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Detection of Biofilm Formation: Evaluation of Congo Red Agar and Crystal Violet Staining Method

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Highlights

- Biofilm was evaluated using Congo Red Agar and Crystal Violet Staining methods.
- CRA and CVS were compared to assess biofilm formation in various microbial strains.
- CVS enabled reliable distinction between weak, moderate, and strong biofilm producers.

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Abstract

This study focuses on the detection of biofilm formation using Congo Red Agar (CRA) and Crystal Violet Staining (CVS) methods. Biofilms are complex microbial communities formed by bacteria adhering to surfaces, surrounded by extracellular polymeric substances, which provide protection against external stressors. Biofilm formation plays a critical role in infection control and combating antibiotic resistance. In this research, the biofilm production capacities of 24 standard bacterial strains, 11 multidrug-resistant (MDR) strains, 9 foodborne isolates, and 24 clinical isolates were evaluated. The CRA method qualitatively identifies biofilm formation through colony morphology, while the CVS assay quantitatively measures biofilm production. The results demonstrated that 66.18% of the 68 microorganisms tested using CRA were biofilm positive, with Gram-positive bacteria generally exhibiting stronger biofilm production. The CVS assay further provided more precise measurements of biofilm production, identifying strong biofilm producers such as *Enterococcus faecalis* ATCC 29212, *Pseudomonas fluorescens* P1, and *Staphylococcus aureus* ATCC 25923. The study found that while CRA is effective in detecting strong biofilm producers, it has limitations in identifying weaker biofilm producers. In contrast, the CVS assay proved to be more sensitive and reliable. The findings also highlight that variations in biofilm production capacity emphasize the significance of biofilm management in clinical infections and the challenges encountered in treatment.

1. INTRODUCTION

Microorganisms have developed various adaptation strategies to sustain their survival and protect themselves against environmental stress factors. One of these strategies, biofilm formation, refers to a complex microbial community formed by bacteria adhering to surfaces or to one another, surrounded by extracellular polymeric substances (EPS) such as polysaccharides, proteins, lipids, and DNA [1]. Biofilms not only provide bacteria with protection against environmental stressors but also render them more resistant to antibiotics, disinfectants, and immune system attacks. This characteristic has made biofilms a significant challenge in both infection control and industrial applications [2].

Biofilm formation typically occurs in four main stages: initial attachment of microorganisms to a surface, followed by cell accumulation and microcolony formation, biofilm maturation, and finally, the dispersion of bacteria into the environment in a planktonic form. The attachment phase is driven by various physical and chemical factors, including hydrogen bonds, van der Waals forces, and hydrophobic interactions. During the maturation phase, microorganisms produce EPS, making the biofilm more resilient and persistent. This process enhances the bacteria's ability to adapt to surrounding conditions while

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simultaneously increasing their resistance to external threats, such as immune responses and antibiotic treatments [3].

Biofilms play a significant role, particularly in infections associated with medical devices. Biofilms formed on catheters, artificial heart valves, orthopedic implants, and other medical devices lead to chronic infections and complicated their treatment. For instance, biofilms produced by pathogens such as *Staphylococcus aureus* and *Staphylococcus epidermidis* are primary causes of catheter-associated infections commonly observed in hospitals. These biofilms can exhibit high levels of resistance to antibiotic treatments and often necessitate surgical interventions for their removal [4,5]. The World Health Organization (WHO) has published a list of antibiotic-resistant bacteria that produce biofilms, including *Acinetobacter, Salmonella, Pseudomonas, Klebsiella, Escherichia coli*, and *Proteus*. These bacteria are responsible for fatal infections and are increasingly resistant to most available antibiotics [6].

Biofilms pose serious challenges not only in healthcare but also in food safety and industrial sectors. In food processing facilities, biofilm formation contaminates equipment and degrades product quality, while in the energy sector, it leads to pipeline blockages and corrosion, resulting in significant economic losses. Controlling biofilm formation is critical to preventing these issues, and research in this area is gaining increasing importance [1, 5].

Various techniques are employed to detect biofilm formation, including tissue culture plates, tube methods, Congo Red Agar (CRA), microplate assays, fluorescence microscopy, and scanning electron microscopy. Among these, methods using crystal violet staining (CVS) are inexpensive and yield reliable quantitative results; however, they have disadvantages such as cell loss and structural disruption of biofilms due to repetitive washing steps. The CRA method, while easy and cost-effective, does not provide quantitative measurements [7, 8].

Recent advancements in biofilm detection methodologies have focused on enhancing the accuracy and reproducibility of assays. For instance, efforts have been made to standardize the crystal violet microtiter plate assay, aiming to harmonize protocols across laboratories and improve the reliability of biofilm quantification [9]. Additionally, innovative staining techniques, such as the dual-staining method utilizing Maneval's stain, have been developed to overcome the limitations of traditional CRA and crystal violet staining, providing clearer differentiation of microbial biofilms [10, 11]. These developments underscore the ongoing evolution of biofilm detection methods, highlighting the importance of integrating such advancements into current research to enhance diagnostic accuracy and clinical relevance.

The primary aim of this study is to evaluate the biofilm formation capacities of different reference and clinical isolates and to compare the efficacy of methods used for detecting biofilm formation. Specifically, the investigation of biofilm formation capacities of multidrug-resistant (MDR) strains seeks to address existing knowledge gaps in this field. The study employs the CRA and CVS methods, both of which are widely used for biofilm detection. By comparing the effectiveness and accuracy of these methods, the study aims to develop strategies to improve infection control and mitigate problems arising from biofilm-associated complications.

2. MATERIAL & METHOD

2.1. Microorganisms

The test panel included 24 standard (STN) strains, comprising 11 Gram-positive and 13 Gram-negative bacteria; 11 multi-drug-resistant (MDR) bacteria, including 8 Gram-positive and 3 Gram-negative strains; 24 clinical isolates (KLI), consisting of 9 Gram-positive and 15 Gram-negative strains; and 9 foodborne isolates (GI), including 3 Gram-positive and 6 Gram-negative bacteria. Additionally, the study incorporated 5 standard, 2 clinical, and 2 food-isolated yeast strains. Clinical isolates used in this study were numbered sequentially (e.g., *S. aureus* 2, *E. coli* 5) to distinguish individual strains within each species. The origin of each isolate is indicated in parentheses as follows: KLI – clinical isolate, GI – foodborne isolate, MDR –

multidrug-resistant isolate. All strains were sourced from the Microbiology Research Laboratory collection within the Department of Biology, Faculty of Science, at Dokuz Eylül University.

2.2. Microbial Inoculum

At this stage, the colony suspension method was employed to prepare standardized microbial inocula. Microorganisms previously cultivated in Tryptic Soy Broth (TSB) (Oxoid, United Kingdom) were streaked onto the surface of Tryptic Soy Agar (TSA) (Oxoid, United Kingdom) and incubated at 37°C for 18–24 hours to obtain isolated colonies. Following incubation, well-isolated single colonies were selected using a sterile inoculating loop and suspended in 5 mL of sterile 0.9% physiological saline solution. The bacterial suspension was vortexed (Wisd, China) briefly to achieve a homogeneous mixture. The turbidity of the suspension was then measured using a densitometer (Biosan, Latvia) and adjusted to match the 0.5 McFarland standard, corresponding to approximately 1.5×10^8 CFU/mL, depending on the species [12]. Inocula were freshly prepared for each assay and used immediately to ensure viability and consistency.

2.3. Biofilm Detection Method Using Congo Red Agar

The CRA method is widely used as a phenotypic method to assess EPS production and hence biofilm formation potential. The Congo Red dye in CRA changes colony morphology by interacting with the extracellular polysaccharide production of bacteria. This is considered a direct indicator of biofilm formation [13]. In this study, the CRA method was used for qualitative analysis.

CRA medium was prepared by incorporating 37 g/L brain heart infusion (BHI) broth (Merck, Germany) with 50 g/L sucrose (Tekkim, Turkiye), 15 g/L agar (Oxoid, United Kingdom), and 0.8 g/L Congo red dye (Merck, Germany). The Congo red dye was first prepared as a concentrated aqueous solution (600 mL) and sterilized via autoclaving (Nüve, Turkiye) at 121°C for 15 minutes. After sterilization, sucrose was added to the autoclaved BHI agar while the medium was maintained at 55°C. CRA plates were then poured and allowed to solidify. Microorganisms, which had been pre-enriched overnight in 5 mL of TSB, were inoculated onto the CRA plates using a sterile inoculating loop. The plates were incubated at 37°C for 24 hours, after which colony morphology and pigmentation were evaluated [14]. The procedure was conducted in triplicate and repeated three times to ensure reproducibility.

