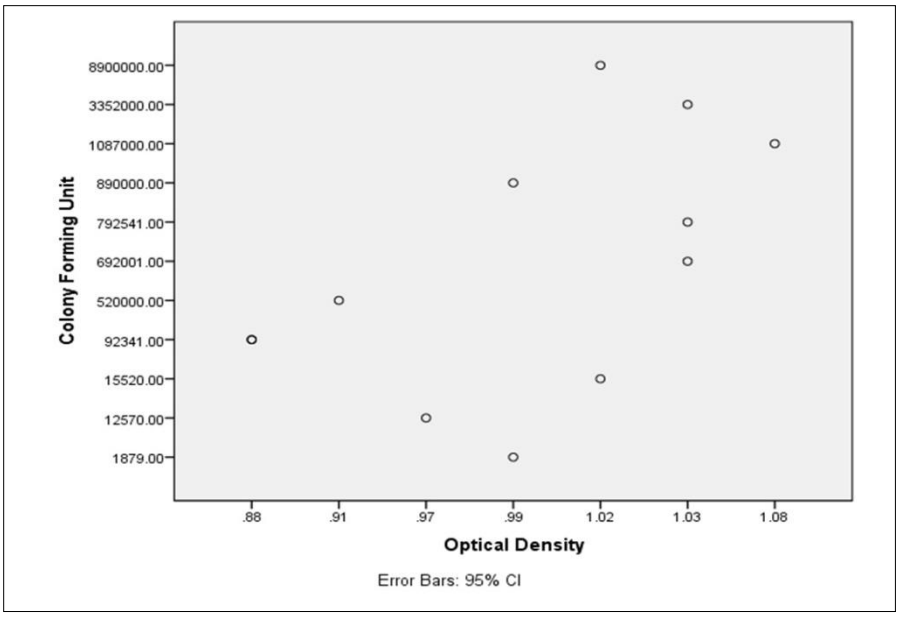

Figure 3: Correlation between Total Colony Forming Units (CFU) and Optical Density (OD) of Multispecies Biofilms, with 95% Confidence Intervals (95% CI).

coli, Bacillus, P. aeruginosa, Enterococci, Salmonella, and Gram-negative rods (OD=1.02, 8,900,000 CFU/cm²). P. aeruginosa was prevalent in multi-species biofilms (83.3%), with E. coli (66.67%), Enterococci (50%), and Bacillus (41.67%) following. S. aureus, Salmonella, and Acinetobacter were present in 25%, while Klebsiella, Listeria, and Vibrio were in 16.67%. A consortium included C. albicans, and some slides featured unidentified strains of gram-positive and gram-negative bacteria (Figures 1, 2, and 3).

3.5 Purification of Isolates

Isolates from the 10 tested strains were separated and purified for individual in vitro biofilm formation studies. P. aeruginosa isolates demonstrated strong biofilm-forming capacity, with 7 out of 10 exhibiting a biofilm OD > 0.90, indicating high biofilm formation. Two isolates showed a biofilm OD > 0.87, signifying a relatively strong biofilm capacity. One isolate displayed a comparatively weaker biofilm formation with an OD of 0.66. Purified E. coli isolates demonstrated weak biofilm-forming capabilities. Out of the 8 tested isolates, 3 had a biofilm OD <0.60, indicating a relatively low biofilm formation capacity.

