Assessment of Novel Antimicrobial Therapy against Methicillin-resistant
Staphylococcus pseudintermedius Biofilm with Conventional Assays and a
Microfluidic Platform

by

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ABSTRACT

Assessment of Novel Antimicrobial Therapy against Methicillin-resistant *Staphylococcus pseudintermedius* Biofilm with Conventional Assays and a Microfluidic Platform

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This thesis is an investigation of methods to remediate methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) biofilms through conventional and microfluidic-based *in vitro* assays. MRSP biofilm related infections are a major concern for veterinary clinicians as they may complicate remediation by the immune system or antimicrobials. Novel antimicrobials that have been found to reduce biofilm growth in other staphylococci were assessed in both mono- and combination therapy against MRSP biofilm. Quantitative assay results (p < 0.05) suggest fosfomycin alone and in combination with clarithromycin can significantly reduce biofilm formation. Morphological examination using scanning electron microscopy and atomic force microscopy further demonstrated the effectiveness of fosfomycin alone on biofilm formation on orthopaedic screws and mica sheets. Fabricated microfluidic assays were utilized to assess multiple concentrations of antimicrobial therapy against pre-formed biofilm under physiologically relevant conditions in a quick and repeatable manner. Results demonstrated the usefulness of microfluidic platforms in determining minimum biofilm eradication concentrations.

**Keywords:** *Staphylococcus pseudintermedius*, Microfluidics, Biofilm, Resistance, AFM, SEM
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<th>Description</th>
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<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
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<td>CLA</td>
<td>Clarithromycin</td>
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<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standards Institute</td>
</tr>
<tr>
<td>CPHAZ</td>
<td>Center for Public Health and Zoonoses</td>
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<tr>
<td>EPS</td>
<td>Extracellular Polymeric Substances</td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility Testing</td>
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<tr>
<td>FICI</td>
<td>Fractional Inhibitory Concentration Index</td>
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<td>FOS</td>
<td>Fosfomycin</td>
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<tr>
<td>G3P</td>
<td>Glucose-3-phosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
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<tr>
<td>MIC</td>
<td>Minimum Inhibition Concentration</td>
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<tr>
<td>MBEC</td>
<td>Minimum Biofilm Eradication Concentration</td>
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<td>MSSP</td>
<td>Methicillin-susceptible <em>Staphylococcus pseudintermedius</em></td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>MRSP</td>
<td>Methicillin-resistant <em>Staphylococcus pseudintermedius</em></td>
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<tr>
<td>OVC</td>
<td>Ontario Veterinary College</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDMS</td>
<td>Poly(dimethylsiloxane)</td>
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<tr>
<td>TSB</td>
<td>Tryptic Soy Broth</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<td>μl</td>
<td>microlitre</td>
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I INTRODUCTION

1.1 Surgical Site Infections in Veterinary Medicine

Surgical site infections (SSIs) are an inherent risk of any surgical procedure and can lead to morbidity, prolonged hospitalization, client frustration, frustration of medical caregivers, and increased treatment costs in veterinary medicine [1]. They have been reported as a complication in 0.8% to 18.1% of operations in dogs and cats, depending on the surgical classification [2-4] and are of particular concern with implanted biomaterials such as orthopaedic implants, suture material, and indwelling medical devices.

1.1.1 Staphylococcus pseudintermedius

*Staphylococcus pseudintermedius* is an opportunistic pathogen that can be found on the skin or in the ears, oral cavity, intestinal tract or other non-sterile sites of a large percentage of healthy dogs [5,6]. In addition to being one of the leading causes of SSIs, staphylococci have a tendency to acquire resistance to antimicrobial agents [7]. Of particular concern is methicillin-resistance, which confers resistance to all beta-lactam antimicrobials.

1.1.2 Emergence of Multi-drug Resistant Bacterium

Extensive use of antimicrobials during the last half century has facilitated the promotion of antimicrobial resistance characteristics in microorganisms. The bacterial response to this pressure is the acquisition and spread of a variety of determinants due to mutations of normal cellular genes, acquisition of foreign resistance determinants from other microorganisms, or a combination of these two genetic mechanisms resulting in difficult to remediate infections. Methicillin-resistant *S. pseudintermedius* (MRSP) has rapidly emerged in companion animals and MRSP infections are being reported with increasing frequency in veterinary hospitals,
becoming a major cause of pyoderma and SSIs in dogs [8-11]. MRSP infections are a tremendous concern in companion animals as they are challenging to eradicate being recalcitrant to traditional antimicrobial therapy, both due to their resistance to beta-lactam antimicrobials and because they typically have also acquired resistance to various other antimicrobial classes [1]. Reasons for the rapid emergence of MRSP are not well understood, however, one potential virulence factor that has received little attention in this bacterium is the ability to form biofilm.

1.1.3 Bacterial Biofilm Implicated Infections

In addition to mutation or acquisition of resistance genes, some bacteria have developed other mechanisms to elude treatment such as creation of biofilm. A bacterial biofilm is defined as a complex community of microorganisms embedded within a self-produced glycocalyx layer formed of high-molecular weight compounds known as extra-cellular polymeric substances (EPS). Biofilm production can facilitate strong bacterial adherence to biological or non-biological surfaces, increase resistance to the immune response and antimicrobials, and provide mechanical stability, viscoelasticity, and resistance to shear forces [12,13]. The metabolic state of the bacteria or microenvironment within the biofilm can also inhibit the efficacy of an antimicrobial [14] wherein the minimum inhibitory concentration (MIC) of biofilm-embedded bacteria can be 10 to 1000 times higher than their non-biofilm-embedded counterparts [15]. Concentrations to remediate biofilm are noted as the minimum biofilm eradication concentration (MBEC). Changes to the metabolic state of biofilm-embedded cells include entering a sessile state [16]. The ability to resist environmental factors may be due to the thickness and chemical composition of the biofilm matrix, preventing penetration of antimicrobial agents [12,17].

Because of the potential impact of biofilm on the development and persistence of infection and the difficulties that may be encountered when treating biofilm-associated
infections, understanding the mechanisms of biofilm production in clinically relevant bacteria and identifying novel strategies to prevent and treat biofilm-associated infections are required. Specifically, biofilm production has been hypothesized as one of the reasons for the emergence of a few successful MRSP clones internationally [18,19], but there has been little study on methods to address this virulence factor [18].

1.1.4 Remediation of Infections

As no methods currently exist for MRSP biofilm remediation this can lead to many complications, especially with foreign implants—a risk factor for biofilm-related SSIs—in veterinary orthopaedic surgery wherein the only way to remediate implant-implicated infections is through device removal [20,21]. With reports on MRSP isolates not susceptible to any antimicrobials authorized for use in veterinary medicine [19,22] there is a potential pressure for veterinarians to use antimicrobials authorized for human medicine [19]. Alternative approaches to traditional monotherapy methods in veterinary medicine are needed and the use of combination antimicrobial regimens could prove useful. Some antimicrobials in combination display synergism, biofilm-reducing properties, as well as decreases in the expression of resistance determinants [23-26].

1.2 Microfluidics

Microfluidic platforms are very useful in experimentation where precise control of the local environment of a sample is critical. Commonly used to analyze micro-organisms, microfluidic platforms can emulate biological phenomena occurring under the various fluid dynamic, geometrical, and heat and mass transport constraints of physiologically relevant micro-environments [27,28]. These systems are able to control fluid composition, fluid flow parameters
(velocity, viscosity, and shear), and many other features. Due to the properties of the hydrodynamics governed at the micron level, control of these features allows for the production of spatio-temporal gradients and precise control of the micro-organism’s environment [29]. Surface attachment and biofilm formation have been associated with many pathogenic bacteria, and it has been shown that both are significantly affected, and even enhanced, by the presence of dynamic flow [30,31]. As biofilm formation is a virulence factor for MRSP based SSIs in veterinary medicine, it would be possible to control and analyze their surrounding environment through flow-based microfluidics in continuous culture. With the zoonotic potential of MRSP [19] there is a further threat to public health as well and therefore rapid and accurate analysis of potential antimicrobial therapies is needed. Microfluidic platforms are expected to play larger roles in the field of clinical microbiology as design and development edges closer to providing identical and reproducible culture conditions that can generate quantitative and statistically valid data, in line with current conventional methods of analysis [32]. Use of polydimethylsiloxane (PDMS) based soft lithography further provides simple ways of quickly fabricating microfluidic devices with attractive properties such as biocompatibility and transparency. Implementation of PDMS based microfluidic devices, with elements to control the micro-environment surrounding biofilm formed within the device could allow for quick fabrication, growth of biofilm, and precise analysis of biofilm-related infection control strategies.

### 1.3 Problem Statement

Developing new strategies to reduce production and adherence of bacteria and biofilm structure is a major goal in the field of clinical microbiology as they play a significant role in the development and persistence of nosocomial infections and facilitate the spread of resistance by
promoting horizontal gene transfer [33]. As biofilm formation is an important property of some MRSP strains, SSIs caused by this bacterium can have a tremendous impact on the management of patient health, often leading to mortality, prolonged hospitalization and increased treatment costs. Currently, no methods exist for therapeutic remediation of SSIs caused by MRSP biofilm. Developing resistance profiles and methods for testing antimicrobial candidates in physiologically relevant platforms is the first step towards finding a potential therapeutic agent for normally recalcitrant SSIs caused by this bacterium.

1.4 **Scope and Objectives**

Various *in vitro* studies have shown the synergistic effect of antimicrobials in combination, but only a few reports have addressed the use against biofilm in animal based infections. Therefore, the aim of the present study was to research the efficacy of notable biofilm reducing antimicrobials alone and in combination against biofilm-forming strains of MRSP *in vitro*. Further assessment of the chosen antimicrobial therapies will be completed through imaging with qualitative/quantitative verification of MRSP biofilm adhesion and growth on clinically relevant abiotic materials. However, given the protective nature and complexity of bacterial biofilms the assessment of growth may not be well represented in conventional methods of analysis. A lack of continuous-culture and dynamic micro-environments commonly found in *in vivo* can confound conventional assessment methods of antimicrobial susceptibility and biofilm growth in standing liquid culture conditions. And although these methods offer statistical breadth, their relevance and transfer to a clinical setting is questionable. As microfluidics can offer an excellent platform in the analysis of bacteria in planktonic and biofilm-embedded states due to their inherent fluidic properties and precisely controllable environments, designing methodology to assess multiple concentrations of antimicrobials alone and in combination to
quickly assess potential remediation therapies against biofilm grown in continuous culture will be attempted. Ultimately, stopping microbial attachment, preventing microbial growth, disrupting cell-to-cell communication or disintegrating the biofilm matrix would help to limit recurring and chronic infections in companion animals.
2 BACKGROUND

2.1 Treating Bacterial Biofilms

Biofilm-forming strains that attach or colonize on surfaces aggregate in a self-produced hydrated polymeric matrix (i.e. EPS), forming inherent resistance to antimicrobial agents and other environmental factors (Figure 1). This can result in a significant growth advantage [34], play a crucial role in microbial population dynamics [35], and in many of these cases result in chronic or increased rates of host infection [36].

![Figure 1 – Bacterial biofilm life cycle.](image)

Studies of biofilm populations have further revealed structured groups of cells with community-like properties, unlike their planktonic counterparts [37]. Advances in the understanding of the genetic basis for this behavior could point to therapeutic targets that may provide a means of control of biofilm related infections. However, these methods would only provide a genetic profile of the infecting bacterial species and lack the direct testing of susceptibility to particular antimicrobials. And, although some types of resistance determinants
have genetic markers, their presence may not always denote resistance; further, they have not been identified for all resistant strains of bacteria. Therefore, susceptibility is best determined by functional assays, especially for bacterial strains with unknown resistance mechanisms, such as MRSP [38].

Currently, no information on eradication therapy exists for MRSP biofilms, but by producing antimicrobial resistant profiles through the quantification of MIC for planktonic and MBEC for biofilm-embedded MRSP, a base of knowledge on this subject and a standardized set of methods can be established. As biofilm formation is also an important virulence factor of methicillin-resistant *Staphylococcus aureus* (MRSA) implant-associated infections in humans [12], the identification of antimicrobial therapy approaches in its reduction could prove fruitful—particularly with those that are not used for the treatment of critically ill human patients.

2.1.1 Clarithromycin

Clarithromycin (CLA), a semi-synthetic broad spectrum macrolide, has demonstrated potent *in vitro* and *in vivo* anti-biofilm activity against Gram-positive *S. aureus* alone and in combination with other antimicrobials, independent of its antimicrobial activity [12,36,39]. This suggests that CLA could be an option for prevention or eradication of MRSP biofilms. However, biofilm formation (and presumably the factors that regulate biofilm formation) varies between bacterial species, and these factors have not been investigated for MRSP. The mechanism of biofilm prevention by macrolides seen in MRSA and other bacteria is also not completely understood but current studies speculate that they also act through modification of the immune system’s inflammatory response to infection, and/or through a direct effect on bacterial virulence [39].
2.1.2 Fosfomycin

In a similar fashion fosfomycin (FOS) has been reported to destroy biofilm and increase penetration of other antimicrobials through biofilm layers in both Gram-positive and Gram-negative bacteria \[25,40,41\]. FOS, an old antimicrobial, has seen a revival in practice in humans in recent years in both mono- and combination therapy \[33\]. Commonly used in urinary tract and gastrointestinal infections, there has been recent expansion in use in other fields \[42\]. Interfering with cell wall synthesis of peptidoglycan, it enters FOS-susceptible bacterium by means of two different transport uptake systems, the L-α-glycerophosphate transport system (GlpT) and the hexose–phosphate uptake system (UhpT) \[43\].