On CRA, biofilm formation was identified by the presence of black, dry, crystalline colonies, which were indicative of positive biofilm production. The extent of biofilm formation was categorized as weakly positive (+), positive (++), and strongly positive (+++). In contrast, smooth colonies exhibiting orange, red, or pink pigmentation were considered negative (-) for biofilm formation [15].

2.4. Determination of Optimal Biofilm Conditions Using Crystal Violet

In this study, the CVS method was used to obtain quantitative analysis results. Biofilms were cultivated in 96-well microtiter plates using a modified version of the method described by Stepanović et al. [16]. Microorganisms capable of biofilm formation were cultured at 37°C for 24 hours before the assay. For each strain, a double-strength TSB supplemented with 2.5% glucose (Merck, Germany) was prepared. The 2.5% glucose concentration was selected based on previous findings demonstrating its effectiveness in enhancing biofilm production across various bacterial species [17]. Although the optimal glucose concentration may vary depending on the microorganism, 2.5% was chosen as a standardized and reproducible condition to induce sufficient biofilm formation for comparative analysis.

A 20 µL bacterial suspension, standardized to 0.5 McFarland turbidity, was added to the wells of microplates and incubated (Nüve, Turkiye) at 37°C for 24, 48, and 72 hours. After incubation, the wells were gently washed twice with pH 7.4 sterile phosphate-buffered saline (PBS) (Thermo Fisher, USA) to remove non-adherent cells. The remaining adherent cells, representing the biofilm, were then fixed by incubating the plates in a dry heat oven (Nüve, Turkiye) at 60°C for 60 minutes. This step ensured the firm attachment of biofilm biomass to the well surface, preventing its loss during the subsequent staining process. Following fixation, each well was stained with 200 µL of 0.5% (w/v) crystal violet solution

(Merck, Germany) for 15 minutes at room temperature. Excess stain was rinsed off with sterile distilled water. To facilitate accurate absorbance measurement, the plates were dried in a 37°C incubator for 30 minutes. Finally, the bound crystal violet was solubilized by adding 200 µl of 33% (v/v) glacial acetic acid to each well, enabling quantification of biofilm biomass by measuring optical density at the appropriate wavelength (Biotek, USA) [18].

Spectrophotometric measurements were recorded at 570 nm to quantify biofilm production. The conditions yielding the highest biofilm production capacity were identified based on the data obtained. All experiments were conducted in triplicate to ensure reproducibility. *Enterococcus faecalis* ATCC 29212 served as the positive control strain [19]. Optical density (OD) ranges were evaluated for all strains and controls, and the cutoff values (ODc) were calculated [20]. The ODc was defined as the sum of the mean OD of the negative control and three standard deviations (SD), as shown in Equation (1).

$$OD_{cutoff} = \text{Mean OD of negative control} + (3 \times \text{SD of negative control}). \quad (1)$$

For each microtiter plate, the OD_c value was calculated independently. Based on the results, strains were categorized into four groups according to their biofilm-forming capacity:

- $OD \leq ODc$: Non-biofilm forming
- $ODc < OD \leq 2 \times ODc$: Weak biofilm producer
- $2 \times ODc < OD \leq 4 \times ODc$: Moderate biofilm producer
- $4 \times ODc < OD$: Strong biofilm producer.

2.5. Statistical Analysis

This study incorporates several common methods for statistical analysis to evaluate biofilm production across different strains and conditions. A one-way ANOVA was used to compare biofilm formation among various groups, helping to identify significant differences in biofilm production levels. The level of statistical significance was set at $p \leq 0.05$. To ensure the reliability of the results, the experiments were repeated three times, ensuring reproducibility and consistency of the data. Additionally, correlation and agreement measures were applied to assess the relationship between different detection methods. The Kappa statistic was used to evaluate the level of agreement between the CRA and CVS, while Spearman correlation was employed to analyze the relationship between biofilm formation measurements across these different methods. All graphs and analyses were conducted using R Studio (version 2024.09.0).

3. RESULTS AND DISCUSSION

3.1. Results Obtained Using the Congo Red Agar (CRA) Method

The biofilm formation capacity of a total of 68 microorganisms was evaluated using the CRA method. Among these, 45 strains (66.18%) were identified as biofilm-positive. Specifically, 15 strains (22.06%) were classified as weakly positive (+), 18 strains (26.47%) as positive (++), and 12 strains (17.65%) as strongly positive (+++), while 23 strains (33.82%) were determined to be biofilm-negative. The results obtained using this colony morphology-based method for standard strains are presented in Figure 1.

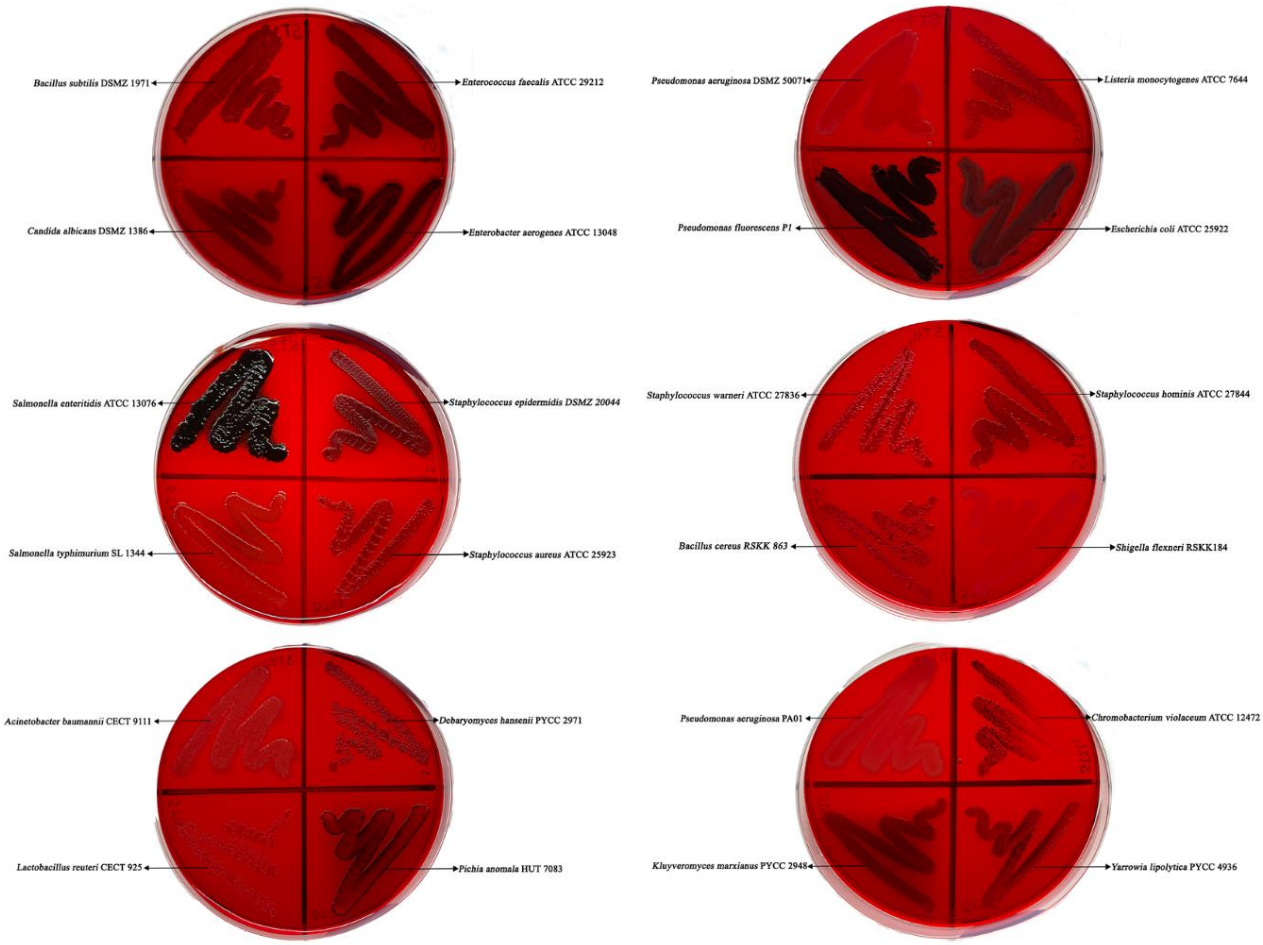

Figure 1. *Results of the Congo Red Agar (CRA) Test Applied to Reference Strains*

Of the 24 reference strains analyzed using the CRA method, 17 were found to be biofilm-positive. Among these, 5 strains exhibited weakly positive biofilm formation, 8 strains were positive, and 4 strains showed strong positive biofilm formation. The colony morphology observed during the test and the corresponding results are detailed in Table 1.

