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A Guideline for Assessment and Characterization of Bacterial Biofilm Formation in the Presence of Inhibitory Compounds

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Abstract

Campylobacter jejuni, a zoonotic foodborne pathogen, is the worldwide leading cause of acute human bacterial gastroenteritis. Biofilms are a significant reservoir for survival and transmission of this pathogen, contributing to its overall antimicrobial resistance. Natural compounds such as essential oils, phytochemicals, polyphenolic extracts, and D-amino acids have been shown to have the potential to control biofilms formed by bacteria, including *Campylobacter* spp. This work presents a proposed guideline for assessing and characterizing bacterial biofilm formation in the presence of naturally occurring inhibitory molecules using *C. jejuni* as a model. The following protocols describe: i) biofilm formation inhibition assay, designed to assess the ability of naturally occurring molecules to inhibit the formation of biofilms; ii) biofilm dispersal assay, to assess the ability of naturally occurring inhibitory molecules to eradicate established biofilms; iii) confocal laser scanning microscopy (CLSM), to evaluate bacterial viability in biofilms after treatment with naturally occurring inhibitory molecules and to study the structured appearance (or architecture) of biofilm before and after treatment.

Keywords: Biofilm assay, Biofilm method, Microtiter plate assay, Antibiofilm compounds, Natural compounds, D-amino acids, Biofilm of *Campylobacter*

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Background

Biofilm formation is considered to be important for the survival and transmission of bacterial pathogens to humans where they are able to cause disease [1]. Inhibition of bacterial biofilms by naturally occurring compounds such as polyphenols, essential oils, and D-amino acids [i.e., serine (D-Ser)] has been previously investigated [2–6] using several methods, such as spectrophotometric or fluorescence-based methods, to quantify total formed biofilms [7–9]. The most common system used to assess biofilm formation is a spectrophotometer, as it does not require overly specialized or expensive equipment, a great quantity of analytical reagents, or a high level of user expertise. This protocol presents a proposed guideline for consistent and reproducible analysis of biofilm formation in the presence of naturally occurring inhibitory compounds using *Campylobacter jejuni* as a model organism.
C. jejuni presents itself as a suitable model for this method as it is an opportunistic pathogen widely believed to be responsible for most cases of bacterial gastroenteritis worldwide. *C. jejuni* is a common commensal bacterium of poultry, especially chickens [10, 11], and has been reported to be able to form mono-species biofilms and to integrate into composite biofilms with other bacterial species, such as *Pseudomonas aeruginosa* [8, 12–18]. The ability of *Campylobacter* to form biofilms plays a critical role in its survival in the environment as well as in the dissemination of infection and the emergence of antibiotic resistance [19–23].