One isolate displayed a biofilm OD <0.50, signifying very weak biofilm production. Two isolates had a biofilm OD <0.70, and 2 other isolates had a biofilm OD <0.80, both suggesting modest biofilm formation ability. Enterococci isolates, when studied individually, exhibited a predominantly weak biofilm-producing capacity. Out of the 6 tested isolates, only one demonstrated strong biofilm formation with a biofilm OD <0.90. Two isolates had a biofilm OD <0.80, indicating a relatively moderate biofilm-forming ability. Three Enterococci isolates displayed a biofilm OD <0.50, suggesting a weak to moderate biofilm formation capacity. Lastly, one isolate had a biofilm OD >0.50, indicating a comparatively weaker biofilm producer. Bacillus species from refrigerators displayed strong biofilm-

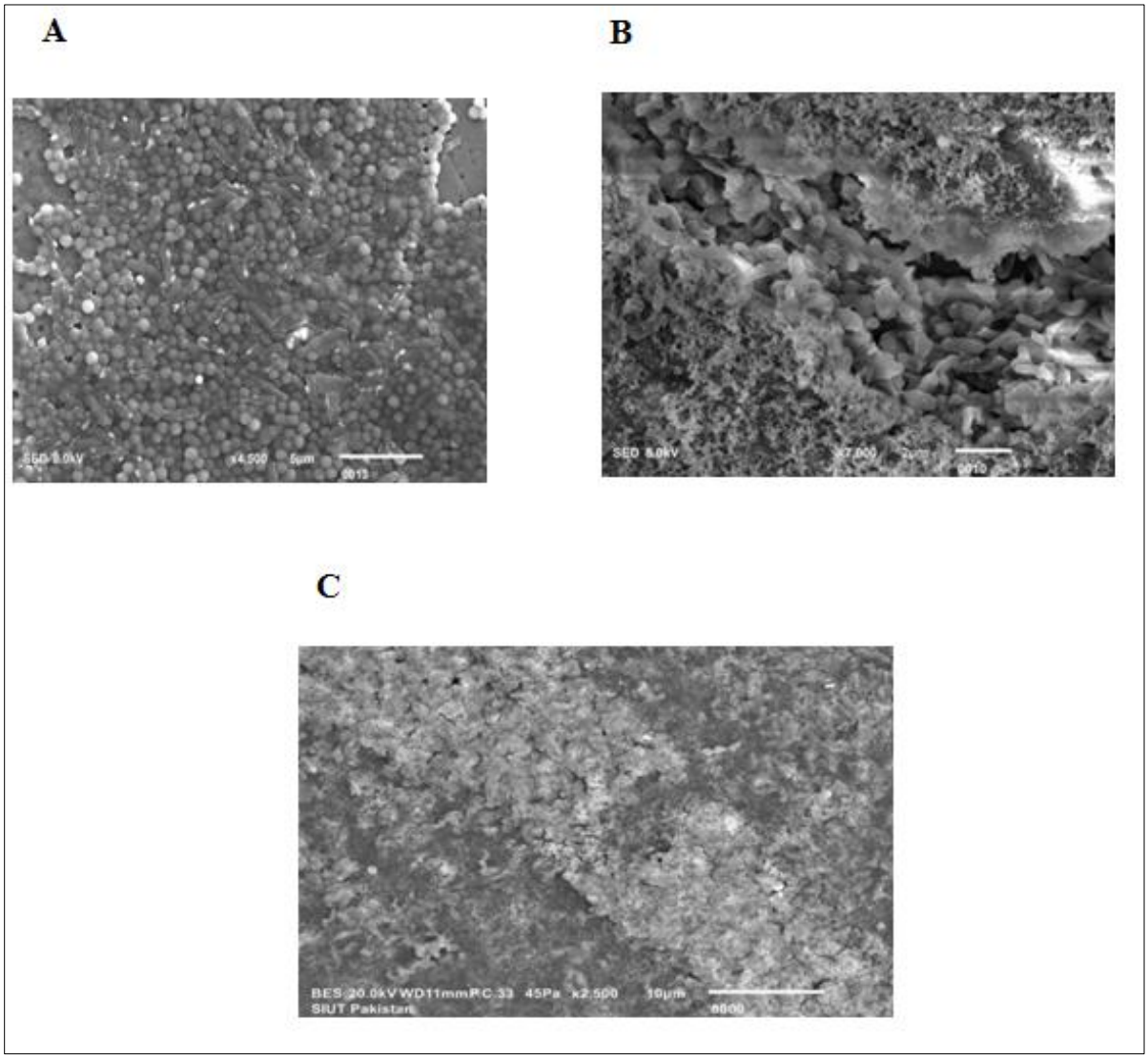

Figure 04: (A) Multispecies Biofilms Showing Adhesion of Rod-Shaped and Cocci Bacteria in Compact Structures after 15 Days of Incubation at 4°C in a Refrigerator. (B) Strong Biofilm Consortia Revealing Multicellular Aggregates of Cells Covered with Matrix Material and Firmly Attached to Solid Surfaces. (C) Slide Surface Covered with Debris Without Bacterial Cells. These Slide Types Were Excluded from the Study.

producing capabilities, both individually and in mixed-species consortia. In single-species biofilms, all four Bacillus isolates consistently demonstrated an OD >0.90, indicating a high level of biofilm formation capacity.The findings from the biofilm OD analysis demonstrated that S. aureus isolates displayed strong biofilm-producing capabilities in single-species studies, while Vibrio, Salmonella, and Klebsiella strains exhibited weak biofilm-forming abilities. Acinetobacter and Listeria strains were categorized as intermediate biofilm producers based on their OD values. In vitro studies at 35°C showed the highest OD after 96 hours of incubation, while none of the

isolates produced biofilm at 4°C even after 15 days of incubation.

3.6 Scanning Electron Microscopy

SEM analysis of biofilm-positive slides revealed strong cell attachment forming a densely packed assembly with abundant extracellular matrix material (Figure 4). Notably, one slide (Figure 2) showed cells enveloped in a substantial matrix layer, indicating significant matrix presence and active production. Even in weak or moderate biofilm consortia, SEM analysis revealed densely packed cells within matrix material, suggesting cohesive structures despite lower biofilm strength. Biofilm-negative slides surprisingly displayed slimy matrix material and a scattered bacterial population. Direct observation revealed a thick layer of matrix material coating the slide's surface, potentially acting as a cementing cover or protective layer for the biofilm structure.