Table 1. *Biofilm formation characteristics of reference strains based on colony color on congo red agar* (*Note: Biofilm intensity levels are indicated as – (negative), + (weak), ++ (moderate), and +++ (strong), based on colony morphology)*

Microorganisms	Colony Morphology	Biofilm Formation	Intensity
Acinetobacter baumannii CECT 9111	Crystallized, pink-red	Negative	-
Bacillus cereus RSKK 863	Dark, partially crystallized	Weak	+
Bacillus subtilis DSM 1971	Dark, partially crystallized	Moderate	++
Candida albicans DSM 1386	Dark, partially crystallized	Moderate	++
Chromobacterium violaceum ATCC 12472	Dark, crystallized	Moderate	++
Debaryomyces hansenii PYCC 2971	Dark, partially crystallized	Weak	+
Enterobacter aerogenes ATCC 13048	Black, crystallized	Strong	+++
Enterococcus faecalis ATCC 29212	Dark, partially crystallized	Moderate	++
Escherichia coli ATCC 25922	Dark, partially crystallized	Moderate	++
Kluyveromyces marxianus PYCC 2948	Dark, partially crystallized	Moderate	++
Lactobacillus reuteri CECT 925	Smooth, Red	Negative	-
Listeria monocytogenes ATCC 7644	Smooth, Red	Negative	-
Pichia anomola HUT 7083	Dark, crystallized	Strong	+++
Pseudomonas aeruginosa DSM 50071	Smooth, Red	Negative	-
Pseudomonas aeruginosa PA01	Smooth, Red	Negative	-
Pseudomonas fluorescens P1	Black, crystallized	Strong	+++
Salmonella enteritidis ATCC 13076	Black, crystallized	Strong	+++

Salmonella typhimurium SL 1344	Smooth, Red	Negative	-
Shigella flexneri RSKK 184	Smooth, Red	Negative	-
Staphylococcus aureus ATCC 25923	Dark, partially crystallized	Weak	+
Staphylococcus epidermidis DSM 20044	Dark, partially crystallized	Moderate	++
Staphylococcus hominis ATCC 27844	Dark, partially crystallized	Weak	+
Staphylococcus warneri ATCC 27836	Dark, partially crystallized	Weak	+
Yarrowia lipolytica PYCC 4936	Dark, crystallized	Moderate	++

The 17 reference strains that tested positive using the CRA method. A comparison with previously reported results in the literature for these reference strains indicates that not all strains have been widely studied using the CRA method. Among these reference strains, *E. coli* ATCC 25922 is commonly used as positive control for both CRA and CVS methods [21, 22]. In this study, *E. coli* ATCC 25922 also demonstrated positive results.

Chromobacterium violaceum ATCC 12472 is a bacterium known for its biofilm formation capacity and quorum sensing (QS) mechanisms. It is often used as a model organism in studies targeting biofilm formation and QS inhibition. In our study, *C. violaceum* ATCC 12472 colonies that tested positive with the CRA method displayed a characteristic shiny red-brown color when bound to Congo red after being incubated on LB-glucose for 72 hours [23]. In contrast, *Pseudomonas aeruginosa* PAO1 tested biofilm-negative in this study.

Interestingly, *Pseudomonas aeruginosa* PAO1—a strain widely acknowledged for its robust biofilm-forming ability—was classified as biofilm-negative using CRA. This inconsistency aligns with previous studies indicating the limited sensitivity of CRA for *Pseudomonas* sp. Lima et al. [24] reported that CRA only detected biofilm in 15% of *P. aeruginosa* isolates. These findings suggest that the extracellular matrix components produced by *P. aeruginosa*, such as alginate, may not interact strongly with Congo Red, thereby reducing staining visibility. Methodological limitations, including CRA's reliance on colony morphology, may lead to false negatives in strains producing non-Congo Red-binding polysaccharides.

Salmonella enteritidis ATCC 13076 is a strain with a high biofilm formation capacity and has been extensively studied using various methods. On CRA media, biofilm-forming strains typically produce red, dry, and rough colonies. In one study, 93% of *S. enteritidis* isolates exhibited red, dry, and rough morphologies on CRA media, along with the formation of a dense pellicle at the air-liquid interface [25]. The CRA method was applied to 44 different clinical and food isolates, and the results based on colony morphology are presented in Figure 2.

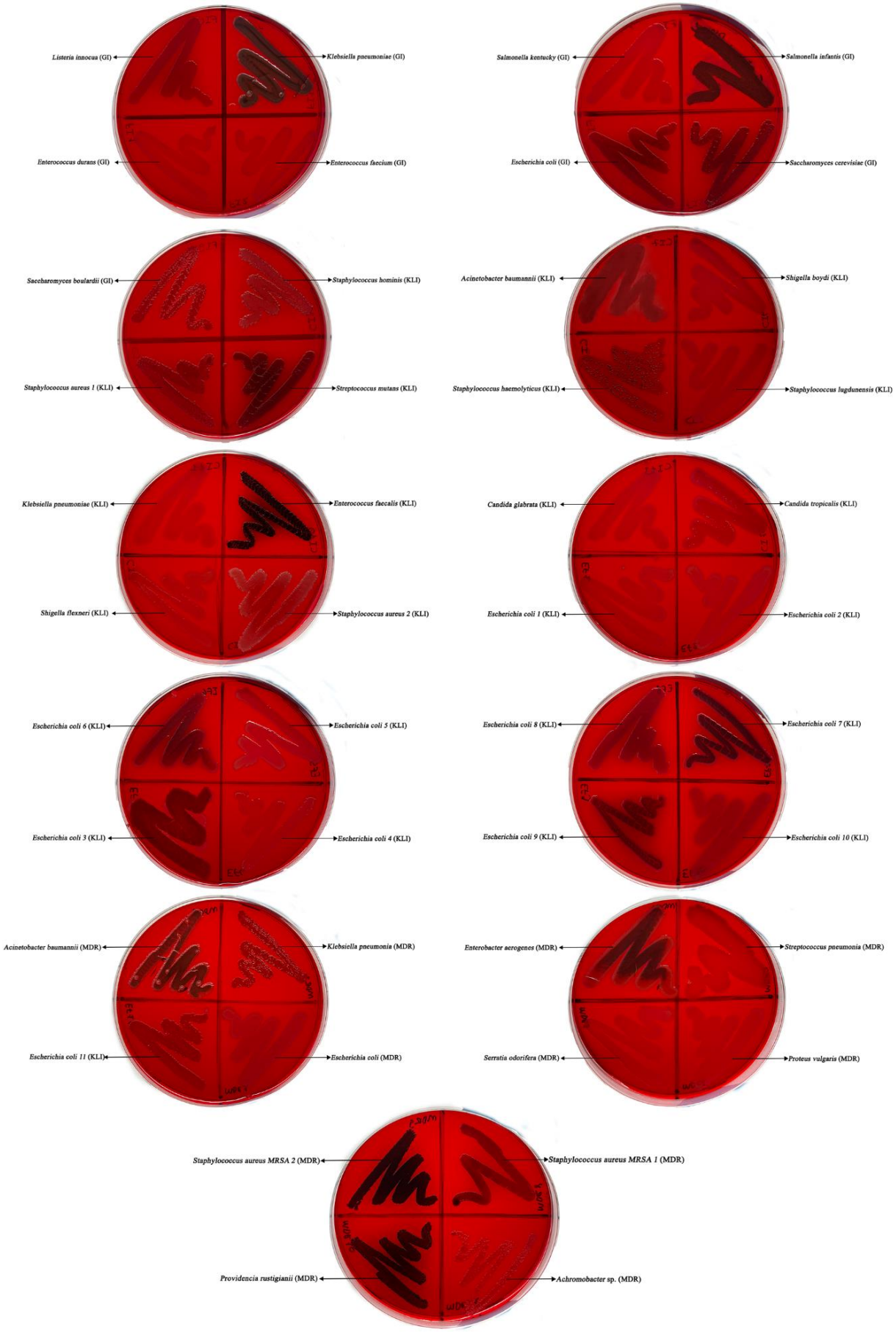

Figure 2. *Results of the Congo Red Agar (CRA) Test on Isolate Strains*

Of the 44 isolate strains analyzed using the CRA method, 28 were found to be biofilm-positive. Among these, 10 strains exhibited weakly positive biofilm formation, 10 strains were positive, and 8 strains showed strong positive biofilm formation. The colony morphology observed during the test and the corresponding results are detailed in Table 2.

