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Biofilm Formation and Antibiotic Resistance in *Arcobacter butzleri* Strains Isolated from Environmental Sources

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Abstract
In recent years, a marked increase in antimicrobial resistance among microorganisms has been reported. One of the key factors contributing to this phenomenon is the formation of biofilms—structured microbial communities that significantly enhance bacterial survival and resistance to external stressors.
The present study aimed to assess the biofilm-forming potential of selected *Arcobacter butzleri* strains isolated from environmental sources. This evaluation included the molecular detection of six biofilm-associated genes (*flaA*, *flaB*, *spoT*, *fliS*, *luxS*, and *pta*), which are involved in bacterial motility, invasiveness, and pathogenicity. All six target genes were detected in 21 strains (42%), while 6 strains (12%) tested negative for all investigated genes. Biofilm formation was quantitatively assessed under static and dynamic conditions using Christensen's colorimetric assay. Under static conditions, biofilm formation was observed in 96% of the strains, with 74% classified as strong or moderate biofilm producers. Conversely, biofilm development was markedly reduced under dynamic conditions, with up to 80% of strains exhibiting limited adhesion capacity.
Keywords: *Arcobacter*, biofilm, microbial resistance, pathogenic potential

1. Introduction

Biofilms are structured microbial consortia that adhere to biotic or abiotic surfaces and interfaces. This complex architecture confers several advantages to the resident microorganisms, including enhanced protection from environmental stressors. Bacteria within biofilms demonstrate significantly increased resistance to host immune responses as well as antimicrobial agents—often exhibiting tolerance levels up to 1000-fold higher than their planktonic counterparts [Donlan and Costerton, 2002]. The development and stability of biofilms are strongly influenced by environmental conditions, which modulate gene expression pathways involved in adhesion, EPS production, and stress response mechanisms. *Arcobacter butzleri* possesses homologues of several genes previously associated with biofilm formation in *Campylobacter* spp., including *flaA*, *flaB*, *fliS*, *luxS*, *pta*, and *spoT*. However, the specific roles of these genes in the biofilm development of *A. butzleri* remain poorly characterized. Given their putative involvement in processes such as motility, *quorum sensing*, and stress adaptation, it is plausible that they also contribute to surface adhesion and biofilm maturation in this species [Salazar-Sánchez *et al.*, 2022]. Elucidating the molecular mechanisms underlying biofilm formation in *A. butzleri* is essential for the development of effective intervention strategies aimed at controlling its persistence in clinical and environmental settings. Therefore, the objective of this study was to investigate the potential involvement of six biofilm-associated genes—*flaA*, *flaB*, *fliS*, *luxS*, *pta*, and *spoT*—in the biofilm-forming capacity of *A. butzleri*, thereby contributing to a deeper understanding of its pathogenicity and environmental adaptability.

2. Material and Methods

2.1. Biofilm-associated Gene Detection

The presence of six biofilm-associated genes (*flaA*, *flaB*, *fliS*, *luxS*, *pta* and *spoT*) was determined by individual PCRs performed on 100 ng of DNA with 1.25 U of *Taq* DNA Polymerase (Top-Bio, Czech), 0.1 mM of each dNTP, 1Xbuffer and 0.25 µM of each primer set [Salazar-Sánchez *et al.*, 2022]. The PCR parameters were 5 min at 95°C; 30 cycles of 94°C for 30 s, annealing temperatures ranging from 50 to 56°C for 30 s and 72°C for 1 min; and 10 min at 72°C. DNA from *A. butzleri* RM4018 was used as the positive control.

2.2. Biofilm Formation in Microtiter Plate

Biofilm formation was assessed using a previously described microtiter plate assay with minor modifications. Briefly, 100 µL of a bacterial suspension standardized to 10^7 CFU/mL in Brain Heart Infusion (BHI) broth (Himedia, Mumbai, India) was inoculated into the wells of sterile, flat-bottom 96-well polystyrene microtiter plates (SPL Life Sciences Co., Ltd., Korea). The plates were incubated under defined conditions (30 °C, 24 h, static or dynamic) to allow biofilm development. Following incubation, non-adherent cells

were removed by washing the wells five times with sterile distilled water, and the plates were subsequently air-dried. Biofilms were fixed with 2% sodium acetate for 15 minutes and stained with 100 µL of 1% crystal violet solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes. Excess stain was removed by repeated washing with distilled water, and the plates were dried. The bound crystal violet was solubilized with 96% ethanol, and the optical density (OD) was measured at 595 nm using a microplate reader (Infinite M200, Tecan, Männedorf, Switzerland).