4. Discussion

Modern household refrigerators, essential for food storage, maintain a temperature range of 37°F (3°C) to 41°F (5°C), ideal for short-term storage of ready-to-eat food, vegetables, and fresh fruits. (Marra et al, 2023; Adamowicz et al, 2018). Furthermore, the majority of bacterial pathogens are unable to thrive at this temperature range (3°C-5°C) (Giaouris et al, 2015). Consequently, storage at this temperature leads to an extension in shelf life and the preservation of product quality (Kamimura et al, 2022). It's important to note that if bacteria are already present, their growth may not cease entirely; rather, it might transition into a dormant state (Kamimura et al, 2022; Ahmed et al, 2022). Nevertheless, there are instances when highly contaminated products are inadvertently stored in refrigerators, leading to the introduction of diverse pathogens into the system (. Wu et al, 2023). Pathogens' ability to endure low temperatures in household refrigerators, operating at around 4°C, allows them to persist and potentially contaminate other fresh products (Adamowicz et al, 2018, Marshall, 2022). Consequently, when food is taken out from the refrigerator and exposed to room temperature, the bacteria have the potential to resume their growth and continue multiplying (Wu et al, 2023; Marshall, 2022). Another significant characteristic of food-borne pathogens is their ability to create biofilms, especially under challenging growth conditions (Sammugam, Lakhsmi, and Visweswara Rao, 2019). This research focuses on the development of multispecies biofilms in refrigerators, addressing the persistent challenge documented in the food and dairy sectors (Shaik, Lubna, and Snehasis, 2022). Pathogens in biofilms resist standard sanitization, posing challenges to their elimination from systems (Marshall, 2022; Sammugam, Lakhsmi, and Visweswara Rao, 2019). Prominent pathogens accountable for biofilm formation under low-temperature conditions in the food and dairy sectors encompass *Listeria, P. aeruginosa*, *Salmonella, Bacillus*, *E. coli*, *Staphylococcus,* and numerous others (Marshall, 2022; Sammugam, Lakhsmi, and Visweswara Rao, 2019; Shaik, Lubna, and Snehasis, 2022). Correspondingly, the current identified key pathogens—*P. aeruginosa*, *E. coli*, Enterococci, and Bacillus—as contributors to biofilm formation in household refrigerators. Other contaminants, including *S. aureus*, Salmonella, Listeria, and various Vibrio species, were also found in multispecies biofilms. Vegetables and contaminated food items emerged as primary sources