Table 2. *Biofilm formation characteristics of isolate strains on Congo Red Agar according to colony color (KLI: Clinical Isolate, GI: Food Isolate and MDR: Multidrug resistant strains. Note: Biofilm intensity levels are indicated as – (negative), + (weak), ++ (moderate), and +++ (strong), based on colony morphology)*

Isolate	Microorganism	Colony Morphology	Biofilm Formation	Intensity
MDR	*Achromobacter* sp.	Dark, crystallized	Weak	+
MDR	*Acinetobacter baumannii*	Black, crystallized	Strong	+++
KLI	*Acinetobacter baumannii*	Dark, partially crystallized	Weak	+
KLI	*Candida glabrata*	Dark red	Negative	-
KLI	*Candida tropicalis*	Dark, partially crystallized	Weak	+
MDR	*Enterobacter aerogenes*	Dark, crystallized	Moderate	++
GI	*Enterococcus durans*	Smooth, pink-red	Negative	-
KLI	*Enterococcus faecalis*	Black, crystallized	Strong	+++
GI	*Enterococcus faecium*	Smooth, pink-red	Negative	-
GI	*Escherichia coli*	Dark, crystallized	Moderate	++
MDR	*Escherichia coli*	Smooth, pink-red	Negative	-
KLI	*Escherichia coli* 1	Dark red	Negative	-
KLI	*Escherichia coli* 2	Dark red	Negative	-
KLI	*Escherichia coli* 3	Dark, partially crystallized	Moderate	++
KLI	*Escherichia coli* 4	Smooth, pink-red	Negative	-
KLI	*Escherichia coli* 5	Smooth, pink-red	Negative	-
KLI	*Escherichia coli* 6	Dark, partially crystallized	Moderate	++
KLI	*Escherichia coli* 7	Dark, crystallized	Strong	+++
KLI	*Escherichia coli* 8	Dark, partially crystallized	Moderate	++
KLI	*Escherichia coli* 9	Black, crystallized	Strong	+++
KLI	*Escherichia coli* 10	Dark, partially crystallized	Moderate	++
KLI	*Escherichia coli* 11	Dark, partially crystallized	Moderate	++
KLI	*Klebsiella pneumoniae*	Smooth, pink-red	Negative	-
GI	*Klebsiella pneumoniae*	Black, crystallized	Strong	+++
MDR	*Klebsiella pneumoniae*	Dark, crystallized	Weak	+
GI	*Listeria innocua*	Dark, partially crystallized	Weak	+
MDR	*Proteus vulgaris*	Smooth, pink-red	Negative	-
MDR	*Providencia rustigianii*	Black, crystallized	Strong	+++
GI	*Saccharomyces boulardii*	Dark, crystallized	Weak	+
GI	*Saccharomyces cerevisiae*	Dark, crystallized	Moderate	++
GI	*Salmonella infantis*	Black, crystallized	Moderate	++
GI	*Salmonella kentucky*	Smooth, Red	Negative	-
MDR	*Serratia odorifera*	Smooth, pink-red	Negative	-
KLI	*Shigella boydi*	Smooth, pink-red	Negative	-
KLI	*Shigella flexneri*	Smooth, pink-red	Negative	-
KLI	*Staphylococcus aureus* 1	Dark, partially crystallized	Weak	+
KLI	*Staphylococcus aureus* 2	Dark, partially crystallized	Weak	+
MDR	*Staphylococcus aureus* MRSA 1	Dark, crystallized	Moderate	++
MDR	*Staphylococcus aureus* MRSA 2	Black, crystallized	Strong	+++
KLI	*Staphylococcus haemolyticus*	Dark, partially crystallized	Weak	+
KLI	*Staphylococcus hominis*	Dark, partially crystallized	Weak	+
KLI	*Staphylococcus lugdunensis*	Dark red	Negative	-
KLI	*Streptococcus mutans*	Black, crystallized	Strong	+++
MDR	*Streptococcus pneumoniae*	Smooth, pink-red	Negative	-

Two isolates of *Acinetobacter baumannii* tested positive. This bacterium, known for its high biofilm formation capacity, plays a significant role in hospital-acquired infections. However, studies using the CRA

method have reported that only a small percentage of isolates (8%) produce black or dark-colored colonies, indicating the method's limited sensitivity. This highlights the need for CRA to be complemented with other methods, such as the microtiter plate assay, for improved detection [26].

Among the yeast strains, *Candida glabrata* tested negative, while *Candida tropicalis* was weakly positive. A study evaluating 120 clinical *Candida* isolates, including non-*C. albicans* species, found that 38.33% were identified as biofilm producers using the CRA method. The variability in detection based on species and biofilm strength underscores the method's limitations [27]. Another study on *Candida* species isolated from diabetic women's urine samples found that only 10% of *C. glabrata* isolates were biofilm-positive using CRA, whereas 96.7% were detected as positive using the tube method [28]. CRA demonstrated lower sensitivity for detecting *Candida* biofilms compared to the tube or microtiter plate methods. For *Candida* species, CRA showed high specificity (99%) but was less effective in detecting weak biofilm producers [29].

A strong biofilm presence was identified in a clinical *E. faecalis* isolate. A comparative study on clinical *E. faecalis* and *E. faecium* isolates found that biofilm production rates were higher in *E. faecalis* (81%) than in *E. faecium* (30%). While CRA demonstrated high sensitivity compared to quantitative methods, it had lower specificity [30]. In urinary tract infection isolates, CRA detected biofilm formation in 76.6% of isolates, but the microtiter plate method provided more accurate results (100%) [31].

For a food-isolated *E. faecium*, the CRA result was negative. In a study on food-derived *E. faecalis* and *E. faecium*, 60% of isolates were weak biofilm producers, and 3.3% were strong producers using CRA [32]. Another study reported that CRA exhibited high sensitivity (100%) but low specificity (6.67%) for detecting *E. faecalis* biofilms, leading to false positives [33]. This emphasizes the variability in biofilm production based on strain and environment, advocating for the combination of CRA with other methods for improved accuracy.

Weak or negative biofilm presence was observed in clinical *Klebsiella pneumoniae* isolates. In contrast, a study on carbapenem-resistant *K. pneumoniae* clinical isolates reported biofilm formation in 65.45% of isolates using CRA [34]. The observed differences could be associated with higher biofilm production in strains with elevated antibiotic resistance. The food-isolated *K. pneumoniae* strain in this study demonstrated strong positive biofilm formation, consistent with Abebe's [35] findings that 73.7% of *K. pneumoniae* isolates from animal-derived foods were strong biofilm producers. These results highlight the potential of CRA in food safety research.

In our study, all four clinical isolates of *S. aureus* tested positive for biofilm production. Knobloch [36] reported that the CRA method detected biofilm production in 55.56% of *S. aureus* isolates; however, its sensitivity was found to be lower compared to the microtiter plate method. Similarly, biofilm production in clinical coagulase-negative staphylococci (CoNS), such as *S. haemolyticus* (57.7%) and *S. lugdunensis* (42.8%), was relatively low [37]. Although these percentages are generally low across staphylococcal species, Mariana [38] demonstrated that using a modified CRA (MCRA) method significantly improved detection stability. This improvement was achieved by enhancing black pigmentation in biofilm-producing strains, suggesting that methodological modifications can enhance the accuracy and reliability of biofilm detection.

A multidrug-resistant *E. aerogenes* isolate tested positive using CRA. However, studies have noted inconsistencies in detecting weak biofilm producers with CRA. Specifically, CRA often fails to distinguish weak biofilm producers from non-producers, highlighting its limitations for strains like *E. aerogenes* [39].

Congo Red binds to curli amyloid fibers and cellulose, which are key components of the extracellular matrix in *E. coli*. Studies have demonstrated that the CRA method effectively detects biofilm formation in pathogenic *E. coli* strains, including Shiga toxin-producing strains. Environmental factors such as elevated temperatures and specific glucose concentrations have been shown to enhance Congo Red staining intensity and promote biofilm development [40, 41]. To interpret CRA results accurately and ensure reproducibility, it is essential to optimize environmental conditions—such as glucose concentration and incubation

temperature—according to the physiological responses of each bacterial species. In our study, among 13 *E. coli* isolates, both positive and negative results were observed, indicating strain-specific differences in Congo Red binding and biofilm production capacities. CRA served as a reliable screening tool for identifying strong biofilm producers. However, combining CRA with quantitative methods such as the microtiter plate assay provided a more comprehensive assessment of biofilm-forming potential. Species-specific characteristics and environmental conditions strongly influenced biofilm detection and production.