Materials and reagents

Materials

1. Media: Mueller-Hinton agar/broth (MHA/MHB) (Thermo Fisher Scientific, catalog number: CM0337) and Luria-Bertani broth (LB) (Oxoid, catalog number: CM0996B)
2. Antibiotic stock: Trimethoprim (2.5 µg/mL) (Sigma-Aldrich, catalog number: T7883-5G) and Vancomycin (10 µg/mL (Sigma-Aldrich, catalog number: 1709007)
3. Strains: *Campylobacter jejuni* NCTC 11168-O [24] and *Pseudomonas aeruginosa* PA0-1
   Culture conditions: cultures of *C. jejuni* are grown microaerobically (85% N_2, 10% CO_2, and 5% O_2) at 42 °C for 36 h while *Pseudomonas aeruginosa* is grown aerobically at 37 °C for 24 h
4. Chemicals: natural compounds that have high anti-biofilm properties such as D-serine (D-Ser) (Sigma-Aldrich, catalog number: S4250)
5. Phosphate-buffered saline (PBS, pH 7.4) (Sigma-Aldrich, catalog number: P4417)
6. Multi-channel pipette (e.g., 100–200 µL, 300–1,000 µL) (Thermo Scientific, catalog number: 4661180N)
7. 15 mL conical tubes or glass test tubes for growing liquid cultures (Thermo Scientific, catalog number: 339650)
8. 24- or 96-well clear flat-bottom plates (Geiner Bio-One, catalog number: 655101)
9. Tray or box slightly larger than a 96-well plate (e.g., tray dimension ~200 mm × 100 mm)
10. 0.1% Crystal violet solution (0.1 g of Crystal violet in 100 mL demineralized water) (Sigma-Aldrich, catalog number: 548-62-9)
11. Modified biofilm dissolving solution (MBDS) [sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: 436143) dissolved to a final concentration of 10% with 80% Ethanol in H_2O) [25]
12. Paper towels
13. Large beaker
14. For confocal laser scanning microscopy (CLSM):
    a. 6-well polystyrene clear flat-bottom plates (Geiner Bio-One, catalog number: 655101)
    b. Glass coverslips (e.g., 22 mm^2 coverslips)
    c. Glass slides
    d. 5% formaldehyde solution (in distilled H_2O)
    e. 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, catalog number: D8417)
    f. Mounting medium (Ibidi GmbH, catalog number: 50001)
    g. Nail varnish
    h. CLSM (Nikon Microscopy, model: Nikon A1R+)

Equipment

1. Plate reader (Tecan, model: Infinite M200 Pro) for measuring optical density (OD) range at 570–600 nm
2. Incubator (SHEL LAB, model: SM12)
3. Laminar flow cabinet (ESCO, model: AHL-4A2)

Software

1. ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA)

Procedure

Protocol 1: Biofilm formation inhibition assay (Figure 1)

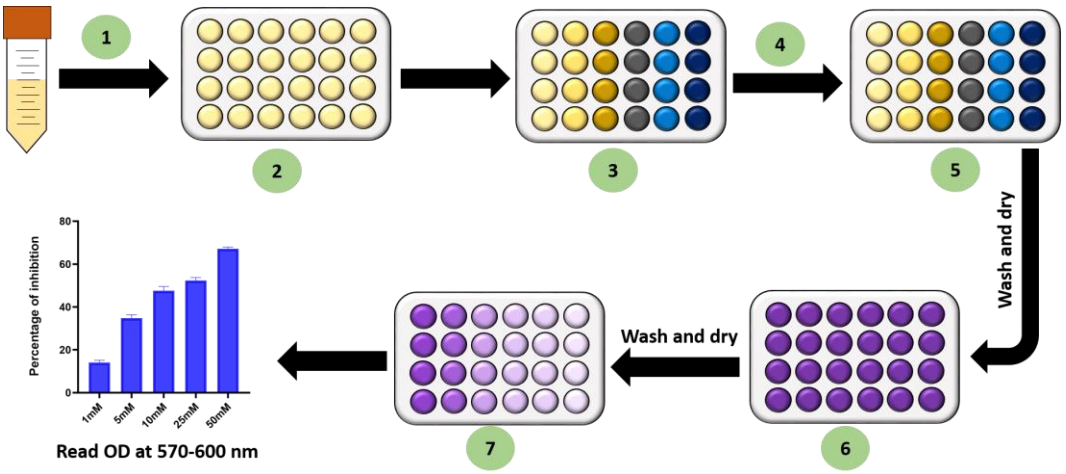


Figure 1. Protocol 1 for screening biofilm formation inhibition. (1–2) Prepare the bacterial suspension and dispense it into a 24-well microplate. (3) Dispense different concentrations of the tested compound (D-Ser) in desired increments into the microplate. (4) Incubate the microplate to generate the biofilm inside the wells. (5) After incubation, the wells are washed, allowed to dry, and then stained with crystal violet solution (6). The stained biofilms are washed to remove excess dye and a volume of MBDS solution is added to the wells to dissolve the crystal violet stained biofilm. (7) The absorbance (OD) of the de-stained MBDS solution is read on a spectrophotometer.