![Figure 2 – Structure of clarithromycin](image)

![Figure 3 – Structure of fosfomycin](image)
Providing good distribution into tissues in clinically relevant concentrations, it is suggested that its high degree of tissue and biofilm penetration is attributed to its low molecular weight of 194 Da (Figure 3), negligible protein binding, and non-reactivity with charged bacterial glycocalyx (a component of EPS) [44,45]. Under the anaerobic conditions of biofilm-embedded cells, the levels of the transport systems affecting FOS increase [46], suggesting that FOS is still transported into sessile cells in the stationary phase, potentially providing an effect against metabolic resistance determinants. Petek et al. [47] also found that FOS can down-regulate surface polysaccharide metabolism genes in S. aureus, supporting its use in the remediation of biofilm. Further, reports on plasmid-based resistance to FOS in staphylococci through fosA and fosB genes are also low suggesting minimal transfer of genetic resistance determinants between strains [48]. These properties indicate that FOS might be a viable treatment option for some MRSP infections, but the susceptibility of MRSP to FOS has not been previously reported.

2.1.3 Antimicrobial Synergism

*In vitro* studies of other staphylococcal strains, such as MRSA, have shown that biofilm-associated SSIs can also be prevented through antimicrobial combination therapy [36] while providing a reduced risk of resistance prevalence as well as previously un-realized synergistic effect between antimicrobials [23-25]. When used in combination, FOS appears to exert substantial antimicrobial activity coupled with clinical effectiveness against infections specifically caused by “problem” Gram-positive cocci pathogens both *in vitro* and *in vivo* [49-52]. Mikuniya *et al.* [25] successfully eradicated MRSA biofilm through combination therapy with prulifoxacin and FOS in a urinary tract infection rat model when compared to mono-therapy results. In their study, scanning electron microscopy (SEM) demonstrated that multilayer
biofilms were destroyed and disappeared after treatment with the combination of prulifoxacin and FOS. Interestingly, synergistic studies have demonstrated that FOS may even decrease the level of penicillin resistance in pneumococci by altering the degree of expression of penicillin-binding proteins [26].

2.2 Susceptibility Analysis with Conventional Methods

2.2.1 Antimicrobial Susceptibility

In order to assess the MIC of antimicrobials across select strains of bacteria in a planktonic state assays are traditionally performed using Kirby-Bauer disk-diffusion or agar dilution. In Kirby-Bauer disk-diffusion methods, a hydrophilic disc is infused with a chosen antimicrobial and placed upon a non-selective and non-differential agar medium streaked with bacterial inoculum. The antimicrobial then diffuses through the agar gel and forms a concentration gradient that inhibits microbial growth close to the disc creating a visual ‘zone of inhibition’ that enables the MIC to be estimated. Though this is technically simple to perform, the subjective and variable nature of estimating an exact value for the MIC is a major drawback. In agar dilution methods, bacteria are first inoculated on agar plates containing growth media and a two-fold serial dilution of an antimicrobial agent. The MIC is then determined by identifying the lowest concentration of antimicrobial that inhibits growth, which is typically measured by visual inspection. Standardized guidelines for these methods are published by the Clinical Laboratory and Standards Institute (CLSI) [53].

2.2.2 Genetic Testing

Through genetic profiling of bacterial strains of a select population it is possible to measure genomic variation and investigate how it relates to virulence factors. Depending on the
desired resolution of these genomic variations several different techniques that can be used; pulsed-field gel electrophoresis (PFGE), *S. aureus* protein A (*spa*) typing, direct repeat unit (*dru*) typing, and multi-locus sequence typing (MLST) [54]. *Dru* typing and MLST through polymerase chain reaction (PCR) techniques have been developed for the sub-typing of *S. pseudintermedius* populations [55]. MLST in particular has proven to be valuable in the examination of isolates collected over large geographic areas and over multiple bacterial generations. Sequencing of major and dominant international clones of MRSP has already been completed using MLST with (most notably) ST68 and ST71, originating in North America and Europe, respectively [19,55]. Further *dru* sub-typing implemented over smaller sub-populations allows for further differentiation, shown to be useful in the characterization of *S. aureus* strains [56]. PCR amplification can also act as an accurate, simple, and rapid method for epidemiological study of specific resistance gene sequences within isolates. As the emergence of MRSP is relatively new, defining both movement and activity of clonal types as well as the resistance genes that they carry holds clinical value, allowing for researchers to expect specific characteristics that help in selecting appropriate antimicrobial therapy and infection control strategies.

### 2.2.3 Characterization of Morphology and Profile

*Scanning Electron Microscopy*

Scanning electron microscopy (SEM) is a standard form of imaging in biological analysis and allows for the imaging of samples on different materials with minimal changes to cellular morphology given correct preparation. SEM allows the examination of surfaces of materials and bulk specimens at much higher magnifications and with much greater depth of field than a conventional light microscope. Providing a topographic view, it can prove useful in the
examination of biofilm development and potential differences in growth on different substrates such as orthopaedic bone screws and other implant devices. Gray-scale micrographs produced through SEM would allow for the analysis of surface coverage of biofilm through delineation of the sample, substrate, and background. An understanding of the differences of biofilm adherence capabilities between substrates could also help in the selection of biomaterials for orthopaedic implants. However, sample preparation requires that the sample under analysis be dried and made conductive before use. In the preparation of bacterial biofilm, dehydration damage can alter the present glycocalyx structures, reducing them to up to 1% of their initial volume, although this does allow for visual indications of bacterial cells within the matrix. To avoid dehydration damage, changes to standardized protocol for preparation can be made, such as air-drying samples vs. critical point drying [57], but this can also produce unwanted charging in SEM micrographs.

*Atomic Force Microscopy*

To further extend the understanding of clinical relevance in recommending potential therapies for treating MRSP biofilms, it is necessary to understand their antimicrobial activity at the nanoscale level. Atomic force microscopy (AFM) imaging techniques overcome the drawbacks of SEM such as the need for a conductive coating on biofilm samples, extensive sample preparation methodology, and the vacuum requirement during imaging. Specifically, the study of nano-conjugated vancomycin in the treatment of *S. aureus* through AFM has demonstrated its use as a potential tool in understanding morphological induced changes to bacteria [58]. Other effective antimicrobial candidates against MRSA have been studied in detail; however to-date there has been no nanoscale investigation of antimicrobial agents on MRSP.
2.3 Microfluidics in Biological Applications

The field of microfluidics involves the precise control and manipulation of fluid (flow-free or free-flow) at the micron level (10µm to 1000µm). With micro-channels at sub-millimetre dimensions fluid flow behaviour is significantly different than macro-scale systems, exhibiting fully laminar flow characteristics which can have a direct impact on shear forces and mixing at surfaces. Fluid shear stress, which denotes the magnitude of force applied tangentially or parallel to a surface, is an important property in the adhesion of biofilm. As an example of a simple microfluidic system, in a straight micro-channel demonstrating fully laminar flow there is no active mixing, and molecular diffusive-based mixing influenced by Stoke’s drag and surface tension effects becomes dominant. With a wide range of applications, the fabrication of these devices is an evolving science, balancing many different techniques including micro-machining processes, polymer based lithography, and soft-lithography (e.g. PDMS microfluidic devices) [31]. They can further incorporate complex MEMS devices such as micro-pumps, micro-valves, and other active mixers [31]. These mini-actuators incorporated into micro-channels can allow for active and passive mixing of reagents and the ability to control fluidic parameters such as flow velocity and flow rate. In laminar environments, fluid flow profiles can be controlled to create spatio-temporal gradients of velocity and chemical composition providing an additional level of control over the micro-environment. In strictly passive devices (no actuators) concentration gradients can be generated using laminar flow phenomena in microfluidic channels and can dilute or mix liquids into linear or logarithmic concentration gradients [59]. In either case the ability to control velocity, shear, and gradients enables researchers to mimic physiological or environmental conditions which are relevant to the organisms being studied. This is different from conventional tests tube or petri dish based experimentation, which has limited relevance to natural conditions.
Chemical gradients that most bacteria experience \textit{in vivo} are generally unsteady (i.e. non-linear flow). Having micro-environmental control over gradients and fluid flow in flow-based microfluidics is therefore an appealing strategy when studying bacterial strains. Because of the laminar flow that generally exists in the microfluidic environment, a lack of turbulence produces dynamic spatio-temporal chemical gradients that are easy to predict. This leads to much more complexity than is currently available to add to the micro-environment and therefore the designs of microfluidic platforms are in constant flux as technology permits greater imitations of \textit{in vivo} conditions \cite{60}. Diffusion-based microfluidics (flow-free) have the benefit of generating steady, arbitrarily shaped chemical gradients but are more commonly used in the analysis of free-swimming single-cell bacteria. The application of microfluidics in quantifying bacterial chemotaxis parameters is ideal, allowing for the analysis of single-cell or population dynamics in laminar flow, with high throughput, and efficiency.

As growth of bacterial biofilm \textit{in vivo} enhances the protection and persistence of infections, \textit{in vitro} study in continuous culture dynamic microenvironments should be preferred over conventional assays used for antimicrobial assessment. The MBEC of bacteria-embedded biofilms can be 10 to 10,000 times that of their planktonic (MIC) counter parts \cite{17}; nevertheless, analysis of antimicrobial susceptibility of biofilm-forming isolates are still routinely found for planktonic phenotypes with conventional methods under standard liquid culture conditions. The MIC commonly determined by microtiter or tube dilution methods also only measures the concentration of antimicrobials required to inhibit growth or kill planktonic bacteria. Microfluidic platforms offer advantages over these conventional assays in testing biofilm by providing clinically relevant physiological environments through dynamic movement of fluid flow on bacterial adhesion. More closely replicating \textit{in vivo} systems with microfluidic
platforms is an attractive option in an attempt to establish guidelines for the treatment dosage of antimicrobials against biofilm-embedded bacteria.

MRSP biofilms could be easily subjected to changes in molecular cues, such as nanomolar concentrations of an antimicrobial within the micro-environment of microfluidic channels. Thus, in using chosen antimicrobials as external stimuli, minute responses in the biofilm can be very effectively studied and the MBEC of biofilm-embedded MRSP strains drawn for various antimicrobials at the nano- or even pico-molar level. There are a few methods currently created to find the MIC of specific bacterial strains using microfluidic devices, many of which focused on reducing traditional assay (such as broth dilution) time for determining MIC or by increasing throughput.

### 2.3.1 Microfluidics Approach to Planktonic MIC

In a study by Cira et al. [61] a self-loading device for determining the minimum inhibitory concentration of antimicrobials was created. The method detailed a set of chambers molded into PDMS preloaded with various concentrations of dried antimicrobial before degassing via vacuum. During operation, degas flow allows the introduction of a bacterial cell suspension into each of the different preloaded chambers without cross contamination (Figure 4). Finally the growth of the bacterial strain is determined by a pH indicator that produces a colour change if growth occurs.
Figure 4 – Microfluidic device design for determining minimum inhibition concentration (MIC) of antimicrobial therapy. Antimicrobial therapy is deposited in separate wells before bacterial inoculum is passed through the device. Growth of bacteria causes pH and colour changes amongst the wells indicating the MIC. Cira et al. [61] Reproduced by permission of The Royal Society of Chemistry

In a study by Boedicker et al. [38]; nano-sized plug-based microfluidics is used enabling rapid detection and drug susceptibility screening, most notably, without pre-incubation times. This method was originally developed in an attempt to reduce the sample size necessary for the bacterial strain’s MIC to be detected against specified antimicrobials. In this way, the detection time is proportional to the plug volume itself. In using this microfluidic/plug-flow hybrid method, this technology determined the antimicrobial resistance profile of MRSA to many antimicrobials in a single experiment (Figure 5).
2.3.2 Microfluidics Approach to Biofilm

As the MBEC of bacteria embedded biofilms can be 10 to 10,000 times that of the MIC of their planktonic counter parts [17], determining which antimicrobials MRSP biofilm is susceptible to, and at what concentration, could assist in both the determination of antimicrobial profiles as well as contribute toward the understanding of how biofilms inherit their resistance. It is also important to understand the physical and chemical parameters that govern the virulence factors behind these mechanisms, especially in systems that mimic the natural environment of these organisms. The initial ability of bacteria to form biofilms and the properties of these films are known to be highly dependent on the initial adhesion of the single cells to a surface which can be affected by the hydrodynamics of the local environment.

A single experiment that could use the laminar properties of microfluidics to test multiple concentrations of an antimicrobial against biofilm would create a rapid and reliable assay. Using stable generation of gradients under flow, pre-formed biofilm grown under continuous culture in a dynamic environment could be subjected to conditions imitating physiological microenvironments such as varying sizes of fluid through the shapes of micro-channels, shear stress applied, and varying fluid pressure and flow rates. A method such as this could also...
provide determine more precisely clinically relevant MBEC dosages and further aid in the design of antimicrobials with rapid screening format.

2.3.3 Passive Free-Flow and Bacterial Analysis

Microfluidic systems can be split up into two general categories, passive and active mixers. Passive mixers allow for easier delineation of gradients produced within a device. In order to more accurately produce steady gradients over a formed biofilm passive devices are the most applicable, using fully laminar flow and diffusive mixing. As mentioned, gradients produced in flow-based microfluidic devices are inherently unstable—in comparison to flow-free diffusive-based devices—but this does not impair relative comparisons amongst experimental conditions when analyzing bacterial parameters. Beneficially, flow also creates a more physiologically relevant environment when compared to purely diffusive chemical gradient production in absence of flow [59,62] while allowing for real-time changes in the chemoeffectors gradients [63,64]. Lin et al. [65] further demonstrated that a simple mixer module or micro-channel networks can be used to generate and manipulate very precise and numerous different gradient shapes with dynamic control. Simply, with the set-up of different styles of fixer modules temporal and spatial gradients were achieved through use of syringe pumps to control the relative flow rates.