The biofilm-positive rate was significantly higher among Gram-positive microorganisms, with 12% classified as weakly positive, 15% as positive, and 8% as strongly positive. In contrast, Gram-negative microorganisms demonstrated a higher biofilm-negative rate (23%), while 10% were weakly positive, 8% were positive, and 4% were strongly positive for biofilm production. Overall, the biofilm-positive rate was markedly higher in Gram-positive strains compared to Gram-negative ones. This difference is likely attributed to the structural characteristics of Gram-positive cell walls, which may provide a greater predisposition for biofilm formation.

3.2. Biofilm Detection Using the Microplate Crystal Violet Method

The CVS method provided a more sensitive and quantitative assessment of biofilm formation. Measurements were conducted considering a 2.5% glucose concentration and various incubation periods (24, 48, and 72 hours). The biofilm formation capacities of different microorganisms were evaluated based on the Cutoff OD values calculated using the results from the negative control wells. The microorganisms included in the study were classified as negative (≤ 0.694), weak (0.694-1.387), moderate (1.387-2.774), and strong (>2.774) biofilm producers. The biofilm formation graph of the reference microorganisms is presented in Figure 3.

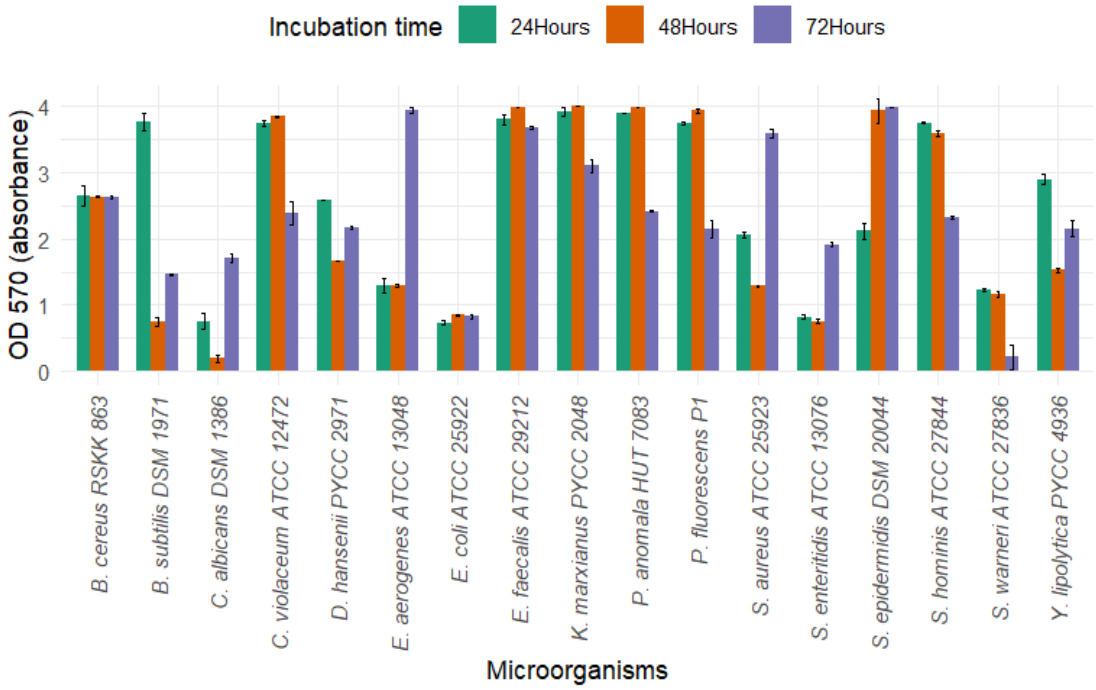

Figure 3. *Quantification of biofilm formation of reference strains using the crystal violet microtiter plate assay (Error bars represent standard deviation (n = 3))*

Among the reference strains, the highest biofilm production was observed in *E. faecalis* ATCC 29212. This strain was classified as a strong biofilm producer, particularly at the 48-hour incubation period. Other strong biofilm producers, *P. fluorescens* P1 and *S. aureus* ATCC 25923, also exhibited strong biofilm formation at the 48-hour mark. *P. fluorescens* has been identified as a bacterium with a strong biofilm-forming capacity in industrial and medical environments [42].

In contrast, species such as *E. coli* ATCC 25922, *S. enteritidis* ATCC 13076, *S. warneri* ATCC 27836, and *E. aerogenes* ATCC 13048 showed consistently low absorbance values across all incubation periods, indicating weak biofilm formation. Detailed OD measurements for all strains are presented in Table 3.

Table 3. *Biofilm formation characteristics of isolate strains on Congo Red Agar according to colony color*

Microorganism	24 Hours	48 Hours	72 Hours
B. subtilis DSM 1971	0.757 ± 0.016	0.748 ± 0.054	1.460 ± 0.109
C. albicans DSM 1386	3.136 ± 0.012	2.197 ± 0.063	1.708 ± 0.013
E. aerogenes ATCC 13048	1.286 ± 0.105	1.296 ± 0.108	3.939 ± 0.012
E. coli ATCC 25922	0.731 ± 0.022	0.845 ± 0.018	0.824 ± 0.047
E. faecalis ATCC 29212	3.801 ± 0.076	3.982 ± 0.001	3.674 ± 0.015
P. fluorescens P1	3.738 ± 0.021	3.963 ± 0.026	2.142 ± 0.127
S. epidermidis DSM 20044	2.113 ± 0.085	3.925 ± 0.006	3.984 ± 0.003
B. cereus RSKK 863	2.639 ± 0.154	2.635 ± 0.177	2.619 ± 0.036
P. anomala HUT 7083	3.893 ± 0.003	3.973 ± 0.001	2.408 ± 0.122
S. aureus ATCC 25923	2.052 ± 0.048	1.282 ± 0.020	3.585 ± 0.069
S. warneri ATCC 27836	1.226 ± 0.014	1.163 ± 0.041	2.220 ± 0.188
S. enteritidis ATCC 13076	0.815 ± 0.002	0.750 ± 0.041	1.912 ± 0.040
S. hominis ATCC 27844	3.744 ± 0.030	3.588 ± 0.122	2.321 ± 0.157
D. hansenii PYCC 2971	2.582 ± 0.007	1.664 ± 0.035	2.162 ± 0.022
K. marxianus PYCC 2948	3.915 ± 0.072	3.996 ± 0.005	3.099 ± 0.068
Y. lipolytica PYCC 4936	2.895 ± 0.083	1.527 ± 0.036	2.154 ± 0.128
C. violaceum ATCC 12472	3.745 ± 0.046	3.844 ± 0.012	2.389 ± 0.179

One of the most consistent findings in our study was the strong biofilm production exhibited by *E. faecalis* ATCC 29212. This bacterium was classified as a strong biofilm producer after a 48-hour incubation period. This finding is consistent with reports in the literature and highlights *E. faecalis*'s capacity to produce robust biofilms, particularly in glucose-rich environments [20, 30]. Similarly, other strong biofilm producers, such as *P. fluorescens* and *S. aureus* ATCC 25923, also demonstrated significant biofilm production, confirming the potential of these species for biofilm formation in various environments [32, 36].

The sensitivity of the method is particularly notable in distinguishing weak and moderate biofilm producers. For example, weak biofilm producers such as *E. coli* ATCC 25922 were consistent with this classification, showing low absorbance values, which validates their limited biofilm production capacity [21]. This level of sensitivity is crucial for understanding the variability of biofilm production among bacterial species, which may have significant implications for clinical infection management, especially for chronic infections and multidrug-resistant (MDR) strains.

Among the clinical isolates, the highest biofilm production was observed in *S. aureus* 2 (KLI) and *E. faecalis* (KLI). These strains were classified as strong biofilm producers, particularly at the 48-hour incubation period, due to their high absorbance values. *S. aureus* 2 (KLI) achieved the highest biofilm production at the 48-hour mark. Similarly, *E. faecalis* (KLI) exhibited high biofilm production, reaching at 48 hours. In contrast, some clinical isolates, especially *E. coli* 9 (KLI) and *S. hominis* (KLI), were found to have low biofilm production capacities. In these species, OD_{570} values were generally below 1. Detailed OD measurements for all strains are presented in Table 4 and visualized in Figure 4.