A. Single-species assay

Note: To investigate the effect of a compound to be tested on biofilm-formed C. jejuni.

1. Recover *C. jejuni* NCTC 11168-O from -80 °C storage, plate the stock on MHA media supplemented with appropriate antibiotics [Trimethoprim (2.5 µg/mL) and Vancomycin (10 µg/mL)], and incubate microaerobically (85% N_2, 10% CO_2, and 5% O_2) at 42 °C overnight.
2. Harvest bacterial cells from the agar plates into 1 mL of MHB using a glass or a disposable plastic rod to lift the cells off the agar plates into a test tube, and transfer ~100–200 µL of the resulting bacterial suspension into 15 mL of MHB (supplemented with appropriate antibiotics). Incubate microaerobically at 42 °C and 125 rpm in a shaker incubator (18–20 h overnight).

3. Dilute overnight culture in fresh MHB (supplemented with appropriate antibiotics) to achieve cell density of OD_{600} of 0.05 at the start of the logarithmic growth phase (~10^7 CFU/mL).
4. Dispense 2 mL of diluted bacterial suspension into each well of a 24-well plate (minimum of three rows of four wells to each sample) or 180 µL per well for 96-well plates. Uninoculated MHB is used as a negative control.
5. To test the inhibitory effect of the tested compound (e.g., D-Ser), add the chosen concentrations (i.e., 1–50 mM) of the compounds to be tested directly to the culture in the wells. For example, 1 M of D-Ser (in PBS) was used as stock solution and 100 µL of stock solution was added to each well, except for negative control.
6. Cover the 24-well plates and incubate them at 42 °C under microaerophilic conditions without shaking (static culture) for 24 h (see Notes 1 and 2). Each plate must include medium-only control in four wells.
7. To quantify biofilm formation, go to section "Assessment of biofilm formation" from Protocol 2.

B. Dual/multiple species assay

Note: To investigate the effect of a compound to be tested on biofilms formed by a mixed culture (in this example: C. jejuni and P. aeruginosa).

1. Grow *P. aeruginosa* cells aerobically in MHB overnight at 37 °C and then adjust the cell density with fresh MHB to OD_{600} of 0.1, at the start of the logarithmic growth phase (10^7 CFU/mL) [26].
2. Grow *C. jejuni* cells as described above (steps A1–A3).
3. Use a ratio of 1:1 of the cell suspensions for both/multiple bacteria for the assay.
4. Repeat steps A4–A6 as described above (see Notes 1 and 2).
5. To quantify biofilm formation, go to section "Assessment of biofilm formation" from Protocol 2.

Protocol 2: Biofilm dispersal assay (Figure 2)