To create more precise and stable gradients of chemoeffectors, comparable to that of flow-free devices, Englert et al. [66] built on the Lin et al. model and developed a precise gradient generator before a bacterium suspension was added to the main chamber (Figure 6). After being tagged with florescence, E. coli cells were imaged with red and green epifluorescence over 20 minute periods after addition to the chemotaxis chamber. Quantified results of the bacterial concentration profile across the main channel demonstrated that
concentration gradient profiles of the added chemoeffectors can be easily modified leading to more precisely controlled (flow-based) environments. However, the addition of the gradient generator did not create an entirely steady gradient in the chemotaxis chamber, since the gradient ultimately still changes with distance downstream. As well, they did not address the implications of hydrodynamic forces induced by the microfluidic channel on single cell and population dynamics.

Figure 6 – Gradient generator based microfluidic device for bacterial chemotaxis analysis. Gradients of chemoeffectors are formed across the chemotaxis chamber. Englert et al. [66] Reproduced by permission of The American Society for Microbiology.

To test the effects of hydrodynamics on bacteria in a microfluidic channel, Lanning et al. [64] observed the impact of bacterial transport in convective flows using a T-sensor. The device was designed to expose bacteria to unsteady gradients and mechanical forces due to fluid-flow in the main microfluidic channel (Figure 7). An important comparison was made by Lanning et al. as experiments were taken and compared under free-flow and flow-free conditions. When operated under flow the bacteria were exposed to an unsteady gradient and the mechanical forces of fluid flow. Under flow-free conditions, the gradient was allowed to diffuse freely across the main channel. Through qualitative imaging analysis a comparison of bacterial distribution at the same channel cross-section over time was made between the two. Observations indicated that
chemotactic migration was due to the attractant gradient and the response was independent of the stream orientation or flow phenomena.

Figure 7 – T-shaped microfluidic device in the assessment of hydrodynamics on bacterial chemotaxis. Set-up for T-Sensor experiments with an impinging flow interface. Experiments included streams of; just E. coli bacteria in both streams, E. coli and lysine in opposing streams, E. coli in both streams and an attractant in one stream, E. coli in both streams and an attractant in the opposite stream of the previous experiment at different flow conditions. Lanning et al. [64] Copyright © 2008 Wiley Periodicals, Inc.

The results of the Lanning et al. study were reinforced with the creation of a Y-shaped channel for chemotaxis to determine preferential chemotactic responses due to channel shape [67]. Kim et al. [67] created a device that enabled highly sensitive analyses of the chemotactic response of motile bacteria cells swimming toward a preferred chemoeffector by sorting and concentrating them (Figure 8). Buffer solution, buffer solution with a fluorescence cell inoculum, and a carbon source chemoeffector were each flowed through different inlet wells. Under high flow rates cells were carried down the middle-stream showing biased chemotaxis in down-stream concentrators due to delayed activation of chemoreceptors (chemotactic response of cells is highly amplified with time). With low-flow rates the cells moved randomly with migration toward both buffer and carbon source streams. This further confirms that the effects of hydrodynamic shear stresses on cells on channel surfaces and vice-versa can be much weaker than originally expected, given proper channel design.
Figure 8 – Y-shaped flow device for bacterial cell sorting through chemotaxis (A) Schematic of the device that can sort and concentrate motile bacterial cells. (B) The fluorescence image of the concentration gradient of FITC (50 μM) across the channel and a concentrator connected with arrowhead-shaped ratchet structures. (C) SEM image of the ratchet structure that can unidirectionally relocate cells from the entrance to the center of the concentrator. Kim et al. [67] Reproduced by permission of The Royal Society of Chemistry

Several studies have examined the effects of hydrodynamics specifically on biofilm development. For example, microfluidic devices were used by Lee et al. [68] to study the influences of hydrodynamics of local microenvironments of S. epidermis biofilm formation. They observed that at high flow velocity the cells formed elongated biofilm morphology, and at low fluid velocity clump-like multilayered biofilms were produced. The results of this study indicate that microfluidic devices with embedded micro-valves can perhaps be used for screening the effects of therapeutic reagents, and as novel tools for developing predictable in vitro models of biofilm related infections. Richter et al. [69] examined the influence of shear stress on the
growth and structure of fungal biofilms using microfluidic systems, increases in shear stress caused significant change in the biofilm formation pattern. This work was further supported by a study by Rupp et al [70], who observed that the compliant viscoelastic nature of *S. aureus* biofilms allows them to resist surface detachment from applied fluid shear. Work by Kumon *et al.* [29] also demonstrated the effect of micro-scale confinement features on the morphology and formation of biofilm along a micro-channel noting the importance of shear forces.

Finally recent studies have shown the use of these gradient producing devices detailed in Lin *et al.* and Englert *et al.* in the determination of MBEC of a chosen bacterial strain in both the planktonic and biofilm state (Figure 9) [71]. Here Kim *et al.* [71] were able to grow fluoresce-expressing *Pseudomonas aeruginosa* biofilm along the main detection channel of the device and flow stable gradients of chemoeffectors over top. The response along the detection channel was then recorded through by an inverted fluorescence microscope allowing for rapid and reliable determination of effective doses of antimicrobials against biofilm-embedded cells.

**Figure 9 – Gradient generator based device for biofilm analysis.** The microfluidic device consists of gradient generator and main detection micro-channel. Kim *et al.* [71] Reproduced by permission of The Royal Society of Chemistry.
Conventional methods for understanding surface morphology, antimicrobial susceptibility, and genetic variances on a base level can prove useful for assessing potential infection control therapy chosen there in. However, the complexity of biofilm and its formation in response to micro-environmental factors can contribute to persistence in SSIs. As MRSP biofilm-related infections present a large challenge in veterinary medicine, addressing ways to analyze and quantify their growth and response to infection control therapies in continuous culture and more physiologically relevant environments is needed. Current studies in free-flow based microfluidic devices show that hydrodynamic forces do not affect single cell or population dynamics of bacterial strains as was once thought. Therefore the use of a device similar to the “steady” gradient generating devices used by Lin et al. and Englert et al. would be ideal in the testing of multiple concentrations of antimicrobials against pre-formed MRSP biofilm in continuous culture. Research by Kim et al. [71] demonstrated a novel way to determine the MIC of preformed biofilms in continuous culture, however to make their assay more effective the device could be expanded, adding more mixing modules in similarity to device employed by Englert et al. This would allow for more precise gradients to be generated and controlled and more precise determination of the concentration of therapy necessary to decrease surface viable biofilm-embedded bacteria. Further optimization of protocol for MRSP will also be needed.
3 MATERIALS AND METHODS

3.1 Traditional Assays

3.1.1 Bacterial Strains and Characterization

MRSP isolates were obtained from clinical samples submitted to the University of Guelph, Ontario Veterinary College. A population of 50 epidemiologically unrelated MRSP isolates from dogs in Canada (n = 35) and the United States (n = 15) were selected and then characterized by sequence analysis of the mec-associated direct repeat unit (dru typing) with dru repeats and types assigned by the dru-typing.org database (http://www.dru-typing.org/search.php) [56]. Major international clones ST68 (n = 27, 54%) and ST71 (n = 17, 34%) accounted for 88% of the isolates. In order to assess the effects of select antimicrobials on bacterial biofilm produced by MRSP, the population was further screened for biofilm production via microtiter plate assay (MPA) [72]. One MRSA isolate (ATTC 29213) was also chosen to serve as a control in the FOS studies.

Following the standardized method set by Stepanovic et al. [72] to determine bacterial biofilm adherence through MPA, strains were first grown in pure culture overnight on Columbia agar plates with 5% sheep blood (MP0351; Oxoid Inc., ON) before suspension at a 0.5 McFarland standard (~10^8 CFU/ml) in tryptic soy broth (TSB) supplemented with 1% glucose (TSB+G). 200 µl of each inoculum was then transferred to a 96-well polystyrene microtiter tissue culture plate (3894; Corning Inc., NY, USA) in triplicate and incubated aerobically for 24 h at 35°C. This was followed by washing of the wells with phosphate buffered saline (PBS) three times to remove non-adherent cells, and heat fixation at 60°C for 1 h. Crystal violet at 0.1% (w/v) was then applied for 15 minutes to dye the cells before washing and drying at room temperature, and finally resolubilization of adhered matrix from the sides of the wells with 95%
ethanol. With an ELISA plate reader (ELx800; BioTek Instruments Inc. VT, USA) optical density (OD) readings—used as an indication of biofilm production—were taken for each of the wells at 570 nm (OD570) and averaged over each strain before subtraction from the OD570 reading of a negative control (wells containing uninoculated media). Strains were classified as biofilm producers if OD570 was >0.200 and further classified as weak (0.600 > OD570 ≥ 0.200), moderate (1.200 > OD570 ≥ 0.600) and strong (OD570 ≥ 1.200) biofilm formers [73].

For the assessment of CLA’s anti-biofilm forming effect, independent of its own antimicrobial activity [12,36,39,74-77], a sub-population of biofilm formers resistant to CLA was determined for further study (Section 4.1). Similarly, to assess the effects of FOS a sub-population of biofilm-formers was chosen and further tested for FOS-resistance. Potential synergistic activity between FOS and CLA was tested on isolates resistant to FOS and CLA to ensure an effect on biofilm formation was independent of their antimicrobial activity.

### 3.1.2 Antimicrobial Susceptibility

Kirby Bauer disk diffusion was used to determine the MIC of CLA on MRSP biofilm forming strains according to Clinical and Laboratory Standards Institute (CLSI) guidelines [78]. Strains were first cultured overnight before suspension in phosphate buffered saline (PBS) at a 0.5 McFarland standard (~10^8 CFU/ml). Inoculation of Mueller-Hinton agar plates with the chosen MRSP isolate was then completed through streaking, ensuring even distribution of the inoculum amongst the medium. Synthetic disks preloaded with CLA were then applied to the surface of the agar plates and incubated aerobically for 24 h at 35°C. MRSP resistance was then categorized as having high-level, intermediate-level, or low-level resistance based on the zone diameter present around the CLA disks.
The MIC of FOS was determined by agar dilution according to CLSI guidelines [53]. 31 biofilm formers of the original population were grown in pure culture overnight before suspension in PBS to achieve a 0.5 McFarland standard (~10^8 CFU/ml). Using a Steer replicator, these suspensions were then inoculated onto plates of Mueller-Hinton agar supplemented with FOS and 25 μg/ml glucose-6-phosphate (G6P). G6P is naturally found in vivo and has been shown to improve FOS uptake [43,79,80]. As no CLSI-approved susceptibility breakpoints exist for FOS against MRSP in dogs and staphylococci in general [78], EUCAST (European Committee on Antimicrobial Susceptibility Testing) breakpoints were used to determine susceptibility with an MIC ≤ 32 μg/ml [81].

3.1.3 PCR Analysis for Resistance Genes

Polymerase chain reaction (PCR) was completed to identify and determine the potential prevalence of a gene associated with plasmid-based FOS resistance in staphylococci, fosB. The sequence of fosB in S. pseudintermedius was obtained from a methicillin-resistant reference strain HKU10-03 (NCBI Reference Sequence: YP_004148680.1). Across the 31 MRSP isolate sub-population, isolates were grown overnight in pure culture before one colony forming unit (CFU) was suspended in 0.5 ml of standard TE buffer mixed with an equal volume of glass beads, each and vortexed for 10 minutes. The cell lysate supernatant was then extracted before the full-length of the fosB gene was amplified by PCR using the corresponding genomic DNA as a template with primer pairs obtained through BLAST sequence alignment tool (http://www.ncbi.nlm.nih.gov/blast/). PCR primers were fosBFwd, 5’ ACC GGT ACT TTA CAA GAG CGT 3’, and fosBRev, 5’ AAC AGC ACC ATC ACT TCC TT 3’. PCR amplification cycling conditions for fosB consisted of 2 min of denaturation at 95°C, followed by 30 cycles of
denaturation at 95°C for 15 s, annealing at 57°C for 15 s and extension at 72°C for 30 s. One PCR product was sequenced to confirm amplification of \textit{fosB} during assay development.

3.1.4 Microtiter Plate Assays

In order to evaluate the effect of CLA on biofilm, independent of its antimicrobial activity, twenty of the MRSP isolates that were found to be CLA resistant by Kirby Bauer disk diffusion (Section 3.1.2) and classified as biofilm producers (Section 3.1.1) were chosen for further study. CLA resistant isolates were chosen to ensure that any impact of CLA on biofilm formation was independent of its antibacterial activity. Standard MPA protocol, as described above, was used comparing biofilm production in TSB-G and TSB-G + 8 µg/ml CLA. A summary of the MPA protocol can be seen in Figure 10. Breakpoint doses for CLA resistance (≥ 8 µg/ml) [78] were chosen to represent a concentration that can be readily achieved \textit{in vitro}. Experimentation assessing biofilm formation and the time-course effect of CLA was also completed. A single high biofilm-forming isolate was chosen from the sub-population and 10 biological replicates assessed using the MPA adjusted with time points at 4, 8, 12, 16 and 24 h.