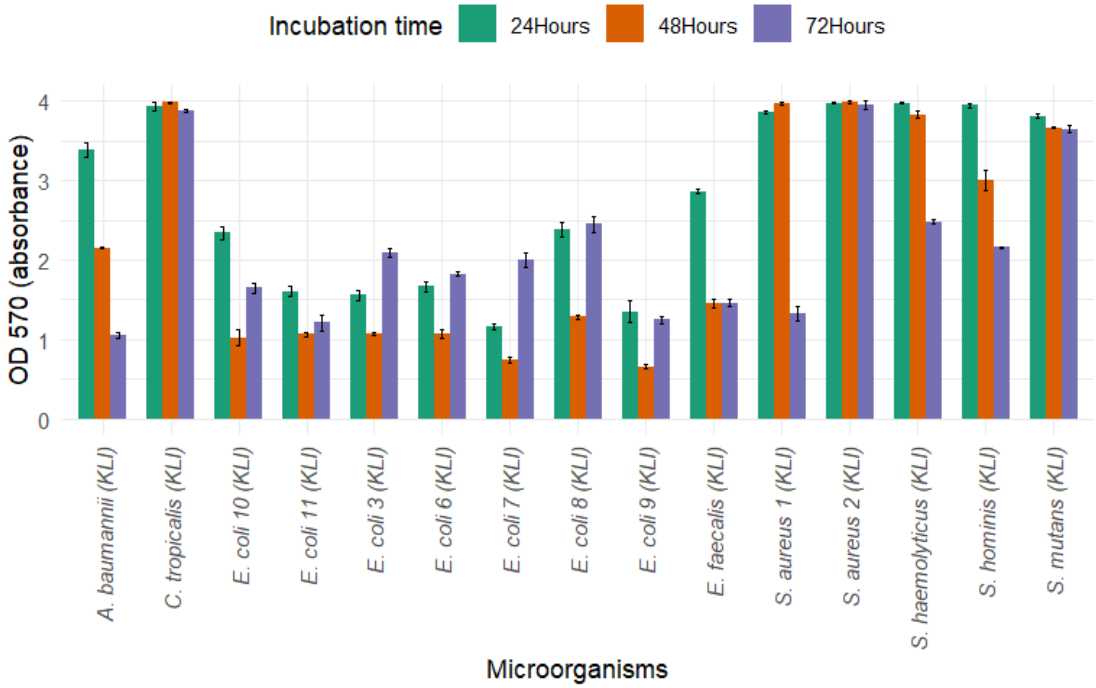

Figure 4. *Quantification of biofilm formation of clinical isolates using the crystal violet microtiter plate assay (Error bars represent standard deviation (n = 3))*

Table 4. *Biofilm formation characteristics of isolate strains on Congo Red Agar according to colony color (KLI: Clinical Isolate strains)*

Microorganism	24 Hours	48 Hours	72 Hours
S. aureus 2 (KLI)	3.979 ± 0.014	3.993 ± 0.001	3.950 ± 0.053
S. mutans (KLI)	3.815 ± 0.023	3.666 ± 0.053	3.652 ± 0.095
A. baumannii (KLI)	3.393 ± 0.090	2.155 ± 0.017	1.051 ± 0.039
S. haemolyticus (KLI)	3.979 ± 0.014	3.829 ± 0.046	2.480 ± 0.026
E. coli 3 (KLI)	1.557 ± 0.067	1.069 ± 0.021	2.095 ± 0.057
E. coli 6 (KLI)	1.668 ± 0.054	1.661 ± 0.065	1.826 ± 0.013
E. coli 7 (KLI)	1.157 ± 0.024	0.746 ± 0.034	1.999 ± 0.089
E. coli 8 (KLI)	2.390 ± 0.041	1.280 ± 0.032	2.452 ± 0.097
E. coli 9 (KLI)	1.331 ± 0.088	0.654 ± 0.028	1.251 ± 0.040
E. coli 10 (KLI)	2.339 ± 0.132	2.024 ± 0.104	1.645 ± 0.060
E. coli 11 (KLI)	1.604 ± 0.066	1.069 ± 0.027	1.208 ± 0.100
S. aureus 1 (KLI)	3.856 ± 0.020	3.979 ± 0.019	1.319 ± 0.094
S. hominis (KLI)	3.953 ± 0.027	3.011 ± 0.125	2.161 ± 0.011
S. haemolyticus (KLI)	3.979 ± 0.014	3.829 ± 0.046	2.480 ± 0.026
E. faecalis (KLI)	2.865 ± 0.031	1.457 ± 0.051	1.461 ± 0.048
C. tropicalis (KLI)	3.936 ± 0.049	3.982 ± 0.007	3.882 ± 0.014

It was observed that incubation time had a significant effect on biofilm formation. During the 24-hour incubation period, *C. tropicalis* (KLI) showed high biofilm production. At 48 hours, strains such as *S. aureus* 2 (KLI) and *E. faecalis* (KLI) exhibited the highest biofilm production. These findings indicate that there are substantial differences in the biofilm production capacities of clinical isolates, and that incubation time is an important factor influencing biofilm formation.

S. aureus strains, particularly MRSA strains, are generally considered moderate to strong biofilm producers. Many studies in the literature suggest that the biofilm production capacity of this species contributes to increased clinical infections and treatment resistance [36]. In this context, our isolates showing weak biofilm production may exhibit lower virulence or may have been influenced by environmental factors. E. faecalis, a pathogen commonly encountered in urinary tract infections, is known for its strong biofilm production. Studies using crystal violet staining have assessed *E. faecalis* biofilm formation, and it has been shown that all tested *E. faecalis* isolates were biofilm-positive. Nitrofurazone-impregnated catheters have been effective in reducing biofilm formation, highlighting the importance of biofilm formation in *E. faecalis* infections [43].

One study evaluated the impact of different culture media on biofilm formation by *E. faecalis, E. coli*, and *S. aureus*. It demonstrated that culture conditions such as oxygen levels and incubation time significantly affect biofilm production, confirming the findings of our study regarding the impact of incubation time on biofilm formation [44].

Among the multidrug-resistant (MDR) isolates, the highest biofilm production was observed in *Achromobacter* sp. and *K. pneumoniae* (MDR). These strains were classified as strong biofilm producers, particularly during the 24-hour incubation period, due to their high OD_{570} values. *Achromobacte*r sp. achieved the highest biofilm production with a high value at 24 hours. *K. pneumoniae* (MDR) reached a high value at 48 hours. In contrast, some MDR isolates, particularly *E. aerogenes* (MDR) and *S. aureus* MRSA 1 (MDR), exhibited lower biofilm production capacities. In these species, OD_{570} values were generally below 2.000. Detailed OD measurements for all strains are presented in Table 5 and visualized in Figure 5.

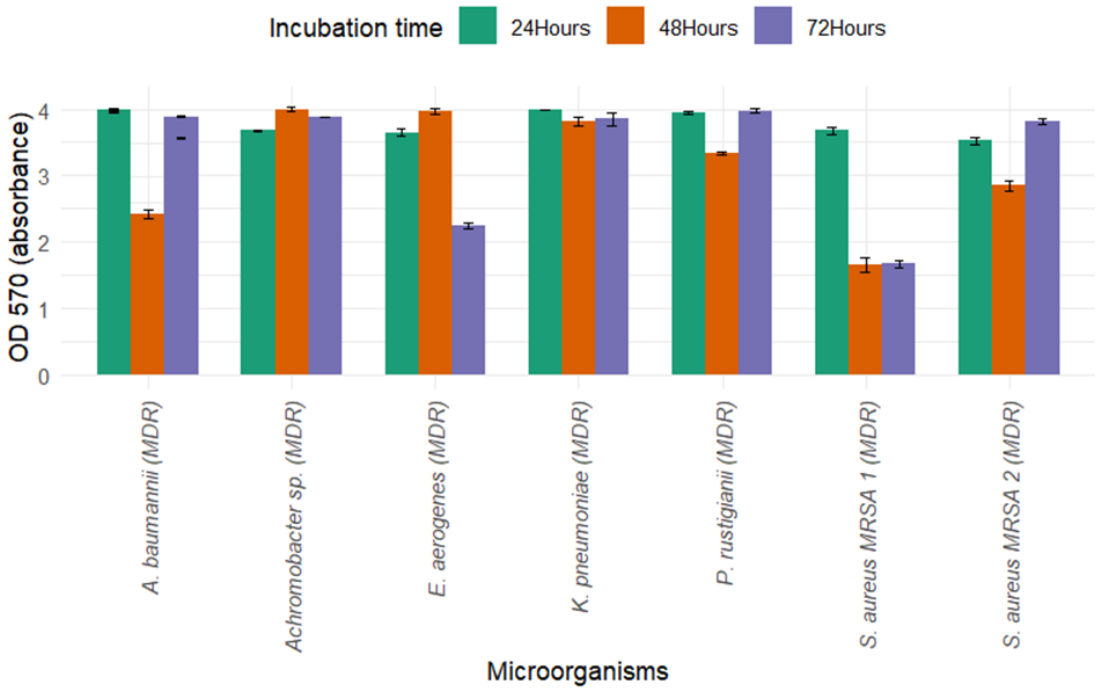

Figure 5. *Quantification of biofilm formation of multi-drug resistance strains using the crystal violet microtiter plate assay (Error bars represent standard deviation (n = 3))*