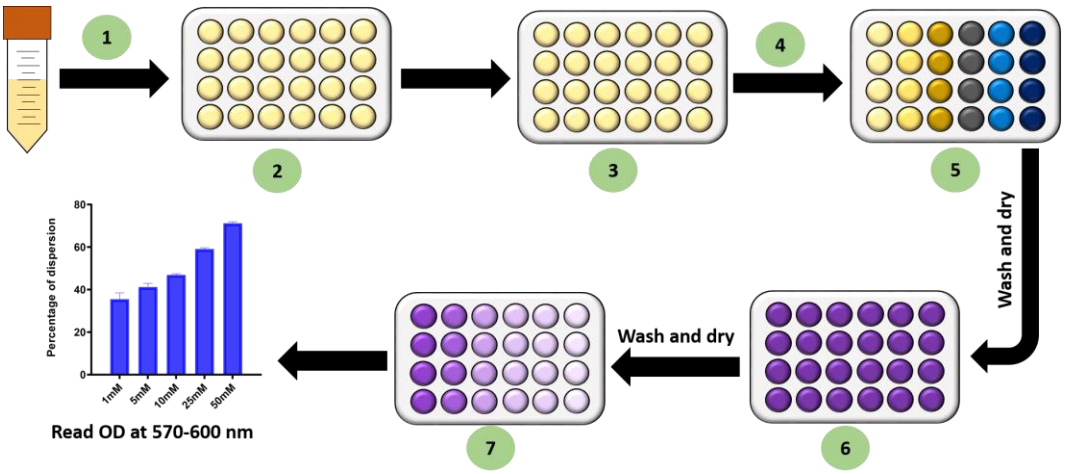


Figure 2. Protocol 2 for screening biofilm formation dispersal. (1–2) Prepare the bacterial suspension and dispense it into a 24-well microplate. (3) Incubate the microplate to generate the biofilm inside the wells. (4) The bacterial culture is then removed, and the wells are washed to remove planktonic cells before dispensing different concentrations of the tested compound (D-Ser) in desired increments into the microtiter plate. (5) After incubation, the wells are washed, allowed to dry, and then stained with crystal violet solution. (6) The stained biofilms are washed to remove excess dye and MBDS solution is added to the wells to dissolve the crystal violet stained biofilm. (7) The absorbance (OD) of the de-stained MBDS solution is read on a spectrophotometer.

1. Repeat steps A1–A6, except step 5.
2. Remove the media from the 24-well plate and add 500 µL of PBS with an appropriate concentration (i.e., 10–50 mM) of the compound to be tested in each well (PBS-only is used as negative control).

3. Incubate the plates at 42 °C under microaerobic conditions without shaking for 24 h (see Notes 1 and 2).
4. Take the supernatants and measure the OD_{600} for each well.
5. To quantify biofilm formation, go to section "Assessment of biofilm formation."

Assessment of biofilm formation

1. Remove the media from plates (by inverting over an absorbent paper towel in a tray) and rinse gently with distilled water twice to remove planktonic cells.
2. Dry plates by gently tapping on a paper towel until no liquid remains in the wells.
3. Additionally, air-dry the plates for 15 min in a laminar flow cabinet.
4. Stain the attached biofilm material by adding 300 µL of 0.1% crystal violet solution (125 µL for a 96-well plate) to each well and let stand for 10 min at room temperature.
5. Remove the crystal violet solution by pipetting out and rinse out unbound crystal violet with distilled water until all wells are free of liquid crystal violet (see Note 6).
6. Tap the plates over the paper towel and leave them face up on the bench overnight at room temperature to dry or air-dry for 15 min in laminar flow cabinet.
7. Add 500 µL (or 200 µL for a 96-well plate) of MBDS to each well to solubilize the crystal violet and incubate for 10 min at room temperature.
8. Mix the MBDS and crystal violet in the wells by pipetting up and down.
9. Transfer 125–200 µL of the MBDS/crystal violet solution from each well into a corresponding well of a flat-bottomed 96-well plate.
10. Quantify OD (570–600 nm) of each well in a plate reader in the flat-bottomed plate.
11. Subtract the measurement for blank wells (MBDS only) from the OD of each well that contained a sample/concentration and calculate the average.
12. Normalize the average to the percentage of biofilm inhibition and dispersion (%) as described in [6, 27, 28]:

    $$\% = (Con - Exp)/Con) \times 100$$

    Where Con = Control (Untreated) and Exp = Experimental (Treated).

CLSM microscopy (Figure 3)

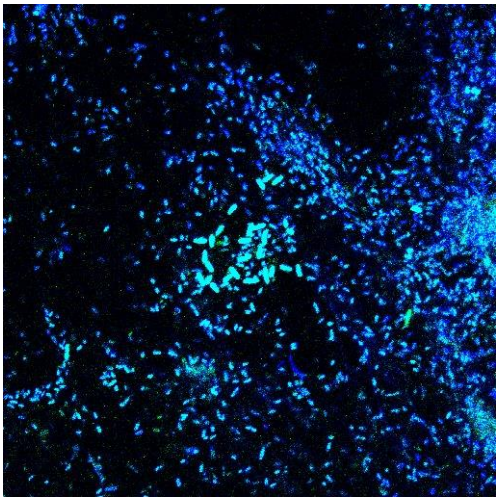


Figure 3. Confocal laser scanning microscopy (CSLM) images of dual-species biofilms with *Campylobacter jejuni* and *Pseudomonas aeruginosa*. *C. jejuni/P. aeruginosa* biofilm (48 h) imaged using dual fluorescence labeling, DAPI (blue), and Thioflavin T (green) (scale bar = 20 µm).