To assess potential use of FOS in monotherapy and synergism with other antimicrobials against biofilm formation, independent of antimicrobial activity, seven biofilm producing (OD\textsubscript{570} >0.200) MRSP isolates resistant to CLA and FOS (Section 3.2.1) were studied. Biofilm formation was evaluated by MPA through comparison of selected isolates treated with the following therapy; no treatment, high FOS (64 µg/ml), low FOS (8 µg/ml), CLA (8 µg/ml), and FOS (8 µg/ml) + CLA (8 µg/ml). Breakpoint doses for CLA resistance (≥ 8 µg/ml) [78] were chosen to represent a concentration that can be readily achieved \textit{in vitro} through CLSI standards.
Antimicrobial synergy was assessed by the fractional inhibitory concentration index (FICI), represented by Equation 1 [82,83]. The FICI formula calculates the potential synergistic relationship between two antimicrobials based on mono- and combination therapy MIC values. FICI values were interpreted as synergistic (FICI ≤ 0.5), synergistic to additive (0.5 < FICI ≤ 1), indifferent (1 < FICI ≤ 4), and antagonistic (FICI > 4) [83].

\[
FICI = \frac{MIC(FOS + CLA)}{MIC(FOS)} + \frac{MIC(FOS + CLA)}{MIC(CLA)}
\]

### 3.1.5 Statistical Analysis

To analyze the effect of CLA alone on MRSP biofilm production against a control through MPA a Student’s t-test was completed. Data from the MPA experimentation assessing FOS and CLA synergism against monotherapy was analyzed through one-way ANOVA with post-hoc Tukey’s Studentized Range test to adjust for multiple comparisons with the control. To make a categorical comparison of FOS resistance with the prevalence of the predominant sequence types (68 versus 71) in the chosen population a Fischer’s exact test was implemented.
Before the data were accepted, examinations of influence and fit diagnostics were also completed to check for leverage of observations on the data sets. In all tests, an adjusted p-value of < 0.05 was used to determine significance. All statistical analysis was performed on commercially available software (SAS 9.2 TS Level 2M3; SAS Institute Inc., NC, USA).

3.2 Bacterial Biofilm Imaging and Validation

3.2.1 Scanning Electron Microscopy

To evaluate the effect of CLA and FOS on MRSP adhesion to a different abiotic and clinically relevant surface, SEM was used to image bacterial adherence and biofilm matrix on inoculated orthopaedic bone screws. Commonly used in canine orthopaedic surgery at OVC, 316 LVM stainless-steel 20 mm orthopaedic bone screws and 316 LVM titanium 20 mm orthopaedic bone screws (Veterinary Orthopaedic Implants, FL, USA) acted as a substrate for biofilm growth in the assessment of CLA and FOS in monotherapy, respectively. One high biofilm producing MRSP isolate (MRSP A12) was chosen from the population and inoculated at a 0.5 McFarland standard suspension in 5 ml of TSB-G + 25 μg/ml G6P. The screws were added to tubes containing the suspension with and without 8 μg/ml CLA or 0.8 μg/ml FOS and incubated at 35°C. In monotherapy with FOS treated samples, the concentration was chosen at a level below the selected strain’s MIC. At time points of 4 through 24 h the screws were washed with PBS, fixed with 2.5% glutaraldehyde for 24 h and rinsed in Sorensen’s phosphate buffer for 15 min three times. This was followed by post-fixation in 1% osmium tetraoxide for 30 min at room temperature, washing in Sorensen’s phosphate buffer for 15 min two times, dehydration through an ethanol gradient (50-100%), critical-point drying, and finally sputter coating with gold.
All samples treated with and without antimicrobials were imaged at 4 levels (3.0, 10, 30, and 100 µm) at two locations—along the head and between the threads of the orthopaedic screws—using a Hitachi S-570 scanning electron microscope. Image acquisition location was standardized across all replicates in relation to the detector beam, with images taken in the top-right quadrant of the screw head, and second screw thread along the minor diameter. Percent particulate coverage of the surface of titanium orthopaedic screws was determined from multiple SEM images of the same region of interest using ImageJ image analysis program (National Institute of Health, Bethesda, USA). The gray-scale SEM images were converted to binary format and the percent white-to-black pixels were calculated for each of the images. The SEM images were also visually ranked for microbial biofilm morphology. As the material properties of biofilm attachment site play a minor role in the susceptibility to and persistence of staphylococci infections [84] control samples for CLA with stainless steel and FOS with titanium were also qualitatively compared.

3.2.2 Sonication and Enumeration

To complete validation of the SEM imaging protocol, enumeration of treated and untreated adhered biofilm grown on stainless steel and titanium screws was completed after removal by sonication. The same high biofilm-forming strain (MRSP A12) from the population was grown over night before inoculation at a 0.5 McFarland standard suspension in 5 ml of TSB-G + 25 µg/ml G6P. Titanium screws were added to the inoculated media with and without 0.8 µg/ml of FOS and incubated for 24 h. After the growth period, the screws were removed from the tubes, washed with PBS to remove non-adherent bacteria, and then transferred to tubes containing fresh TSB-G. Samples were then sonicated for 2 minutes (Branson Ultrasonic Cleaner Model 2510; Emerson Electric Co., MO, USA) and vortexed for 15 seconds to disperse
previously adhered biofilm amongst the media. Serial dilutions of $10^{-1}$ through $10^{-5}$ of the inoculum for each screw were plated and colony forming units (CFU) counted after overnight growth.

3.2.3 **Statistical Analysis**

Mean particulate coverage on SEM images in two areas of the screws were assessed with Kruskal–Wallis one-way ANOVA. Enumeration profiles of biofilm adhered to screws was analyzed using Student’s t-test to compare biofilm growth between antimicrobial treatment and the control. In all tests, an adjusted p-value of $< 0.05$ was used to determine significance.

3.2.4 **Atomic Force Microscopy**

AFM imaging was also implemented to qualitatively assess adhesion properties and characterize MRSP biofilm structure on mica sheet surfaces under high resolution. AFM can be used to investigate the adhesion force on single live cell surfaces and determine the surface charge and growth patterns of chosen strains of MRSP. Methods below outline preparation of biofilm growth on mica sheets needed to proceed with AFM protocol. For morphological studies, one high biofilm-forming isolate (MRSP A12) was chosen from the population and inoculated at a 0.5 McFarland standard suspension in 10 ml of TSB-G + 25 μg/ml G6P and grown to late mid-log phase. Samples were also treated with concentrations of FOS below the selected strain’s MIC. The cells in a 1 ml sub-sample were centrifuged in a Scilogex Model D3024 (CT, USA) microfuge at 3500 rpm for 3 min at room temperature, and washed 3 times with sterile analytical-grade water. The pellet was again suspended in deionized distilled water and the concentration of the bacteria was measured by a spectrophotometer (ELx800; BioTek Instruments Inc. VT, USA) at 420 nm. Freshly cleaved atomically flat mica sheets were added to petri-dishes containing the bacterial cell culture suspension with and without 0.8μg/ml FOS and
incubated for 4 h through 24 h at 35°C (Figure 11). Growth on mica sheets allows for imaging of the surface morphology or adhesion kinetics of a sample with little interference from substrate undulations.

Upon incubation, the mica sheets were gently removed using fine tip tweezers, washed in free-flowing nano-pure water to remove freely attached cells and dried at room temperature for 3 h. AFM imaging was then carried out for both the control samples and those treated with FOS. Analysis was done with duplicate cultures for each time point with cells imaged in air with a tapping mode atomic force microscope (Dimension Icon SPM; Bruker, CA, USA). AFM height, amplitude and phase images were obtained in AC mode on the air-dried mica substrates. A triangular Si cantilever tip (Bruker AFM Probes, CA, USA) with a spring constant of 0.35 N/m and a resonance frequency of 18 kHz was used. A scan speed of 0.7-1.5 Hz was set and resulted in a final resolution of 512 by 512 pixels.
3.3 Microfluidic Assay for Biofilm Analysis

3.3.1 Microfluidic Passive Flow Devices

The characteristics of passive mixing in microfluidic platforms was utilized to create a gradient forming device capable of studying MRSP biofilm formation and remediation by a range of antimicrobial concentrations in one experiment. Laminar flow present in passive devices allows for stable and predictable gradients to be formed between two or more solutions, unaffected by turbulence [85]. In a study by Kim et al. [71] they utilized gradient forming modules to successfully test varying antimicrobial concentrations on green fluorescent protein (GFP) expressing Pseudomonas aeruginosa biofilm. Here two solutions were passed through a gradient generator before reaching a single straight channel—where mixing is then dominated solely by molecular diffusion—with preformed biofilm. To create a similar assay for MRSP biofilms a larger mixing module was implemented allowing for a smoother gradient to form across the straight channel of the device (Figure 12A). Protocol was also optimized for MRSP biofilm growth in reference to previously completed conventional studies (Section 3.1). With growth of MRSP biofilm along the main channel of the device, antimicrobial therapy and a buffer solution can be passed through the mixing module and into the observation module to discern the effect of multiple concentrations of therapy (Figure 12B). Syringe pumps (HA1100; Harvard Apparatus, USA) were utilized to flow the solutions and inoculum through the micro-channels. Capable of precisely controlling flow rates independent of fluidic resistance, they are ideal for producing steady and predictable gradients across the main channel of the device.

3.3.2 Device Design and Fabrication

Device master molds used in these experiments were designed and fabricated at the Centre for Nanophase Materials of the Oak-Ridge National Laboratory (TN, USA) using e-beam
photolithography processing to define a microfluidic template on SU-8(50) thick-film photoresist onto which negative polymer casting can be completed. E-beam photolithography, in the design of microfluidic devices, involves the removal of select parts of a polymer on substratum through UV radiation before coating with SU-8(50). This process allows for minimum feature sizes ranging from a few to hundreds of microns with high-aspect ratios resulting in less resistance in micro-channels by surface alterations. Thick-film photoresists also remain structurally stable after processing, adhere well to surfaces, and one wafer can be cast hundreds of times before disposal; making them ideal for this application.

Figure 12 – A passive mixing gradient forming device for bacterial biofilm analysis. (A) Gradient formation by the mixing module is stable in the straight channel (B) Set-up of a passive mixing device for biofilm analysis with gradient formation
The device utilized for these experiments contains 8 rows of mixing modules allowing for 10 different concentration ratios of the inlet solutions to flow into the observation module as can be seen in Figure 12. The device design includes two sections, consisting of a mixing module and an observation module with the following dimension details:

1) Mixing Module: Eight rows of mixers with micro-channels of 20 micron in depth, 100 microns in width and a total length of 18,750 microns in length (across all 8 rows).

2) Observation Module: Where the biofilm is grown and observed, it is 1000 microns in width, 40 microns in depth, and 12000 microns in length (between the inlet and outlet).

PDMS is deposited on the SU-8(50) master mold wafer etched with this design to create a negative copy of the device (Figure 13). PDMS is a widely used organic silicone polymer in the field of microfluidics [27] made of vinyl and hydrosiloxane groups that, when a cross-linking curing agent is introduced, catalyzes the formation of long polymer chains and solidifies the compound. Transparent, biocompatible, and moldable before curing makes it ideal for microfluidic platforms designed for chemotaxis and other forms of optical detections [86]. In comparison to other polymers, PDMS’s biocompatible properties have been found to have less influence on bio-sample analysis [87,88]. Created with Sylgard 184 PDMS kits (Dow-Corning, USA), the silicone elastomers and curing agents from were mixed in a 10:1 ratio before being poured onto the SU-8(50) master wafers (Figure 13A), desiccated to remove air-bubbles, and then baked at 60 °C for 4 hours. After curing/baking the PDMS devices were carefully cut and mechanically peeled off of the SU-8(50) master wafers and a biopsy punch (Harris Uni-core 15072; Ted Pella Inc., USA) was used to form 0.75 mm diameter inlet and outlet holes with which to connect tubing (Figure 13B and Figure 13C). The PDMS surface containing the micro-channels and a glass cover slip were then surface-treated prior to bonding with oxygen plasma
(PDC-002; Harrick, USA) at 1 kV with 300 mTor of oxygen for 1 minute. This treatment was found to be the most reliable, rapid, and robust bonding technique for microfluidic devices by Eddings et al. [89].

**Figure 13 – PDMS molding process on SU-8(50) master wafers** (A) Device design is etched onto SU-8(50) photoresist (B) PDMS is poured over top and cured before being cut off and holes punched (C) PDMS device is bonded to a glass cover slip to form the micro-channels and inlet/outlet tubes are added.

### 3.3.3 Device Characterization

As the assay method involves the assessment of pre-formed biofilm reduced by a gradient of antimicrobial concentrations, characterization of the concentrations across the width of the channel needs to be determined. To characterize this gradient, 50mM fluorescein (07217; Sigma Aldrich, USA) and PBS were passed through the device and imaged with an inverted epi-fluorescence microscope (LumaScope™ 500; Etaluma Inc., USA) (Figure 14A). By normalizing a linear profile of the fluorescence intensity across the width of the channel, a relation can be formed with other solutions used in testing, such as antimicrobials (Figure 14B). Here PBS and
50 mM of fluorescein in double-deionized water were added to separate 1 ml 4.64 mm syringes (Becton, Dickinson and Company, NJ, USA), connected to the inlets of a prefabricated PDMS device (Section 3.3.2) with teflon tubing (SWTT-24-50; Zeus, USA) and subsequently passed through with a syringe pump (HA1100; Harvard Apparatus, USA). Images were then taken in total darkness with the inverted epi-fluorescence microscope at varying spots along the main channel. The excitation LED is optimized to image at a single excitation/emission wavelength (488 nm) which matches that of the fluorescence.

