SYMCEL

WOUND IN A CUP – ISOTHERMAL MICROCALORIMETRY AND CHRONIC WOUND MEDIUM FOR A WOUND MODEL

INTRODUCTION

Chronic wounds represent a major and increasing public health challenge worldwide. Conditions such as diabetic foot ulcers, pressure ulcers, and venous leg ulcers affect millions of patients annually, leading to prolonged hospitalizations, reduced quality of life, and increased healthcare costs[1,2]. The prevalence of chronic wounds is rising, particularly as the global population ages and metabolic disorders become more widespread. Chronic wounds are often associated with delayed healing due to a combination of factors, including impaired vascularization, persistent inflammation, and bacterial colonization[3].

One of the most significant barriers to chronic wound healing is the presence of bacterial biofilms. These structured microbial communities embed themselves in an extracellular polymeric matrix, protecting them from host immune responses and antimicrobial agents[4]. Bacteria in biofilms are significantly more resistant to antibiotics compared to planktonic cells, necessitating higher doses or combination therapies to achieve bacterial clearance[5]. Despite their clinical significance, biofilm-related infections in chronic wounds remain difficult to study due to the lack of appropriate *in vitro* models that accurately mimic the wound environment.

Current *in vitro* biofilm models often rely on simple agar plates or artificial surfaces that fail to capture the dynamic and heterogeneous nature of chronic wound infections[6]. These models lack the complex biochemical conditions found in chronic wounds, such as fluctuating oxygen levels, inflammatory mediators, and the presence of host-derived proteins. Additionally, most biofilm assays require disruptive sample processing, such as mechanical disruption or chemical extraction, which can alter biofilm structure and underestimate viable but dormant bacterial subpopulations[7].

To overcome these limitations, more advanced *in vitro* models have been developed to simulate the microenvironment of chronic wounds. One such approach involves the use of SynthBiome's chronic wound medium, a chemically defined medium designed to replicate the biochemical composition of chronic wound exudates. This medium appearance is dark in color and features precipitation, making it challenging to use other methods like OD. Utilizing this medium in combination with isothermal microcalorimetry (IMC) makes it possible to monitor bacterial activity *in situ*, preserving biofilm integrity while obtaining real-time metabolic data.

MATERIALS AND METHODS

Pseudomonas aeruginosa PAO1 and a clinical isolate from a chronic wound (courtesy of Mads Holm Christensen and Tim Holm Jacobsen, Costerton Biofilm Center, University of Copenhagen) were grown in chronic wound medium (CWM2) (SynthBiome inc.) within the calorimetric inserts of the CalScreener calorimeter (Symcel AB). The medium was specifically tailored to replicate chronic wounds' microenvironmental and chemical conditions. Over a pre-incubation period of 24 hours, single cells grew into mature biofilm aggregates. In parallel, biofilms were grown as biofilm aggregates in 0.5% LB agar.

Antimicrobial Treatments: Mature biofilms were exposed to ciprofloxacin at up to 10 times the minimum inhibitory concentration (MIC). Treatments were conducted directly in the chronic wound medium and in the agar-based models.

The metabolic activity of the biofilms was continuously monitored using the CalScreener. Heat flow measurements provided real-time data on bacterial activity before, during, and after treatment, without disturbing the biofilm or extracting bacteria from the media.

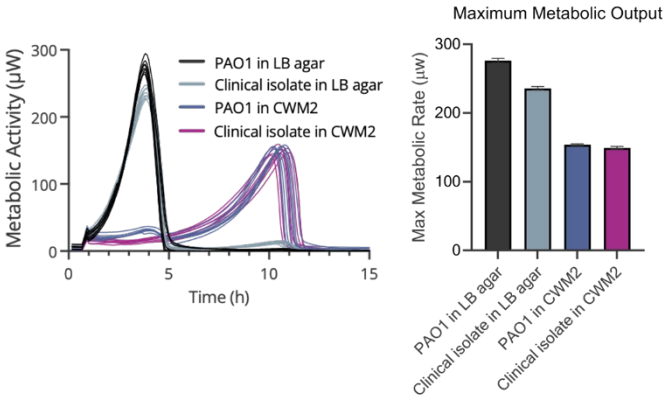

Figure 1 Metabolic thermogram first 15 hours of the overnight biofilm aggregate pre-growth in either LB agar or CWM2 when seeded with either *P. aeruginosa* PAO1 or a clinical wound isolate. Mean with SEM.