2.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of isolates to amikacin (30 µg), amoxicillin–clavulanic acid (30 µg), ampicillin (10 µg), aztreonam (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), doxycycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), oxacillin (1 µg), penicillin G (10 IU), streptomycin (10 µg), and tetracycline (30 µg) was evaluated using the disk diffusion method as previously described. Briefly, bacterial isolates were cultured on blood agar at 30 °C under aerobic conditions for 48 hours. Subsequently, bacterial suspensions were prepared in physiological saline, and their turbidity was adjusted to a concentration of 10^8 CFU/mL. The suspensions were evenly spread onto Mueller–Hinton agar supplemented with 5% defibrinated horse blood and 20 mg/L β-nicotinamide adenine dinucleotide (β-NAD; bioMérieux, France). Antimicrobial discs were then applied to the inoculated agar surface, and the plates were incubated aerobically at 30 °C for 48 hours. Following incubation, inhibition zones were measured and interpreted using a Bacmed 6iG2 automated reader and analyzer (Aspiag, Czech Republic). Classification of the isolates was based on clinical breakpoint values recommended by the Clinical and Laboratory Standards Institute (CLSI).

3. Results and Discussion

Of the 50 *Arcobacter butzleri* strains analyzed, 21 (42%) were positive for all six target genes, while 6 strains (12%) lacked all of the detected genes. The genes *flaA*, *flaB*, and *spoT* were present in 29 strains (58%), with *flaA* and *flaB* always co-occurring. The gene combination *fliS*, *luxS*, and *pta* was identified in 44 strains (88%). These findings are largely consistent with the study by Martinez-Malaxetxebarria *et al.* (2022), who reported universal presence of *fliS*, *luxS*, *pta*, and *spoT*, while 67.9% of strains lacked *flaA* and *flaB*. The main discrepancy lies in the prevalence of *spoT*, suggesting potential geographical or environmental variation. In contrast, Salazar-Sánchez *et al.* (2022) exclusively analyzed PCR-confirmed strains carrying all six genes. Collectively, the results emphasize the genetic heterogeneity of *A. butzleri*.

Biofilm-forming ability was assessed using the Christensen microtiter plate method under both static and dynamic conditions. Under static cultivation, 96% of strains formed biofilms on plastic surfaces, with 74% classified as strong or moderate producers. Cultivation under dynamic conditions, which better simulate natural environments, resulted in decreased biofilm density in 80% of strains, likely due to reduced cell adhesion.

These findings highlight the variability in biofilm production among *A. butzleri* strains and its association with genetic factors. Given the role of biofilms in persistence, virulence, and antimicrobial resistance, these results contribute to a deeper understanding of the pathogen's environmental adaptability and potential public health impact.

Antimicrobial susceptibility testing revealed that 99% of *Arcobacter* isolates exhibited resistance to at least one antibiotic. High resistance rates were observed for β-lactams, including ampicillin (79%), amoxicillin-clavulanic acid (28%), cephalothin (72%), and aztreonam (91%). A similarly high resistance was detected for the lincosamide clindamycin (96%). In contrast, the aminoglycosides amikacin, gentamicin, and tobramycin demonstrated the highest efficacy against the tested strains.

4. Conclusion

This study presents a comparative analysis of biofilm formation among environmental *A. butzleri* isolates from the Czech Republic. Nearly all strains demonstrated the capacity to form biofilms, though production levels varied depending on environmental conditions such as oxygen availability and cultivation duration. These findings support the relevance of biofilm formation in the persistence and pathogenic potential of *A. butzleri*, underlining the importance of ongoing surveillance in relation to food safety and public health.

References

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