![Diagram of fluorescein and PBS flow](A)

**Figure 14 – Ideal characterization of a two-source gradient across the main channel of the device using fluorescein** (A) Inlet set-up for the device (B) Ideal normalized fluorescence gradient formed across the width of the device

Stability in the flow rate and gradient formed may be affected by small changes micro-tube diameter and pulsatile flow created by syringe pumps at low flow rates [90]. Syringe pumps utilized here are specifically designed not to exhibit pulsatile flow however, the micro-tubing
may still impose an effect. To ensure gradient formation was unaffected during characterization assessment of the two-source gradient generator was made as the flow evolved and maintained a steady state profile over 12 hours.

3.3.4 **MRSP Inoculum Preparation**

One clinical isolate of MRSP from the population was selected to test the microfluidic assay based on its biofilm forming capability as determined previously by MPA (Section 3.1.1). The chosen isolate was subcultured on Columbia agar plates with 5% sheep blood under pure 24h growth at 35°C. Cultured bacteria were then used to inoculate 5 ml TSB-G tubes at a 0.5 McFarland standard (~10^8 CFU) and cultured again at 35°C and 140 rpm for 4 h to reach mid-log phase. This second growth period was used to encourage cellular adhesion to the main channel of the microfluidic device upon application. The bacterial suspension was then concentrated down to 1 ml by centrifugation and washed with PBS at 5000 rpm for 5 minutes 3 times to remove the TSB media from the PBS inoculum. Before application into the main channel of the device the cells were stained with SYTO-9 fluorescent from a LIVE/DEAD® BacLight™ bacterial viability kit (Molecular Probes, USA) by the manufacturer’s recommendations. Staining procedures do not affect cell viability [91] but effects on biofilm growth formation have not been elucidated. Stained bacterial samples were then tested for fluorescence using the inverted epi-fluorescence microscope in darkness and a focus level obtained for the cells in preparation for the microfluidic experimentation.

3.3.5 **Antibiotic Preparation**

FOS was used at 3 set concentrations (16 µg/ml, 32 µg/ml, 64 µg/ml) in analytical grade double-deionized water to determine minimum MBEC needed to remediate preformed biofilm. As the MBEC is notably higher than that of planktonic cells [17,92] a range reaching up to 100
times that of the MIC of the selected isolate were applied (Section 3.1.2). Different inlet concentrations of FOS were created through serial dilution.

### 3.3.6 Microfluidic Biofilm Assay Protocol

The fabricated PDMS microfluidic device (Section 3.3.2) is first pre-conditioned with PBS at a set flow rate of 10 µl/h for 4 h to remove air-bubbles from the micro-channels and remove the surface hydrophobicity of the glass slide substrate. Air-bubbles or other obstructions can negatively affect the formation of the gradients in the mixing module. PBS filled syringes were connected to the inlets of the device (Figure 13C) by tubing and pumped through the micro-channels by the syringe pump. After 4 h, the flow was stopped, an inlet was replaced with a syringe containing FOS therapy, and a syringe containing the prepared bacterial inoculum was connected to the bacterial inlet (Figure 15). As MRSP biofilm production was incited within 4 h [93] the inoculum was introduced into the main channel at low flow rates (3 µl/h) for 4 h at 35°C with a heating pad attached to the stage of the microscope.

![Microfluidic experimentation set-up](image1)

**Figure 15 – Microfluidic experimentation set-up**
Fresh TSB+G was then passed through the main channel from the bacterial inlet for 20 h at 35°C to facilitate growth. After a total elapsed time of 24 h, the syringe pump was turned off and restarted with the syringes containing PBS and the desired FOS concentration (Figure 15). Images were taken using the inverted epi-fluorescence microscope in darkness in series every minute during application of the antimicrobial gradient to the pre-formed biofilm.

3.3.7 Image Analysis of Adhered Biofilm

After both the biofilm culturing and antimicrobial therapy application periods, taken images of the main channel were analyzed for their fluorescence intensity profiles. The fluorescence intensity across the width of the main channel was used as an indication of the remaining biofilm and correlated to the normalized fluorescence profile determined in Section 3.3.3 to infer the MBEC. OTSU thresholding was employed to remove the background from the region of interest to aid in the calculation of the linear intensity profile across the width of the channel [94].
4 RESULTS AND DISCUSSION

4.1 Clarithromycin Effect on S. pseudintermedius Biofilm Formers

4.1.1 Population Studied

In order to assess the impact of the anti-biofilm effect of CLA on MRSP, not simply from inhibition of bacterial growth, 20 strains determined to be CLA resistant by Kirby Bauer disk diffusion were chosen for this study. The population contained 3 different *dru* types, corresponding to the two main international MRSP clones (Table 1) [95]. Of the 20 selected isolates 15%, 35%, and 50% were categorized as having strong, moderate, or low biofilm adherence properties, respectively (Table 1). The Clinical and Laboratory Standards Institute interpretive breakpoint for clarithromycin resistance (> 8 µg/ml) [78] was also chosen to represent a breakpoint concentration that is readily achieved through *in vitro* studies.

4.1.2 Anti-biofilm Effect of CLA on MRSP

The ability of MRSP to form biofilm is an important virulence factor [18,19] and while MRSP is closely related to MRSA, results suggest that there are important cross-species differences. While biofilm production was common amongst this collection of isolates from different major clones and geographic regions, there was no evidence that CLA inhibits biofilm formation, in contrast to previous reports on MRSA [12,39,96]. Through analysis by MPA, twenty CLA-resistant isolates had OD$_{570}$ readings ranging from 0.206 to 2.64 (Figure 16). There was no significant impact of CLA on MRSP biofilm formation on polystyrene, with a mean OD$_{570}$ ± SD of the 20 MRSP isolates with and without CLA of 1.0 ± 0.63 and 1.1 ± 0.59, respectively (p = 0.5216). Accordingly, results do not support the use of CLA for prevention of biofilm formation, and since there was no significant impact on biofilm formation regardless of classification, it is unlikely that CLA would have any impact on biofilm eradication. Biofilm
formation by high biofilm forming strain, MRSP A12, was evident by 4 hours of incubation and increased over time until 18 h (Figure 17) demonstrating that infection remediation is time sensitive.

Table 1 – Study isolates’ origin, biofilm adherence capabilities, sequence type, direct repeat unit (dru) for isolates chosen for CLA study.

<table>
<thead>
<tr>
<th>Isolate Selected</th>
<th>Adherence Capabilities</th>
<th>Dru Typing</th>
<th>Sequence Type</th>
<th>Origin Location</th>
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</thead>
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<tr>
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<td>LOW</td>
<td>9a</td>
<td>71</td>
<td>U.S.A</td>
</tr>
<tr>
<td>A12</td>
<td>STRONG</td>
<td>10h</td>
<td>68</td>
<td>U.S.A</td>
</tr>
<tr>
<td>A23</td>
<td>STRONG</td>
<td>10a</td>
<td>68</td>
<td>U.S.A</td>
</tr>
<tr>
<td>A46</td>
<td>MODERATE</td>
<td>9a</td>
<td>71</td>
<td>U.S.A</td>
</tr>
<tr>
<td>A56</td>
<td>LOW</td>
<td>9a</td>
<td>71</td>
<td>U.S.A</td>
</tr>
<tr>
<td>A92</td>
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<td>9a</td>
<td>71</td>
<td>U.S.A</td>
</tr>
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<td>9a</td>
<td>71</td>
<td>Canada</td>
</tr>
<tr>
<td>SP88</td>
<td>LOW</td>
<td>9a</td>
<td>71</td>
<td>Canada</td>
</tr>
<tr>
<td>SP90</td>
<td>STRONG</td>
<td>9a</td>
<td>71</td>
<td>Canada</td>
</tr>
<tr>
<td>SP91</td>
<td>LOW</td>
<td>9a</td>
<td>71</td>
<td>Canada</td>
</tr>
<tr>
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<td>LOW</td>
<td>9a</td>
<td>71</td>
<td>Canada</td>
</tr>
<tr>
<td>SP102</td>
<td>LOW</td>
<td>11a</td>
<td>68</td>
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</tr>
<tr>
<td>SP104</td>
<td>MODERATE</td>
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</tr>
<tr>
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<td>MODERATE</td>
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<td>Canada</td>
</tr>
<tr>
<td>SP114</td>
<td>LOW</td>
<td>9a</td>
<td>71</td>
<td>Canada</td>
</tr>
<tr>
<td>SP115</td>
<td>LOW</td>
<td>9a</td>
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<tr>
<td>SP116</td>
<td>LOW</td>
<td>9a</td>
<td>71</td>
<td>Canada</td>
</tr>
</tbody>
</table>

Dru typing of chosen biofilm forming isolates of MRSP reveals a representative population of strains commonly found in veterinary hospitals across North America. Adherence capabilities were determined based on the model developed by Stepanovic et al., 2000
**Figure 16 – Effect of Clarithromycin on MRSP after 24 h.** Biofilm forming potential of 20 MRSP strains and the effect of clarithromycin after 24 h as revealed by Crystal Violet Microtiter Assay. Mean OD570 ± SD of the MRSP isolates with and without clarithromycin were 1.0 ± 0.63 and 1.1 ± 0.59, respectively. Clarithromycin had no significant effect (p = 0.5126) on MRSP biofilm formation.

As current studies speculate that macrolides also act through modification of the immune system’s inflammatory response to infection, and/or through a direct effect on bacterial virulence [75,77,97] it cannot be excluded that CLA may be effective *in vivo*, however *in vitro* studies involving MRSA still support a preventative effect on biofilm formation [12]. It has also previously been shown that macrolide antibiotics affect quorum sensing—the initial mechanism behind bacterial biofilm formation and cell-cell communication—within the biofilm leading to reduced polysaccharide synthesis and instability of biofilm architecture [21,76,98]. So, it is assumed that there would be a detectable effect.
**Figure 17 – Time course study of the effect of clarithromycin on MRSP.** Biofilm forming potential of MRSP A12 strain and the effect of clarithromycin over time as revealed by Crystal Violet Microtiter Plate Assay. CLA had no significant effect on MRSP biofilm formation between 4 and 24 h.

It is possible that CLA does not impose a preventative biofilm forming mechanism on MRSP, as seen in MRSA, due to genetic variances not yet revealed between the two species. Currently, ica is considered to be the major operon responsible for staphylococcal biofilm formation [84] but its study in MRSP strains has not been performed. Alternative pathways for quorum sensing could also cause the mitigation of the demonstrated effect of macrolides. Dru typing results suggested a varying geographic distribution and representative chosen isolate population across the two current internationally predominant MRSP clones, ST68 and ST71 (Table 1) [22]. These results suggest that there are genetic differences and therefore the effect of CLA on different strains of MRSP are likely minimal however; in vivo studies may prove useful.
4.1.3 Scanning Electron Microscopy Analysis

Qualitative evaluation of micrographs produced by SEM on surgical 316 LVM orthopaedic bone screws revealed the ability of MRSP to form biofilm on the surface of and between the screw threads. Adherent bacteria were evident by 4 h of infection with EPS in variable amounts (Figure 18A and Figure 18D). Visually, CLA did not appear to inhibit MRSP adherence and biofilm formation, supporting results from the MPA. Non-homogenous biofilm formation was evident, with focal biofilm accumulation and circular deposition of biofilm evident on screw heads (Figure 18C and Figure 18F). Time assessed biofilm development, while only one isolate was studied, suggests that biofilm formation occurs rapidly in vitro, since adherent bacteria and EPS matrix were evident within 4 h by both the crystal violet MPA and through qualitative SEM evaluation of growth on surgical screws. Particulate image analysis comparing surface area coverage between 4 h and 24 h showed no statistical difference between the control (15.2% and 20.4%) and CLA treated (16.3% and 19.7%) samples across the growth time points (Figure 19). Assessment of the impact of CLA on biofilm formation on screws conforms to the crystal violet MPA results, and provides further evidence of a lack of efficacy of CLA for prevention of biofilm formation. The irregular biofilm patterns on screws—most notably the circular biofilm accumulations on screw heads—is consistent with preferential biofilm adhesion to invisible surface defects or irregularities in the machining process. This suggests that minor surface alterations, either from inherent defects or damage to implants during placement, could facilitate biofilm attachment in vivo.

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Figure 18 – SEM images of biofilms formed on canine orthopaedic bone screws treated with addition of clarithromycin (A)(D) Showing MRSP biofilm production at 4 h and (B)(E) showing increased EPS (extracellular polymeric substance) and morphological changes characteristic to biofilms at 24 h with and without CLA. (C)(F) Biofilm adherence around circular striations from machining on canine orthopaedic bone screws with and without CLA at 24 h growth period.
Figure 19 – Percent MRSP biofilm coverage on orthopaedic screw surface over 4 and 24 h time periods. Image analysis of particulate coverage of SEM images demonstrates no significant difference (p > 0.05) exists between treated and untreated samples. Extra-cellular polymeric substances and adhered and biofilm-embedded cells were highlighted against the background in the same locations across both samples.

Figure 20 – Deflation of biofilm matrix through SEM sample preparation methods. Cocci cells can be clearly seen embedded in the EPS matrix

Figure 20 denotes dehydration damage due to sample processing to present glycocalyx structures with cells visible under the biofilm layer. This may negatively affect the surface coverage assessment through image analysis and interpretation of the biofilm reduction by
clarithromycin. However further, enumeration (Table 2) of biofilm loosened from stainless-steel screws (without SEM preparation methods) through sonication confirms the quantitative and qualitative inferences of the micrographs with treatment of CLA having no significant effect on biofilm formation on screws ($p > 0.05$).