Table 5. *Biofilm formation characteristics of isolate strains on Congo Red Agar according to colony color (MDR: Multidrug resistant strains)*

Microorganism	24 Hours	48 Hours	72 Hours
S. aureus MRSA 2 (MDR)	3.523 ± 0.057	2.851 ± 0.081	3.819 ± 0.039
K. pneumoniae (MDR)	3.995 ± 0.005	3.821 ± 0.068	3.850 ± 0.096
A. baumannii (MDR)	3.961 ± 0.018	2.431 ± 0.136	3.565 ± 0.008
E. aerogenes (MDR)	3.647 ± 0.054	3.968 ± 0.041	2.252 ± 0.045
S. aureus MRSA 1 (MDR)	3.675 ± 0.060	1.658 ± 0.112	1.675 ± 0.056
P. rustigianii (MDR)	3.949 ± 0.014	3.337 ± 0.013	3.978 ± 0.039
Achromobacter sp. (MDR)	3.999 ± 0.001	3.997 ± 0.003	3.885 ± 0.008

Multidrug-resistant (MDR) isolates have shown significant variations in their biofilm production capacities. Notably, *Achromobacter* sp. and *K. pneumoniae* (MDR) isolates were classified as strong biofilm producers. These strains exhibited high absorbance values after a 24-hour incubation period, and their biofilm production capacities remained at elevated levels, correlating with antibiotic resistance. Furthermore, studies utilizing crystal violet staining have shown that MDR isolates exhibit stronger biofilm formation compared to non-MDR isolates. Despite the presence of high resistance levels, the biofilm biomass in MDR strains is considerably greater [45,46].

Among the foodborne isolates, the highest biofilm production was observed in *K. pneumoniae* (GI). This strain was classified as a strong biofilm producer, particularly at the 48-hour incubation period, due to its high absorbance value. In contrast, some food isolates, particularly *E. faecium* (GI) and *L. innocua* (GI), exhibited lower biofilm production capacities. In these species, absorbance values were generally below 1.000. Detailed OD measurements for all strains are presented in Table 6 and visualized in Figure 6.

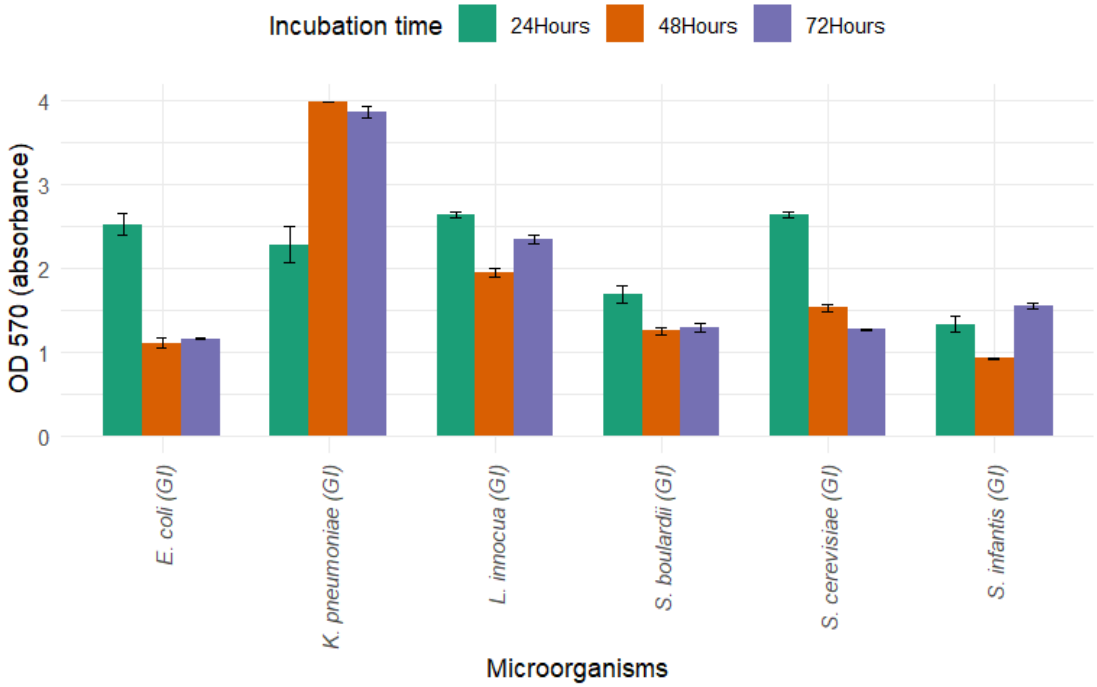

Figure 6. *Quantification of biofilm formation of foodborne isolates using the crystal violet microtiter plate assay (Error bars represent standard deviation (n = 3))*

Table 6. *Biofilm formation characteristics of isolate strains on Congo Red Agar according to colony color (GI: Food Isolate strains)*

Microorganism	24 Hours	48 Hours	72 Hours
K. pneumoniae (GI)	2.287 ± 0.210	3.998 ± 0.001	3.869 ± 0.063
L. innocua (GI)	2.640 ± 0.038	1.947 ± 0.050	2.356 ± 0.054
S. infantis (GI)	1.336 ± 0.023	0.925 ± 0.010	1.559 ± 0.037
S. boulardii (GI)	1.689 ± 0.100	1.256 ± 0.043	1.296 ± 0.046
E. coli (GI)	2.529 ± 0.136	1.110 ± 0.060	1.162 ± 0.008
S. cerevisiae (GI)	2.644 ± 0.041	1.535 ± 0.041	1.276 ± 0.008

The effect of incubation time on biofilm formation was evident. During the 24-hour incubation period, *S. cerevisiae* (GI) showed high biofilm production. At 48 hours, *K. pneumoniae* (GI) reached its peak biofilm production. After 72 hours of incubation, species such as *S. boulardii* (GI) and *S. infantis* (GI) also exhibited biofilm production, demonstrating that the biofilm production capacity of foodborne isolates increases over time. In conclusion, the biofilm production capacities of foodborne isolates vary by species, and the incubation time has a significant impact on this capacity.

Pathogenic *L. monocytogenes*, *S. aureus*, *E. coli*, and *B. cereus* strains isolated from retail foods in Poland were tested for biofilm formation using the crystal violet assay. The results showed that both incubation time and medium composition significantly affected biofilm formation, with *B. cereus* demonstrating the strongest biofilm production after 48 hours. This study provides valuable insights into biofilm formation in foodborne pathogens, contributing to food safety knowledge [47]. Also, *E. coli* isolates from vegetables were tested for biofilm formation at different temperatures using the crystal violet assay, revealing significantly higher biofilm production at 37°C and 25°C, and showing a notable temperature-dependent variation in biofilm production among the strains. This suggests that *E. coli* biofilms in food environments may be temperature dependent [48]. Consequently, the biofilm production capacities of food isolates are an important factor to consider for both food safety and public health.

The effect of incubation time on biofilm formation was found to be significant. At the 24-hour incubation period, the highest biofilm formation was observed in *C. albicans* DSM 1386. During the 48-hour period, the highest biofilm production was observed in *K. pneumoniae* (GI) and *S. aureus* 2 (KLI) isolates. After 72 hours of incubation, the strongest biofilm formation occurred in *E. aerogenes* ATCC 13048 and *S. aureus* ATCC 25923.

Among Gram-positive microorganisms, the biofilm-positive rate was more pronounced. When evaluated based on biofilm formation capacity, approximately 40% of the microorganisms tested in this study were classified as strong biofilm producers, 35% as moderate, and 25% as weak biofilm producers. The results revealed significant differences in biofilm production capacity depending on the microorganism species, and incubation time showed variable effects on biofilm formation. These findings provide crucial data for a better understanding of the role of biofilm formation in microbial pathogenesis, particularly in infection control, biotechnological applications, and antibiotic resistance.