1. Prepare the cells as described in steps A1–A3.
2. Place a coverslip into each well of the 6-well plate.
3. Dispense 2 mL of diluted bacterial suspension into 2 wells of the 6-well plate to enable the formation of biofilm on the coverslip.
4. Incubate plates at 42 °C under microaerobic condition without shaking for at least 24 h and up to 72 h (see Notes 1 and 2).
5. Remove the media from plates over a tray and rinse gently with PBS twice to remove planktonic cells.
6. Fix the cells on the coverslips using a 5% formaldehyde solution for 1 h at room temperature, rinse gently with 2 mL of PBS, and stain with fluorescent dyes (e.g., DAPI) that label DNA [6].
7. DAPI is usually used to visualize bacterial cell distribution in the biofilm [29] and is often included in the anti-fade mounting medium.
8. Remove the coverslips using forceps, carefully hold the coverslip edge to avoid disrupting the biofilm, and invert onto a glass slide containing a drop of the anti-fade mounting medium with DAPI (10 µg/mL) (see Note 7).
9. Seal coverslips mount on glass slides using transparent nail varnish. Two coverslips per sample/concentration from at least two separate experiments should be examined microscopically.
10. All images are processed using ImageJ [30]. ImageJ was used to combine/overlay two images [e.g., DAPI and Thioflavin T (green)] into a single image to generate a representative image as shown in Figure 3. Briefly, open the images in ImageJ and adjust the contrast (go to *Image* > *Adjust* > *Brightness*). To combine two/three images into a single-color image, go to *Image* > *Color* > *Merge channels*; each image represents one of the three channels (red, green, and blue) and the merge channels box will appear. Then, select the channels that you want and click OK to create the composite image; save it as .tiff file.