**Table 2 – Average number of MRSP bacterial colonies grown from stainless-steel screws treated with and without clarithromycin.**

<table>
<thead>
<tr>
<th>Dilution Factor</th>
<th>Average Number of Bacterial Colonies (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>$1 \times 10^1$</td>
<td>322 ± 12.3</td>
</tr>
<tr>
<td>$1 \times 10^2$</td>
<td>32 ± 1.1</td>
</tr>
<tr>
<td>$1 \times 10^3$</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>$1 \times 10^4$</td>
<td>0</td>
</tr>
<tr>
<td>$1 \times 10^5$</td>
<td>0</td>
</tr>
</tbody>
</table>

### 4.1.4 Limitations

Large variances in both the amount of bacteria and antimicrobial in suspension in the 200 µl sample before addition to the microtiter plate could add to uncertainty in crystal violet microtiter plate assay results as previously described [72]. Optical determination of 0.5 McFarland and quick doubling-time of *S. pseudintermedius* could also contribute to the large standard deviations found for each averaged isolate OD$_{570}$ reading (Figure 16). Microtiter plate washing techniques, as described in Stepanovic *et al.* [72], are not clear and more rigorous washing than intended could lead to the removal of transient bacterial biofilms, further adding to the variance in quantitative results for each isolate. Although the two currently most internationally prevalent MRSP strains were represented (ST68 and ST71) other biofilm forming strains of MRSP susceptible to the biofilm prevention mechanism in CLA might exist.
The *in vitro* evaluations of CLA on MRSP biofilm formation were performed on polystyrene and one orthopedically relevant abiotic biomaterial providing additional potential limitations to this study. Although no significant inhibitory effect of CLA on these materials was found, material properties of biofilm attachment sites could play a minor role in the susceptibility to and persistence of staphylococcal infections [84]. The SEM study also only accounts for one of 20 screened isolates across 11 biological replicates so the analysis is only preliminary and images are not representative of strains seen in the MPA. Because of the potential benefit to biofilm formation prevention and the safety of macrolides shown in long-term randomized macrolide therapy, further study and use of CLA in combinational therapy and on varying biomaterials is still warranted [21]. Notably, combinational therapy with CLA and varying antimicrobials has been shown to have appreciable effects against MRSA biofilm formation [12,39,96].

4.2 *Fosfomycin Resistance*

4.2.1 Population Studied

In this study, the effectiveness of FOS was analyzed across 31 MRSP strains through agar dilution tests using Clinical and Laboratory Standards Institute guidelines outlined in Section 3.3 [53]. A convenience sample of 31 epidemiologically-unrelated MRSP isolates from dogs from Canada (n = 21, 65%) and the United States (n = 10, 35%) were studied, characterized by sequence analysis of the mec-associated direct repeat unit (dru typing) (Table 3). Of the 31 selected MRSP isolates 17%, 35%, and 48% were categorized as having strong, moderate, or low biofilm adherence properties, respectively. Sequence type analysis had 28% of the population relating to the Europe originating clone ST71, and 54% of the population as ST68, originating in North America.
### Table 3 – Isolate origin, sequence type, and direct repeat unit (dru) analysis

<table>
<thead>
<tr>
<th>Isolate Selected</th>
<th>Origin Location</th>
<th>Sequence type</th>
<th>Dru type</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>U.S.A</td>
<td>71</td>
<td>9a</td>
</tr>
<tr>
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<td>n/a</td>
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</table>

Origin and Dru typing of MRSP isolates reveals a representative population. Fosfomycin susceptibility and the presence of fosB resistance gene were determined by agar dilution and PCR, respectively.
4.2.2 Fosfomycin Resistance MIC through Agar Dilution

The prevalence of chromosomally and plasmid based resistance to current antimicrobial therapy in bacterial infections is a growing problem that is causing clinicians to look to generally uncommon or older drugs for new effective treatments. FOS is an old antimicrobial that has seen a revival in its use in recent years due to its unique mechanism of action—which reduces the chance for cross-resistance—with low resistance rates and resistance determinants reported despite extensive use in Europe.

Structurally unrelated to other antimicrobials, and with its small size allowing for diffusivity through tissue in high concentrations, FOS inhibits the first step of peptidoglycan biosynthesis in bacterial cell walls by binding to UDP-N-acetyl-glucosamine enolpyruvate transferase \[43\]. *In vitro* study through agar dilution of the chosen population confirmed the effectiveness of FOS as an inhibitor against MRSP with MICs up to 64 µg/ml across all strains and as low as 0.125 µg/ml for 60% of the population (Figure 21). No CLSI-approved susceptibility breakpoints exist for the FOS against staphylococci so EUCAST breakpoints were used to determine susceptibility with an MIC ≤ 32 µg/ml \[81\]. Based on EUCAST breakpoint susceptibility for FOS with staphylococci (MIC ≤ 32 µg/ml) 84% of the chosen population was defined as susceptible, with an MIC\(_50\) of 0.125 µg/ml and MIC\(_90\) of 64 µg/ml (Figure 21). A Fischer’s exact test as well suggests a significant difference in susceptibility between the major clones, with 100% of ST68-associated *dru* types being susceptible compared to only 20% of ST71-associated types (p < 0.05).
Figure 21 – Distribution of estimated minimum inhibitory concentration (MIC) of fosfomycin (FOS) by agar dilution. Showing the distribution of estimated MIC for FOS against 31 MRSP isolates and 1 MRSA control. S = Susceptible, R = Resistant. Line denotes EUCAST breakpoint \( \text{MIC} \leq 32 \, \mu\text{g/ml} \).

Despite these favorable results demonstrating the effectiveness of FOS in monotherapy \textit{in vitro}, clinical data indicates that FOS is excreted in high-levels in urine suggesting the possibility of increased breakpoints for systemic infections \textit{in vivo} [99]. If systemic application levels for FOS are lower, the resistance breakpoint for systemic infections may also be lower as susceptible isolates may be clinically resistant as it will not encounter FOS in high concentrations in the body. However, recent studies have highlighted the clinical use of FOS alone and in combination being associated with a high-rate of clinical success for the treatment of nosocomial infection for other Gram-positive and Gram-negative bacteria [100,101].

The MIC of a high biofilm-forming strain MRSP A12 was determined with greater resolution through crystal violet MPA for use in morphological studies through SEM and AFM (Section 4.2.4). Results (Figure 22), suggest that the MIC of MRSP A12 for FOS is 0.8 \( \mu\text{g/ml} \), reducing the OD\textsubscript{570} below that of the negative control.
Figure 22 – Minimum inhibitory concentration (MIC) of fosfomycin (FOS) against MRSP A12 by microtitre plate assay (MPA). 0.8 µg/ml of FOS reduces the amount of biofilm produces through MPA by MRSP A12 below the negative control.

4.2.3 PCR Results

Among clinical strains of bacteria, use of this antimicrobial has resulted in the selection of mutant strains showing high-level chromosomal and plasmid-based resistance when used in mono-therapy for systemic infections in vivo [102,103]. In staphylococci, plasmid-encoded resistance results in enzymatic modification of FOS through the fosB gene after uptake into the cell [104]. Plasmid-based resistance to FOS has been regarded as a significant problem in the treatment of infections with this antimicrobial, mainly due to its capacity for dispersion [48] and the increased chromosomal plasmid data transfer that exists in biofilm environments. However, despite long-term use, it has been reported that microbial FOS resistance has not substantially increased in staphylococci [24] and Gram-negative bacteria [105-107].

In this study, PCR analysis identified the presence of fosB in 28 of 31 strains (87.5%) including those demonstrating low MICs, on average through agar dilution, with 20 of 24 (83%)
FOS-susceptible isolates and 7 (100%) FOS-resistant isolates holding the gene. Uncommon use in practice in North America suggests an extremely low likelihood that any of the host-animals from which the chosen MRSP isolates originated have been treated with FOS and there is little reported data on its use in clinical animal studies against S. pseudintermedius worldwide. However, the Fisher’s Exact test comparing FOS resistance and ST reported an association between determined FOS-resistance by agar dilution and biofilm-forming strains of the major clone ST71 (p < 0.05). This clone originated in and is still predominately found in Europe where FOS has been used in systemic infections of other Gram-positive and Gram-negative bacteria through intravenous administration [18,107]. Therefore, this presence of gene encoded FOS resistance in Gram-positive staphylococci microbes indicates potential resistance gene transmission between different bacterial strains or species.

The prevalence of *fosB* in both phenotypically FOS-resistant and FOS-susceptible isolates is surprising. This discrepancy could be a result of *fosB* being non-functional or repressed in these isolates, further indicating that additional study into the mechanisms of FOS resistance in MRSP is required. This would include investigating other FOS resistance genes that have been found in Gram-negative bacteria and potential chromosomal mutations. The detection but lack of effect of *fosB* seen here might be due to amino-acid residue mutations of binding sites of MRSP. Active-site residues Arg119, Lys90, Ser94, and Tyr100 are found to be important for catalysis of substrate binding, forming hydrogen bonds to phosphonate groups of FOS [108]. Illustrating the key role that Arg119 plays in Gram-negative *Pseudomonas aeruginosa* plasmid-encoded resistance gene *fosA* has been shown to bind to FOS and other phosphonates with the residue Arg119 [108]. The residue is conserved in other FOS resistance genes such as *fosB*, with Tyr100 and Arg119 being within hydrogen-bonding distance to two phosphonate oxygen of that
of FOS in \textit{fosA} [109]. In \textit{P. aeruginosa}, mutation of the Arg119 residue affecting the enzymatic function of \textit{fosA} abolishes FOS resistance as well as persistence found in cells [109]. Finally, Arg119 is one of two positively charged residues (Arg119 and Lys90) that help shield against the negative charge of phosphonate moieties, and it has been suggested that this may be another resistance factor against FOS nucleophillic attack [110].

In other species the mediation of FOS resistance could be two-fold as reduced plasmid transfer has been shown in FOS-resistant strains [111] wherein the possibility of acquiring and transferring plasmids carrying resistance determinants with adaptive mutations may contribute to survival [112]. In Gram-negative bacteria chromosomal mutants are incapable of transporting FOS through cell walls owning to an impairment of the L-\(\alpha\)-glycerophosphate or the glucose-6-phosphate uptake systems, which can cause profound alterations in bacterial physiology [113]. Such impairments have resulted in, (i) slower rates of growth in comparison to sensitive parental strains through biochemical poisoning by \(\alpha\)-glycerophosphate accumulation; (ii) reduced cellular adherence to indwelling biomaterials; and (iii) biochemical poisoning through physiochemical modifications to outer cell structure by surface hydrophobicity alterations [113-117]. In contrast to that of Gram-negative bacteria, there is little study describing chromosomally-mediated FOS resistance in clinical Gram-positive staphylococci and none for \textit{Staphylococcus pseudintermedius} [118]. This or receptor mutations may explain the low reports of both chromosomal and plasmid-based FOS resistance for other clinical staphylococci strains.
Figure 23 – Characteristic cell morphologies of MRSP biofilms and its surface coverage on titanium orthopaedic screws. The effect of fosfomycin against MRSP A12 strain on titanium orthopaedic screws was assessed microscopically. Scanning electron micrographs of 4 and 24 h old MRSP biofilms on orthopaedic screws are shown without (A), (C) and treated with fosfomycin (B), (D) respectively.
4.2.4 Surface Coverage and Morphological Effects of Fosfomycin

In the assessment of the efficacy of FOS against MRSP biofilm on a clinically relevant abiotic material, monotherapy with concentrations of FOS below the selected strain’s MIC were found to reduce adherence and biofilm structure on titanium orthopaedic screws. Objective study on percent particulate (clusters of biofilms) on the orthopaedic screw surfaces indicated significant decrease (p < 0.05) in adherence between control and FOS treated samples. In agreement with SEM images of the stainless steel screws, control samples had growth of complicated fibrous structures, biofilm-embedded cells, and colonies of bacteria noted as early as 4 h with increasing amounts of surface coverage after 24 h of growth (Figure 23A and Figure 23C). Comparisons between the samples indicate that surface area coverage by MRSP biofilm decreased from 13.9% to 0.8% due to FOS treatment over 4 h and from 18.2% to 0.3% over 24 h (Figure 24). Marked change in EPS production and density of adherent bacteria and biofilm structures was also noted at 4 h in samples treated with 0.8 µg/ml of FOS (Figure 23A and Figure 23B).

Figure 24 – Percent biofilm coverage on orthopaedic screw surface over 4 and 24 h time periods. Image analysis of particulate coverage of SEM images demonstrates that a significant difference (p < 0.05) exists between treated and untreated samples. Extra-cellular polymeric substances and adhered and biofilm-embedded cells were highlighted against the background in the same locations across both samples.
The image analysis shows a large statistical difference in biofilm coverage between the control and FOS treated samples, indicating higher efficacy and potential prevention of MRSP adhesion on clinically relevant surfaces. Enumeration (Table 4) of biofilm loosened from titanium screws through sonication confirms these inferences with treatment of FOS at below-MIC levels for the selected strain significantly decreasing formed biofilm (p < 0.05).

Table 4 – Average number of MRSP bacterial colonies grown on titanium screws treated with and without fosfomycin.