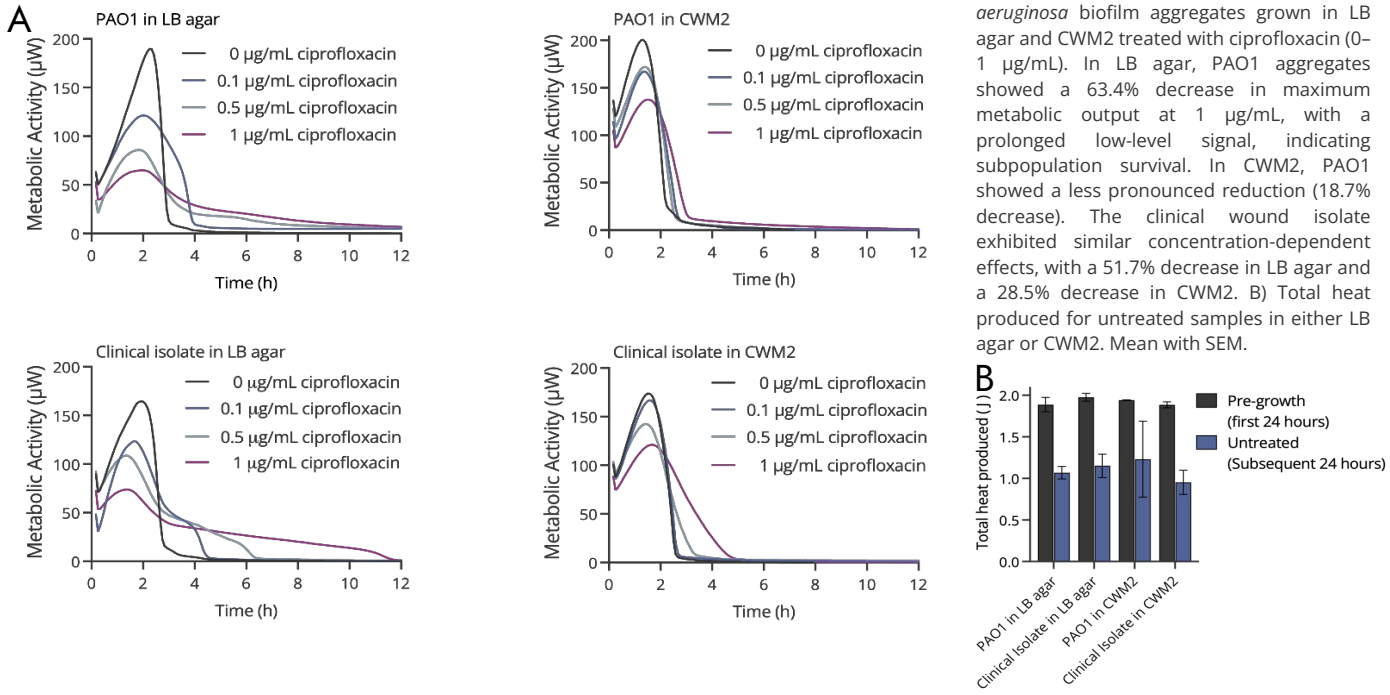

Figure 2 A) Metabolic thermograms of *P. aeruginosa* biofilm aggregates grown in LB agar and CWM2 treated with ciprofloxacin (0–1 µg/mL). In LB agar, PAO1 aggregates showed a 63.4% decrease in maximum metabolic output at 1 µg/mL, with a prolonged low-level signal, indicating subpopulation survival. In CWM2, PAO1 showed a less pronounced reduction (18.7% decrease). The clinical wound isolate exhibited similar concentration-dependent effects, with a 51.7% decrease in LB agar and a 28.5% decrease in CWM2. B) Total heat produced for untreated samples in either LB agar or CWM2. Mean with SEM.

RESULTS

Biofilm Metabolism in Complex Media: *P. aeruginosa* biofilm aggregates exhibited distinctly different metabolic patterns in LB agar (0.5%) compared to CWM2 (Fig. 1). In LB agar, biofilms showed a rapid metabolic peak, reaching approximately 300 µW within the first five hours, followed by a sharp decline. Notably, the PAO1 laboratory strain displayed a higher peak compared to the clinical isolate, indicating strain-specific growth dynamics. PAO1 in LB agar peaked at 283 µW after 3.8 hours, with the clinical isolate at 237 µW after 4 hours. In CWM2 PAO1 peaked at 150 µW after 10.3 hours, with the clinical isolate at 153 µW after 10.6 hours. In contrast, biofilms grown in CWM2 exhibited

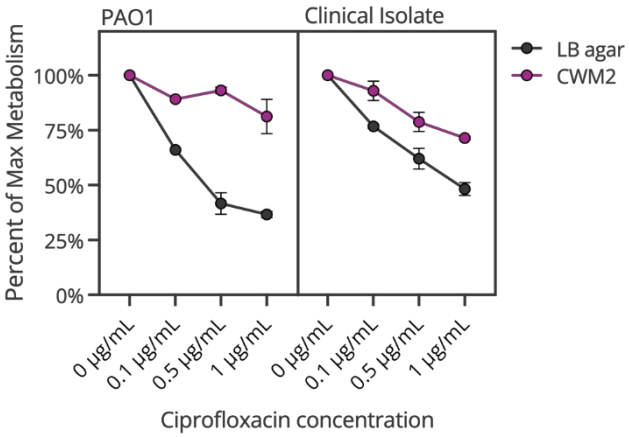

Figure 3 Mean maximum heat from biofilm aggregates in either CWM2 or LB agar when treated with ciprofloxacin as a percentage of the maximum heat from the untreated biofilm aggregates. On the left, aggregates of PAO1 in either LB agar (black) or in CWM2 (purple). On the right, the Clinical wound isolate. Mean with SEM.

a slower and more sustained metabolic increase over 12 hours, with a steep decline thereafter (Fig. 1). The metabolic profiles of the PAO1 and clinical isolates were more similar in CWM2 compared to in the LB agar. These results may indicate that CWM2 supports more resilient biofilm growth, reflecting chronic wound-like conditions, whereas LB agar promotes rapid, nutrient-driven growth. The total metabolic heat produced within the first 24 hours of aggregate pre-grown was the same for both strains and in both media. The same was true for the untreated aggregates during the subsequent 24 hours (Fig. 2B).