Additionally, the microplate crystal violet method offers advantages in terms of experimental efficiency. Its ability to analyze multiple strains simultaneously under standardized conditions provides reproducibility and consistency, which is a significant advantage in cross-study comparisons. This feature greatly aids researchers in large-scale studies or when tracking changes in the biofilm production capacities of clinical isolates over time [24]. Additionally, the CVS method offers flexibility in experimental design, allowing for the exploration of factors such as incubation time and nutrient availability, and their effects on biofilm formation.

Although the CVS method is a robust technique in terms of sensitivity and providing quantitative data, it has some limitations, particularly when considering the variability in biofilm production between bacterial species and strains. In our study, some strains exhibited distinctly different biofilm production capacities

under the same conditions, indicating that biofilm dynamics can vary strain-specifically and that environmental factors may influence this production. Furthermore, the reproducibility and sensitivity of the CVS method make it an ideal candidate for investigating the effects of environmental factors (such as pH, temperature, and nutrient availability) on biofilm formation [1].

In conclusion, the microplate crystal violet method remains one of the most effective and versatile techniques for biofilm detection. Its quantitative nature and high sensitivity make it an indispensable tool in clinical and research settings, particularly for studying biofilm formation in pathogenic organisms and multidrug-resistant (MDR) strains. However, to better detect weaker biofilm producers and to understand the complex dynamics of biofilm formation, it is recommended that this method be combined with other techniques.

3.3. Comparison of CRA and CVS Methods

This study compared the qualitative CRA method with the quantitative CVS microplate assay to evaluate the biofilm-forming capacities of 68 microbial strains. CRA detected biofilm positivity in 66.18% of tested strains, while CVS revealed greater sensitivity, especially for weak and moderate biofilm producers.

Discrepancies between CRA and CVS results were also noted for *Enterobacter aerogenes* (MDR). While CRA classified it as a moderate biofilm producer, CVS revealed strong biofilm formation at 48 and 72 hours (OD > 3.9). This reinforces the known challenge of detecting weak or intermediate-level biofilm producers with CRA and underlines the necessity of combining qualitative and quantitative methods.

In addition, some isolates in this study demonstrated weak or negative biofilm activity, even though previous literature has identified them as biofilm producers. These discrepancies can be explained by multiple factors. First, strain-level genetic variation can lead to significant differences in biofilm-regulating genes and pathways, even within the same species. Second, environmental conditions—including nutrient composition, glucose concentration, pH, temperature, and oxygen availability—are known to influence biofilm development. Third, methodological differences between studies (e.g., CRA vs. tube or flow-cell methods) impact detection sensitivity. Moreover, phenotypic plasticity enables bacteria to modulate their biofilm-forming behavior in response to environmental cues, meaning that some strains may not express biofilm-associated traits under standardized laboratory conditions. These differences highlight the need for environmental optimization and method integration when evaluating biofilm formation.

Environmental conditions, including temperature, incubation duration, and glucose supplementation, significantly influenced biofilm formation. In our study, 2.5% glucose was used based on prior research [17] and optimized to induce robust biofilm formation. However, species-specific responses to glucose concentration suggest that optimization studies tailored to each strain are essential for accurate biofilm profiling.

Although *E. coli* ATCC 25922 was positive in both assays, several *E. coli* clinical isolates showed weak or negative CRA results despite being reported as biofilm producers in other studies. These inconsistencies can be attributed to strain-level genetic variability, stress response differences, and methodological sensitivity. Similar discrepancies were seen in Candida glabrata, which tested negative using CRA but has previously demonstrated biofilm activity under alternative conditions such as the tube method [28].

Statistical analyses revealed a significant difference in biofilm formation between Gram-positive and Gram-negative strains at 24, 48, and 72 hours of incubation ($p < 0.001$), with Gram-positive strains exhibiting higher levels of biofilm production. consistent with their structural characteristics, such as a thick peptidoglycan layer that facilitates adhesion and EPS retention. In contrast, Gram-negative strains demonstrated a higher rate of biofilm negativity, which may be due to variations in outer membrane composition and lower affinity for Congo Red.

Regarding the MDR (multidrug resistance) factor, no statistically significant differences were observed between MDR and non-MDR strains at 24 hours ($p = 0.592$), 48 hours ($p = 0.071$), or 72 hours ($p = 0.414$)

of incubation. For the source factor, a significant difference in biofilm formation was found between clinical and food isolates at all time points—24, 48, and 72 hours ($p < 0.001$)—with clinical isolates demonstrating significantly greater biofilm production. Similarly, reference strains also showed a significant difference in biofilm formation between clinical and food origins at 24, 48, and 72 hours of incubation ($p < 0.001$), with both groups exhibiting elevated biofilm-forming capacity.

Quantitative analysis via CVS revealed incubation time as a critical factor. Biofilm production peaked at 48 or 72 hours for several strong producers, such as *E. faecalis*, *K. pneumoniae*, and *S. aureus* (MRSA). This aligns with previous literature indicating that biofilm maturation and EPS accumulation intensify over extended incubation periods [43, 44].

Statistical analysis confirmed significant differences in biofilm production between groups ($p < 0.05$), and strong correlations between methods were supported by a high Spearman correlation ($r = 0.95$) and Kappa agreement value of 0.86. These values reflect a strong consistency in overall biofilm detection but highlight that CVS is more precise in distinguishing between intermediate production levels.

This study has several limitations. Firstly, all assays were conducted under static, in vitro conditions, which may not fully represent in vivo biofilm behavior. Secondly, strain-level genotypic differences were not analyzed; thus, regulatory gene expression influencing biofilm formation remains unexplored. Additionally, CRA's dependence on colony morphology introduces subjectivity, especially in borderline cases. These factors may limit the broader generalizability of the results, especially to complex clinical or environmental settings.

While previous studies have compared CRA and CVS biofilm detection methods, our study introduces key novel elements that enhance its scientific value. First, the inclusion of a comprehensive set of 68 microbial strains—including standard strains, multidrug-resistant (MDR) isolates, clinical pathogens, and foodborne isolates—provides a broad-spectrum analysis not commonly addressed in earlier works. This study uniquely applies both methods under a uniformly optimized glucose concentration (2.5%), and biofilm formation was assessed across multiple incubation periods (24, 48, and 72 hours), offering dynamic insights into biofilm maturation. Statistical analysis using one-way ANOVA and post-hoc evaluation revealed significant differences ($p < 0.05$) in biofilm formation capacity among Gram-positive vs. Gram-negative strains, MDR vs. non-MDR isolates, and clinical vs. food-derived strains—an aspect often lacking in prior reports. Finally, the direct correlation ($r = 0.95$) and agreement (Kappa = 0.86) between CRA and CVS methods, validated across these diverse microbial groups, strengthen the reliability and clinical relevance of our findings. Together, these points distinguish this work as a valuable contribution to biofilm diagnostics and comparative phenotypic methodology.

4. CONCLUSION

This study demonstrates that both CRA and CVS methods offer valuable yet distinct advantages for biofilm detection. CRA, a rapid and low-cost phenotypic assay, effectively identified strong biofilm producers based on colony morphology. However, its limited sensitivity, particularly for weak producers and certain Gram-negative strains, reduces its standalone reliability. In contrast, the CVS method enabled precise, reproducible quantification of biofilm biomass and was especially effective in detecting moderate and weak biofilm producers.

The findings support the use of a hybrid approach, combining CRA for initial screening with CVS for confirmatory and quantitative evaluation. This strategy allows for a more comprehensive interpretation of biofilm-forming potential across a diverse array of strains, including multidrug-resistant clinical isolates and foodborne pathogens.

Our comparative analyses further revealed significant strain-level differences in biofilm formation. Gram-positive bacteria exhibited significantly greater biofilm production than Gram-negative strains. No statistically significant difference was observed between MDR and non-MDR strains. Clinical isolates demonstrated higher biofilm formation compared to foodborne isolates. Moreover, both clinical and food

isolates formed significantly more biofilm than reference strains across all incubation periods. These findings underscore the importance of considering microbial taxonomy, resistance profiles, and source origin in biofilm-related assessments.

Based on our findings, CRA is more appropriate for quick, high-throughput screening of strong biofilm producers, especially in resource-limited environments. In contrast, CVS is recommended for detailed comparative studies, clinical assessments, or research requiring sensitivity and quantification.

Beyond biofilm detection, both methods have potential applications in antimicrobial screening, medical device testing, and environmental monitoring, where biofilm formation is a critical factor.

Future studies should incorporate gene expression analysis and flow-cell systems to simulate dynamic conditions and provide deeper insights into the molecular regulation of biofilm development. Additionally, standardized optimization of assay conditions—such as nutrient composition, glucose concentration, and incubation duration—will enhance the reproducibility and comparability of results across laboratories.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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