introducing these pathogens into the refrigerator system. (Baptista et al, 2020). The rapid shift from an open environment to an enclosed, cool system induces growth inhibition and dormancy in bacteria. This metabolic adjustment, a survival strategy, conserves energy under unfavorable conditions. The study identifies *P. aeruginosa*, Enterococci, *S. aureus*, and Bacillus as adept at transitioning from planktonic to biofilm states. In mixed-species biofilms, these pathogens not only protect themselves but also shelter other consortium pathogens, showcasing intricate microbial interactions that influence collective behavior and resilience. This assertion is substantiated by published research as well (Gallo et al, 2020). It is evident that a significant portion of biofilms discovered in natural environments does not arise from a solitary species; instead, they commonly manifest as collaborative creations involving two or more species of microorganisms (Bell et al, 2021). *P. aeruginosa* excelled in solo biofilm formation, while *E. coli* had limited proficiency. In mixed scenarios, *P. aeruginosa* and *E. coli* frequently coexisted. Listeria, *S. aureus*, and Enterococci individually showed modest biofilm formation but formed robust biofilms synergistically in consortia. Scanning electron microscopy confirmed *P. aeruginosa*'s advantage in adapting to the biofilm mode, especially in the refrigerator's cold environment. These pathogens, capable of dormancy at lower temperatures, exhibit a higher propensity for biofilm formation in persistent strains (Alvarez-Ordóñez et al, 2015). A significant proportion of food-borne pathogens possess the ability to adhere to and create biofilms on various surfaces within food processing and storage environments (Allata, Valero, Benhadja, 2017). Both the existing literature (Gallo et al, 2020; Osafo et al, 2022) and the current study proved that *P. aeruginosa* and Bacillus in household refrigerators shelter other pathogens in biofilms, posing a persistent threat. Public awareness and proper sanitation education are crucial to prevent contamination and infections from these resilient pathogens.

MMoreover, the low prevalence of biofilm formation in refrigerators, despite the presence of potential biofilm-forming pathogens, suggests that additional factors may influence the development of biofilms (Thi, et al, 2020; Sadiq et al, 2021). Factors such as the composition of the refrigerator environment, food handling practices, and cleaning procedures can impact the initiation and growth of biofilms (Liu et al, 2023). Further exploration is necessary to gain a comprehensive understanding of the conditions and mechanisms that contribute to biofilm formation in refrigerators (Mevo et al, 2021; Sionov et al, 2022).

5. Conclusion

This study highlights diverse biofilm formation in household refrigerators, emphasizing the importance of regular cleaning to address potential risks to food safety and hygiene. The robust methodology strengthens findings, underscoring the significance of public health and environmental considerations.

Conflict of Interest

The authors declare that they have no competing interests.

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Study Approval
This study was approved by the Department of Microbiology, University of Karachi, Pakistan.

Consent Forms
NA.

Authors Contribution
RS, SU, Zam, NH, and SN carried out all the data collection, bench work, initial draft, and manuscript writing by AAM, AS, AK, and MNK. ABK, TS, and YR helped in data collection and statistical analysis. YR and ZAM conceptualized and supervised the study.

Data Availability
Data is available upon reasonable request from the corresponding author.

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