Metabolic thermograms of preformed biofilm aggregates grown in either LB agar or CWM2 when treated with 0 – 1 µg/mL ciprofloxacin: Aggregates of PAO1 in LB agar exhibited a distinct concentration-dependent lowering of the metabolic output (Fig. 2A). Increasing doses of ciprofloxacin resulted in a prolonged but lowered metabolic signal, with 1 µL/mL being the longest and lowest. The maximum metabolism was 63.4% lower compared to the untreated sample (Fig. 3). When PAO1 was treated in CWM2, the effect was diminished compared to LB agar, with less of a decrease in metabolic output as ciprofloxacin concentrations increased. With a treatment of 1 µg/mL the maximum metabolism was lowered 18.7% compared to the untreated.

Aggregates formed by the clinical wound isolate exhibited the same concentration-dependent metabolic dynamics as PAO1 in both types of media (Fig. 2). The maximum metabolic rate was reduced by 51.7% in LB agar, whereas it was reduced by 28.5% in CWM2 (Fig. 3). In LB agar, when treated with 1 µL/mL the aggregates produced a long but low metabolic signal, indicating survival of subpopulations inside of the aggregates.

DISCUSSION

The results of this study reveal significant differences in metabolic activity and treatment response between biofilm aggregates formed in LB agar and those grown in Chronic Wound Medium (CWM2). Notably, there was little difference in the total metabolic output between the two media when untreated, indicating that the initial biofilm formation and growth were comparable. However, the application of ciprofloxacin revealed a markedly greater impact on biofilms in LB agar compared to CWM2. Specifically, the metabolic activity in LB agar decreased by over 60% for the PAO1 strain, while the same treatment in CWM2 resulted in only an 18% reduction. Also, there was no significant difference in total heat between the four combinations for the untreated aggregates in the subsequent 24 hours, suggesting any difference in metabolism as a result of treatment was due solely to the media composition.

These findings highlight the risk of misleading conclusions when evaluating antimicrobial efficacy using simplistic *in vitro* models, such as LB agar with laboratory strains. If tested solely in LB agar, one could incorrectly conclude a high efficacy of ciprofloxacin against *P. aeruginosa* biofilms. This discrepancy is particularly pronounced with laboratory strains like PAO1, which displayed high susceptibility in LB agar but demonstrated significant metabolic resilience in the more clinically relevant CWM2. This could lead to an overestimation of antimicrobial potency and an underestimation of biofilm tolerance, resulting in costly failures during clinical translation and, ultimately, ineffective patient treatments.

Furthermore, the results challenge the conventional interpretation of reduced metabolic output as an indicator of biofilm eradication. In CWM2, the relatively smaller decrease in metabolic activity suggests the survival of highly tolerant subpopulations, potentially contributing to chronicity and recurrent infections. If only the simpler LB agar model were used, this tolerant phenotype would be overlooked, leading to an inaccurate understanding of biofilm persistence and drug tolerance.

These findings underscore the importance of using physiologically relevant biofilm models for antimicrobial testing. The combination of isothermal microcalorimetry with chemically defined chronic wound medium enables real-time, non-destructive monitoring of bacterial metabolic activity within a clinically relevant microenvironment. This approach more accurately reflects the *in vivo* conditions of chronic wound infections, providing critical insights into biofilm dynamics and antimicrobial tolerance. By bridging the gap between *in vitro* studies and clinical reality, this method enhances the predictive value of preclinical research, guiding the development of more effective antimicrobial therapies for chronic wound management.

EXPERIMENTAL WORKFLOW

Day 1: Bacterial Culture Preparation
- Prepare overnight cultures at 37°C with shaking.

Day 2: Biofilm Inoculation and Growth
- Dilution: Dilute the overnight culture 1:50 in both CWM2 and LB agar (0.5%).
- Inoculation: Vortex the diluted cultures and transfer 200 µL into calScreener vials.
- Attach lids and load the machine as per standard protocol.
- Incubate inside the calScreener for 24 hours.

Day 3: Biofilm Treatment and Monitoring
- After 24 hours, pause the experiment and remove the calPlate from the calScreener.
- Open the vials and treat them with 100 µl of fresh CWM2 or LB with a final concentration of antibiotics in the vials:
  - 0, 0.1, 0.5, or 1 µg/mL ciprofloxacin.
- Close the lids and reload the machine.
- Resume the experiment and incubate overnight.
- Analyze the data using calData for total heat and maximum metabolic rate.

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SYMCEL°

Symcel AB
Tomtebodavägen 6
171 65, Stockholm, Sweden
symcel.com