<table>
<thead>
<tr>
<th>Dilution Factor</th>
<th>Average Number of Bacterial Colonies (CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>1:10^1</td>
<td>468 ± 16.7</td>
</tr>
<tr>
<td>1:10^2</td>
<td>47.2 ± 1.5</td>
</tr>
<tr>
<td>1:10^3</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>1:10^4</td>
<td>0</td>
</tr>
<tr>
<td>1:10^5</td>
<td>0</td>
</tr>
</tbody>
</table>

To further elucidate the effect of FOS on MRSP cell wall synthesis and MRSP adhesion, AFM was employed. Assessment of the effect of FOS on MRSP biofilm through AFM showed distinct morphological variations when comparing large clusters of cocci shaped biofilms in untreated controls and treated samples (Figure 25A and Figure 25C). The cocci or spherical shape is evident in the control sample; while the cells are lysed in the FOS treated sample (strong modifications in cellular morphology with collapsed cell shape indicate lysis of the MRSP cells with FOS treatment). Untreated (control) MRSP biofilms grown over 4 h on mica sheets had a significantly larger diameter (1 µm) compared to the FOS treated MRSP biofilms with an average of 97 nm. In the treated sample, MRSP cells were well dispersed and isolated, appearing to be damaged with greatly lowered height.
Figure 25 – MRSP biofilm surface height profiles with corresponding AFM deflection mode images. (Scale = 5 µm) (A)(B) 50 µm² scan of MRSP A12 showing clusters of biofilms with extended chains exhibiting stable nanoscale morphology (C)(D) Fosfomycin treated MRSP biofilms exhibits greater deviation in nanoscale morphology and reduced height indicating the efficacy of fosfomycin. The cellular ultrastructure has significantly altered with less surface coverage and a smaller cell diameter.
Figure 26 – Time dependence of fosfomycin effects on MRSP imaged by AFM. Three dimensional orthogonal projection images (derived from the height data) of untreated MRSP cells (A)(C)(E), and MRSP cells treated with 0.8 μg/ml fosfomycin (B)(D)(F). Images were acquired following the treatment of the MRSP cells for 4 h (A)(B), 8 h (C)(D) and 12 h (E)(F). Total scanning area for the 8 and 12 h treated MRSP A12 images are 20 X 20 μm².
The effects of FOS on the MRSP cells over various periods of treatment are shown in Figure 26. The nature and degree of outer membrane damage on the MRSP cells caused by FOS treatment is demonstrated over 4, 8, and 12 h with substantial topographical changes such as shrunken cell size and reduced surface coverage. Increased incubation time and FOS treatment are correlated well with the bacterial outer membrane damage as observed by the decreased height from Figure 26 (B), (D), and (E). In addition, in similarity to the SEM results, the total number of cells present on the treated mica sheets was substantially reduced and the remaining cells displayed a tendency to scatter. The AFM image analysis clearly indicates that the effect of FOS on MRSP biofilms was intense indicating the possibility of cell-wall degradation.

In these three studies only one isolate was selected for analysis, yet the SEM and AFM image analysis data conform to the agar dilution data and provide further evidence of FOS’s direct bioactivity against MRSP growth in vitro.

4.2.5 Limitations

The SEM and AFM studies only accounted for one screened isolate across 6 biological replicates and are not representative of the chosen population and those data should be considered preliminary. Glucose-6-phosphate was used in preparation of the growth media for the agar dilution tests, which has been shown to facilitate FOS uptake, and is naturally found in vivo. However, there are no current studies demonstrating this effect directly with S. pseudintermedius as there are with other staphylococci [43]. Other factors affecting FOS uptake and effectiveness—demonstrated in vivo—were not possible to replicate and may affect results. In vitro activity of FOS against Gram-positive staphylococci was significantly greater under fully anaerobic conditions [80,119] of which was not used here. Under the anaerobic conditions, the transport system that delivers FOS into bacterial cells also increases providing further effects
against sessile cells and cells with low growth rates [46]. A study by Cao et al. [120], further demonstrates activation of \textit{fosB} in the presence of specific metal ions. Therefore, there is the possibility for improved \textit{in vivo} efficacy in areas with decreased oxygen levels or increased resistance \textit{in vivo}.

### 4.3 Fosfomycin Synergy against \textit{S. pseudintermedius} Biofilm Formers

As FOS can be used in combinational therapy to achieve synergism with aminoglycosides, quinolones, or \(\beta\)-lactams, to reduce biofilm matrix and potentially mitigate transfer of plasmid-based resistance it was paired with CLA [103,107,121-124]. With enhanced antimicrobial activity against biofilm embedded bacteria it has been shown to break-up biofilms and enhance the permeability of other antimicrobials [25,40,125]. Its low molecular weight (194.1 Da) and non-reactivity with negatively charged bacterial glyocalyx allows for efficient diffusion into tissues and multilayers of biofilm matrix may explain this activity [45].

#### 4.3.1 Population Studied

Through MPA the synergistic effects of FOS and CLA in combination were assessed against isolates demonstrating high FOS and CLA resistance as determined in previous sections. A sub-population of seven selected isolates were chosen with an additional strain (MRSP A12) chosen to act as a control for FOS susceptibility. As agar dilution testing demonstrated, this sub-population has a heavy bias toward ST71 MRSP clones, with strong, moderate, and low biofilm formers represented.

#### 4.3.2 Fosfomycin and Clarithromycin Synergism

Divided into five groups; no treatment, high FOS (64 \(\mu\)g/ml) treated, low FOS (8 \(\mu\)g/ml) treated, CLA (8 \(\mu\)g/ml) treated, and FOS (8 \(\mu\)g/ml) plus CLA (8 \(\mu\)g/ml) treated isolates MPA
results identified synergism between CLA and FOS in direct effect to biofilm production. FICI values revealed fractional synergy (FICI ≤ 0.5) of 0.31 to 0.56 in the FOS and CLA resistant strains in reduction of biofilm. As a set 1:1 combination of FOS and CLA (breakpoint doses for CLA resistance is ≥ 8 μg/ml) was chosen, the FIC may lower based on specific MICs against biofilm for each strain (Table 5).

Table 5 – Interaction of FOS and CLA against MRSP biofilms by microdilution arrays

<table>
<thead>
<tr>
<th>Isolate Selected</th>
<th>Sequence Type</th>
<th>Drug type</th>
<th>Adherence Capabilities</th>
<th>FOS (µg/ml) MIC</th>
<th>CLA (µg/ml) MIC</th>
<th>FICI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A12</td>
<td>68</td>
<td>10h</td>
<td>STRONG</td>
<td>≥0.8</td>
<td>≥256</td>
<td>NA</td>
</tr>
<tr>
<td>A46</td>
<td>71</td>
<td>9a</td>
<td>MODERATE</td>
<td>≥64</td>
<td>≥256</td>
<td>0.31</td>
</tr>
<tr>
<td>A56</td>
<td>71</td>
<td>9a</td>
<td>LOW</td>
<td>≥32</td>
<td>≥256</td>
<td>0.56</td>
</tr>
<tr>
<td>A92</td>
<td>71</td>
<td>9a</td>
<td>MODERATE</td>
<td>≥64</td>
<td>≥256</td>
<td>0.31</td>
</tr>
<tr>
<td>SP90</td>
<td>71</td>
<td>9a</td>
<td>STRONG</td>
<td>≥32</td>
<td>≥256</td>
<td>0.56</td>
</tr>
<tr>
<td>SP106</td>
<td>71</td>
<td>9a</td>
<td>LOW</td>
<td>≥64</td>
<td>≥256</td>
<td>0.31</td>
</tr>
<tr>
<td>SP112</td>
<td>71</td>
<td>9a</td>
<td>LOW</td>
<td>≥64</td>
<td>≥256</td>
<td>0.31</td>
</tr>
<tr>
<td>SP113</td>
<td>71</td>
<td>9a</td>
<td>LOW</td>
<td>≥64</td>
<td>≥256</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Adherence capabilities were determined based on the model developed by Stepanovic et al., 2000. Fosfomycin and Clarithromycin susceptibility was determined by agar dilution and Kirby Bauer disk diffusion, respectively.

One-way ANOVA with a post-hoc Tukey’s against the control samples found that low doses of FOS at 8 µg/ml (p > 0.05) and CLA at 8 µg/ml (p > 0.05) independently produced no significant reduction in biofilm production, whereas treatment with FOS and CLA in combination resulted in a significant reduction (p < 0.05) in the number of viable bacteria (Figure 27) in one-way ANOVA models. To ensure this impact was anti-biofilm, not simply bacterial growth inhibition, strains demonstrating both FOS resistance (≥ 64 µg/ml) and CLA resistance (≥ 256 µg/ml) were chosen. As the strains tested are resistant to FOS in high doses, and MRSP has been found to resist the anti-biofilm effect of CLA in mono-therapy [93], this demonstrated synergism between FOS and CLA in vitro study is particularly interesting.
Figure 27 – Enhanced antibacterial activity of fosfomycin (FOS) and clarithromycin (CLA) against MRSP after 24 h. Biofilm forming potential of one ST68 strain (A12) and seven ST71 strains (A46, A56, A92, SP90, SP106, SP112, SP113) and the effect of FOS and CLA in mono and combination therapy. Mean OD\textsubscript{570} ± SD of the MRSP isolates with and without treatment were as follows; control, 1.82 ± 0.34; FOS\textsubscript{64 µg/ml}, 0.15 ± 0.03; FOS 8 µg/ml, 1.51 ± 0.37; CLA 8 µg/ml, 1.79 ± 0.41; FOS 8 µg/ml + CLA 8 µg/ml, 0.74 ± 0.26. Combination therapy had a significant effect (p < 0.05) while low doses of FOS and CLA alone had no significant effect (p > 0.05) on MRSP biofilm formation.

4.3.3 Potential Mechanism of Synergism against MRSP

The mechanism behind this synergism is unknown; however, some well documented reasons could explain the effect of FOS and CLA on MRSP biofilm. In \textit{S. aureus}, cellular adhesion is mediated by adhesive matrix molecules, which are covalently anchored to the cell wall peptidoglycan, and extracellular matrix fibronectin can serve as bridging molecule between
several bacterial species and a variety of host type cells [124]. In the case of *S. aureus*, it has been shown that fibronectin-dependent infections are mediated by the cell surface-expressed fibronectin-binding protein and the host cell integrin [126]. *S. pseudintermedius* expresses surface proteins that resemble those from *S. aureus* and has the capacity to bind to fibrinogen, fibronectin and cytokeratin of host cells. It also produces an immunoglobulin-binding protein called staphylococcal protein A (spa), similar to that of *S. aureus* [127]. It is possible that FOS might have influenced these two binding mechanisms by interfering with peptidoglycan biosynthesis of the bacteria, and inducing an increase in the penetration of CLA through pre-formed biofilm. As these strains tested in combinational therapy were less sensitive to FOS, it may also be able to maintain drug concentrations of CLA above the MIC used and allow it to destroy biofilm or reduce cells at the planktonic state.

Quorum sensing—the initial mechanism regulating biofilm formation and cell-cell communication—may also be influenced by the combined antimicrobials against MRSP biofilms. Accessory gene regulator (agr) quorum sensing and signal transduction has been described in *S. aureus* [128] and recently found in *S. pseudintermedius* [129]. Quorum sensing in Gram-positive bacteria have been found to regulate a number of physiological activities including induction of virulence factors in *S. aureus*. Macrolide antimicrobials affect quorum sensing within biofilm leading to reduced polysaccharide synthesis and instability of biofilm architecture [21,76] and it is possible that FOS may also influence the quorum-sensing signals of these strains.

### 4.3.4 Combination Therapy Benefits

Synergistic approaches have also been shown to reduce the possibility of resistance gaining in systemic therapy and has proven effective in reducing this occurrence for MRSA in
both *in vitro* testing and *in vivo* trials [83,103]. In addition, development of cross-resistance to FOS through the use of other antimicrobial agents has been regarded as insignificant, likely due to its unique bioactivity against bacteria [102]. For these reasons the use of FOS/CLA in combination therapy may prove effective for MRSP biofilm-forming strains in a clinical setting to reduce recurrent SSIs on indwelling biomaterials. However, additional *in vitro* studies using biofilm models across larger populations of strains and *in vivo* studies are warranted.

**4.3.5 Limitations**

As an *in vitro* study, a limited number of isolates was studied so inter-strain variation could not be clearly evaluated. However, the findings regarding the synergistic activity of FOS in combination with CLA indicate that further *in vitro* and *in vivo* study of this potential treatment approach is needed. Time-kill studies could further assess whether the synergistic effect seen here is purely due to an independent anti-biofilm effect. It should be mentioned that the use of FOS in the treatment of established clinical infections has not been adequately evaluated in large-scale randomized studies, other than for urinary tract infections despite combinational therapy being widely used [130]. Further limitations in the MPA protocol follow that of section 4.14 in the evaluation of CLA on MRSP biofilm
4.4 Microfluidic Platform Analysis of MRSP Biofilm

Microfluidic platforms were used to develop an assay for the application of multiple concentrations of antimicrobial therapy (alone or in combination) on pre-formed MRSP biofilm along a micro-channel. This would allow for rapid analysis of potential remediation therapies for normally recalcitrant SSIs formed by MRSP biofilms.

4.4.1 Generation of the Antimicrobial Gradient

In order to correlate the concentration of antimicrobial therapy applied to the biofilm-embedded cells across the observation module (Figure 12A), it was necessary to characterize the concentration gradient formed by the mixing module. To visualize the concentration gradient formed, 50 mM of fluorescein and PBS were introduced into the main inlets of the device at a flow rate of 10 µl/h (Figure 28) and images then taken down the length of the observation module. The stability of the linear gradient was also assessed to check for potential differences caused by diffusive mixing along the length of the module. This was completed by taking images at the connection point (0 mm) of the mixing module into the observation module and at increasing distances (3, 6, 9 mm) before measurement of the linear intensity profile across the width of the channel. Ten streams of varying (linear) concentrations were formed across the mixing module and injected into the observation module of the device. At 0 mm it was easy to delineate between the streams but as the gradient moves along the length of the channel it became stable through diffusive mixing and the streams became indistinguishable.
Characterization of a fluorescein gradient formed across a gradient forming microfluidic device at 0 mm along the observation module. Fluorescein and PBS are passed through the gradient generator and into the observation module.