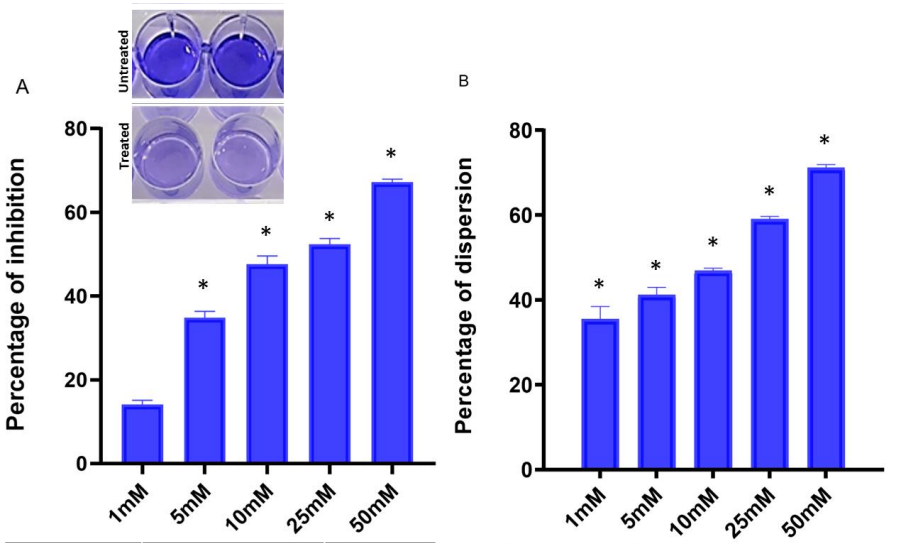
Notes

1. To create a humid environment for the biofilm assay when using 24-well plates, place a small container of water and a stack of wet paper towels around the plate during incubation.
2. If using a 96-well plate, add 200 µL of sterile water to each outer well to prevent drying.
3. All solutions should be prepared in ultrapure water and all reagents should be stored at room temperature (unless otherwise stated).
4. It is important to note that there are factors that can affect the results of this assay, such as the ability of the organism to adhere to the surface. For example, *C. jejuni* 81116 forms more abundant biofilms than *C. jejuni* 11168-O. In addition, *C. jejuni* strains display flagellin phase variation, and it is important to make sure that the culture is motile (e.g., check the cell motility by using “wet drop” light microscopy) prior to seeding of the plates, as non-motile variants do not attach nor form biofilms. Briefly, the wet mount is made by placing a drop of water on a microscopic slide and suspending a single colony of the selected strain into the water drop. The suspension is then covered with a cover slide and examined via light microscopy.
5. Each test plate should contain both a strong positive control (i.e., a wild-type strain, untreated sample) and a negative control (e.g., inoculated medium). Higher variability can appear following the incubation due to dryness or overwashing (detaching of cells).
6. Make sure that the only remaining crystal violet is bound to the biofilm at the bottom of the well. A crystal purple ring around the well is not indicative of biofilm formation and should be rinsed again as overstaining can affect assay results.
7. Using a sterile needle and forceps, carefully remove the coverslip from the 6-well plate and fix it with 5% formaldehyde solution for 1 h at room temperature.

Data analysis

Expected results

Here, we introduce two assays—inhibition and dispersion assays—to evaluate the effect of natural compounds (e.g., DAs). Treatment of *C. jejuni* culture with the DA D-Ser showed a significant inhibitory effect ($p < 0.001$) on biofilm formation (Figure 4) in a dose-dependent manner. Also, D-Ser had a significant disruptive effect on the existing biofilm ($p < 0.001$), up to 71% at 50 mM (Figure 4). Both assays can be used to screen the inhibitory and dispersal effect of selected compounds on biofilms and determine the right concentration for any further investigations. We have tested the *C. jejuni* 81–176 (81–176) and *C. jejuni* 81116 (81116) [6] strains with this protocol.



Concentration	Percentage (mean±sd)	n
1mM	14.1±2.5	3
5mM	34.85±3.7	3
10mM	47.6±4.9	3
25mM	52.4±3.35	3
50mM	67.2±1.8	3

Concentration	Percentage (mean±sd)	n
1mM	35.5±7.2	3
5mM	41.2±4.35	3
10mM	46.9±1.5	3
25mM	59.05±1.65	3
50mM	71.2±1.8	3

Figure 4. Effect of D-serine (D-Ser) on *Campylobacter jejuni* 11168-O biofilm. A) Inhibition of biofilm formation in the presence of D-Ser at different concentrations. B) Dispersion of the existing biofilm induced by different concentrations of D-Ser. The asterisk (*) indicates a statistically significant difference using the unpaired Student's *t*-test, $p < 0.05$. At least three biological replicates were included for each experiment and each biological replicate included at least three technical repeats.

Validation of protocol

This protocol or parts of it has been used and validated in the following research article(s):

- Elgamoudi et al. (2020). Inhibition of *Campylobacter jejuni* Biofilm Formation by D-Amino Acids. *Antibiotics* (Figures 2 and 3). Data are representative of three independent experiments (n = 3), and values are presented as mean ± standard errors. Statistical significance of data generated in this study was determined using two tailed Student's *t*-test. $p \geq 0.05$ was considered statistically significant.

Acknowledgments

This protocol was derived from and validated in the original research papers (Elgamoudi et al., 2020 [6]; Elgamoudi et al., 2022 [31]). The work has been partially supported by Griffith University.
Author contributions: Bassam Elgamoudi: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing: original draft, Writing: review & editing. Victoria Korolik: Conceptualization, Writing: original draft, Writing: review & editing.

Competing interests

The authors declare no conflicts of interest.

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