Moving from the injection point (0 mm) to further down the micro-channel, the gradient became stable and uniformly maintained between 3 and 6 mm showing little difference in the linear intensity profile (Figure 29). As there is potential for the gradient to produce instability due to changes in flow caused by non-conformities [65], the gradient was also assessed in one location over 12 h. However, no significant changes to the linear profile were noted (p > 0.05). Characterization of the device suggests that a stable linear gradient was formed along the observation module which was used to calculate the concentration of antimicrobials used to determine the MBEC against formed biofilm. Figure 30 relates the normalized fluorescence intensity to the potential antimicrobial concentration across the width of the channel.
Figure 29 – Characterization of the steady gradient formed across the gradient generator microfluidic device along the observation module. A steady gradient is formed between along the observation module between 3 mm and 6 mm.

Figure 30 – Relation of normalized fluorescence intensity and fosfomycin (FOS) (µg/ml) therapy across the channel. The device’s characteristic linear intensity was normalized to represent 16 µg/ml FOS.
4.4.2 Quantification of MBEC against FOS

Growth of MRSP biofilm in the observation module of the microfluidic device under continuous culture allows for assessment of therapies in a dynamic spatio-temporal environment with more physiological relevance to wound infections. To quantify the MBEC of these biofilm, a suspended strain was stained with fluorescein before washing and being passed through the observation module of the device under continuous culture.

The MBEC was determined as the borderline between the normalized fluorescence intensity emitted by the biofilm and that of the background across the width of the channel of processed images (Figure 31). The concentration needed to reduce the biofilm to this point (i.e. the MBEC) was then interpolated from the characterized fluorescence intensity profile across the width of the channel found in Figure 30. As antimicrobial concentrations needed to reduce biofilm implicated infections can be much higher than their planktonic counterparts, concentrations of FOS well above the chosen strain’s MIC from the agar dilution study (Section 4.2) were initially chosen. FOS therapy at 16, 32, and 64 µg/ml were used to investigate the remediation of biofilm (Figure 31) representing concentration up to 80 fold of the MIC. Interpolations found the average MBEC of 16, 32, and 64 µg/ml therapies to be 8.6 ± 2.1 µg/ml. Recognized cell concentration (by a factor of fluorescence intensity) appeared stable in areas of low FOS therapy in contrast to areas of high FOS therapy, where cells appeared detached and washed away. These results support the potential use of this microfluidic assay for the rapid analysis of antimicrobial susceptibility of biofilm-embedded cells and further study of FOS for biofilm-implicated infections. With further optimization of this platform for physiological relevance, results may be more applicable to in vivo testing than conventional testing.
In comparing MIC values obtained through agar dilution (Table 5), a 10 fold difference from the MIC to MBEC is apparent for this tested strain (MRSP A12). This indicates a less dramatic difference between FOS concentrations needed to reduce planktonic and biofilm-embedded cells than other antimicrobials [17]. As the chosen strain was a high biofilm former (OD of 2.65 ± 0.02, Figure 16) but the susceptibility was low (0.8 µg/ml, Table 5) this effect may be due to slow diffusion through the biofilm matrix. And, although uptake systems are increased in favour of FOS binding in anaerobic environments the effect against sessile, low growth cells within biofilm will still be lower than that of planktonic cells [111]. Visual assessment notes little to no biofilm matrix present in areas of high FOS concentrations. Down-
regulation of surface polysaccharide metabolism genes in *S. aureus* [47] and further remediation of biofilm-embedded cells may explain this effect, as quorum sensing also plays a role in the attachment and growth of biofilms. These results further support the ability of FOS in the penetration and detachment of biofilm matrix seen in other species of bacteria [25]. However, the smaller difference in MIC vs. MBEC for FOS in contrast to other antimicrobials may be specific to this strain with different metabolic, genetic, or physiochemical properties in comparison to the rest of the chosen population. It is valuable, in this case, to test other high biofilm forming strains; especially those used in the synergistic study displaying high FOS-resistance. If FOS merely reduces biofilm matrix and cellular adherence, this would still leave infection areas susceptible to treatment with other antimicrobials.

In relating these results to that of the MPA study (Section 4.3) FOS is not effective at removing preformed biofilm at the low concentration noted to prevent biofilm production. Along with the potential down regulation of surface polysaccharide production by FOS [47], there may be changes to the morphology and physiochemical properties of biofilm in the dynamic fluid environment within the observation module. Although hydrodynamics of local environments play a smaller role in single cell and population dynamics than originally thought there are demonstrated effects in in the production of biofilm. Under high shear conditions biofilm become more strongly attached to substrates, demonstrating increased mechanical strength in comparison [131,132]. In continuous culture/dynamic environments the viscoelastic nature of *S. aureus* biofilms was shown to resist surface detachment by applied fluid shear [70]. Here, biofilm clusters exhibited “rolling migration behaviour” meaning that the biofilm responded to the hydrodynamics exerted upon it while staying attached to the given substrate by viscoelastic tethers. These properties are prevalent in a wide range of biofilm producing microorganisms and
they even share similar stress relaxation times that allow for further resistance of local forces of approximately 18 minutes [133]. As biofilm-embedded cells can modify their physiochemical properties in response to these micro-environmental stressors the need to test potential biofilm-remediation therapies in more physiologically relevant platforms than conventional testing is underscored. A further understanding of the physical properties of MRSP biofilms will also be needed to properly assess potential therapies.

4.4.3 Utility

The utility of this microfluidic assay may be adjusted to evaluate the effectiveness of therapies over time as a function of accumulative culture time. Using the standard protocol mentioned in Section 3.3, preliminary experiments had 100 μg/ml of FOS passed through the inlets of the mixing module with images of the observation module taken over 4 hours. In relating the change in surface coverage area (by function of average fluorescence intensity across the channel) over time, it was noted that appreciable differences in biofilm adherence was observed by 4 hours of incubation in comparison to a control test (double-deionized water added to the inlets for 4 hours with images taken at similar time points). This measure adds another level to the assessment of the chosen therapy; however, it cannot be related to that of proper pharmacokinetic and pharmacodynamics studies as the antimicrobial is applied directly to the source in consistent concentrations.
4.4.4 Validation

To further validate the microfluidic assay, the repeatability of the reduction in surface coverage of MRSP biofilm by a single concentration of FOS was evaluated. Figure 33 demonstrates the change in surface coverage across three devices with 16 µg/ml of therapy and PBS applied at the inlets under standard protocol detailed in Section 3.3. Across three successive experiments the MBEC of MRSP A12 with FOS was interpolated as 8.1 ± 0.9 µg/ml, indicating lower variation than tests comparing a range of inlet concentrations (8.6 ± 2.1 µg/ml). These results further support the use of the assay for the rapid assessment multiple concentrations of therapy on MRSP biofilms. In each test, it was further noted that biofilm production was more prevalent along the center of the channel, affecting average fluorescence intensity on the channel walls.

Figure 32 – Comparison of surface coverage through average fluorescence intensity after application of FOS therapy (100 µg/ml) through both inlets.
Figure 33 – Validation of results at across 3 subsequent experiments. Results indicate concentration of FOS to remediate biofilm-embedded cells of MRSP A12 to 8.1 ± 0.9 µg/ml. Fluorescence images are unprocessed for demonstration purposes.

4.4.5 Limitations

As there are no current studies detailing the analysis of MRSP with microfluidic platforms there are still challenges with this assay that need to be overcome. Through MPA analysis (Section 4.1) it is apparent that there are stark differences in the adherence capabilities of biofilm producing isolates across this population. The strength and coverage of these biofilms may be increased in dynamic microenvironments [131] but adherence in the observation module may still be affected making the standardization of protocol across multiple strains difficult. Differences in adherence strength will also affect accumulative surface coverage making it
difficult to discern the true borderline between the biofilm and background fluorescence intensity boundary.

A study by Choi et al. [134] correlated the fluorescence intensity in a channel to cell density through inoculum passed through the device. In this way, an approximation of the thickness of the biofilm could be made for this assay. However, as the biofilm-embedded cells in this assay are adherent to a substrate, confocal imaging or AFM may be the best course of action to determine average biofilm height across specific areas of the device. Correlating the thickness to the fluorescence intensity through similar methods would allow for the assessment of the effect of FOS on multilayer biofilm grown over larger and more relevant time frames. As MRSP biofilm infections are often detected in out-patients, the biofilm-matrix formed will be more mature with multiple layers and stronger connections between cells, than those used to evaluate antimicrobial therapy in these experiments. Further analysis of FOS in the remediation of more mature biofilm should be completed to further assess clinical relevance. Additionally, through confocal imaging/AFM knowledge of the morphology/topology of the biofilm could also give insights into the effects of surface undulations on micro-mixing of the linear gradients along the channel, which may affect the interpolation of the MBEC.

During experimentation with the microfluidic assay only one flow speed was analyzed in the production of the linear gradient across the formed biofilm. However, different flow rates can affect the stability of the gradient over the length of the micro-channel [90]. The chosen flow rate is generally accepted as maintaining physiologically relevant shear forces to bacterial cells [135], however, shear forces in wound models have been found to be much higher. If increases in flow do not detach biofilm from the micro-channel then they may further strengthen them, possibly
affecting the MBEC detected. Further analysis of biofilm production under varying flow rates and shear forces may also be completed to validate the assay.

FOS has also been shown effective in combination therapy in successive application to wound models [41,136]. Late addition of the antimicrobial, in combination therapy, was found to increase the efficacy of other therapeutic agents. However, these experiments were run for 12 hours in monotherapy only. As a synergistic effect was noted between FOS and CLA in the MPA experimentation it can be inferred that the same effect would be seen in the microfluidic assay. Further increases in the therapeutic susceptibility of biofilm would also support the use of FOS as a potential therapeutic agent for normally recalcitrant MRSP biofilms and it is therefore suggested that staggered combination studies be considered.
5  **CONCLUSIONS AND RECOMMENDATIONS**

Results reveal that CLA does not inhibit MRSP biofilm formation independent of antimicrobial activity when evaluated through MPA on polystyrene substrates. Qualitative and quantitative SEM imaging results suggested that adhesion and formation of MRSP biofilm begin within 4 h of infection on stainless steel with no inhibitory effect of CLA. However, CLA may inhibit MRSP biofilm growth *in vivo* due to enhanced immune system effects. As a synergistic effect was noted with FOS it is recommended that further *in vitro* and *in vivo* trials be completed to explore this potential mechanism of biofilm reduction.

The effectiveness of FOS was studied across 31 strains through agar dilution tests. Prevalence of the FOS-resistance determinant gene in staphylococci, *fosB*, was evaluated through PCR analysis of the chosen population. Results demonstrate effective use of FOS against MRSP *in vitro* with 84% of strains deemed susceptible. However, PCR analysis is in contrast to studies reporting low incidences of FOS resistance in clinical staphylococci and Gram-negative strains, with 87% expressing it. The differences seen between the prevalence of genetic markers of the plasmid-based FOS resistance determinant in staphylococci and prevalence of FOS resistance further underscores the need to direct testing of antimicrobial resistance and, in the case of MRSP, to further determine other potential FOS resistance mechanisms. SEM imaging on titanium screws and AFM imaging on mica sheets of MRSP biofilm further confirmed the effectiveness of FOS on biofilm adherence (*p* < 0.05). However, to further support the use of FOS in MRSP based infections (planktonic) a larger population should be assessed.

In testing the synergism of FOS and CLA in combination a significant effect on biofilm formation *in vitro* was found, independent of their antimicrobial activity and in contrast to monotherapy results (*p* < 0.05). The results support the potential combinational use of FOS and
CLA in the treatment of MRSP biofilms. Interestingly, some form of biofilm remediation in CLA was triggered in these experiments, or the susceptibility to macrolide antimicrobials may have been lowered. However, *in vivo* and further *in vitro* trials evaluating the effect of these two antimicrobials in combination on other surfaces and environments with a larger population are also warranted.

A bio-analytical approach using microfluidics enhanced the understanding of the multi-drug resistant cell’s susceptibility to treatment. By determining the *in vitro* characteristics and activities of MRSP isolates in continuous culture against antimicrobials, more accurate models of MRSP biofilm-related infections can be made. In addition, MBECs obtained from the microfluidic assay could contribute to the improvement of the clinical management of SSIs and apply to the development of new infection control therapies. The results of the microfluidic assay demonstrate that this *in vitro* platform is successful in studying MRSP biofilm showing that low concentrations of FOS are able to penetrate and break up biofilms formed after 24 h of growth. To increase the relevance of the assay, however, longer growth periods to induce multi-layer biofilm growth are recommended. As well, using the synergistic effect noted between FOS and CLA through the MPA could prove useful, with further experimentation across a larger population.

Through determination of the MIC and MBEC of MRSP through both microfluidics and conventional assays, both eradication and prevention profiles could be made for SSI related infections in orthopaedic surgeries in veterinary hospitals across North America. In all studies, however, additional *in vitro* studies evaluating the effect of these chosen antimicrobials on MRSP biofilm formation on the surfaces of biomaterials are warranted. Determining the effect of novel antimicrobial combinations on the biofilm activity of MRSP in an *in vitro* setting is the
first step towards finding a potential therapeutic agent for normally recalcitrant SSIs cause by this infection.

Ultimately, in the short-term, identifying an effective antimicrobial regime to treat these MRSP biofilm-related infections could significantly decrease mortality and reduce the cost of treating patients with SSI related MRSP infections. This strategy also enables us to gain multi-parametric information although genetic and biochemical studies are needed to identify the precise mechanism of antimicrobial susceptibility of biofilms.
REFERENCES


