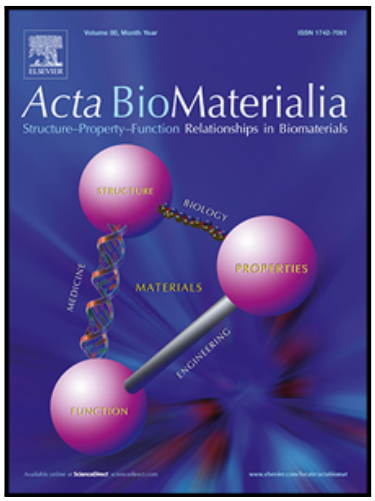

Repeated exposure of nosocomial pathogens to silver does not select for silver resistance but does impact ciprofloxacin susceptibility

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Repeated exposure of nosocomial pathogens to silver does not select for silver resistance but does impact ciprofloxacin susceptibility

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Abstract

The rise of antimicrobial resistant bacteria coupled with a void in antibiotic development marks Antimicrobial Resistance as one of the biggest current threats to modern medicine. Antimicrobial metals are being developed and used as alternative anti-infectives, however, the existence of known resistance mechanisms and limited data regarding bacterial responses to long-term metal exposure are barriers to widespread implementation. In this study, a panel of reference and clinical strains of major nosocomial pathogens were subjected to serial dosage cycles of silver and ciprofloxacin. Populations exposed to silver initially showed no change in sensitivity, however, increasingly susceptibility was observed after the 25th cycle. A control experiment with ciprofloxacin revealed a selection for resistance over time, with silver treated bacteria showing faster adaptation. Morphological analysis revealed filamentation in Gram negative species suggesting membrane perturbation, while sequencing of isolated strains identified mutations in numerous genes. These included those encoding for efflux systems, chemosensory systems, stress responses, biofilm formation and respiratory chain processes, although no consistent locus was identified that correlated with silver sensitivity. These results suggest that de novo silver resistance is hard to select in a range of nosocomial pathogens, although silver exposure may detrimentally impact sensitivity to antibiotics in the long term.

Keywords: Antimicrobial silver, Antimicrobial resistance, Ciprofloxacin, Nosocomial pathogens

1. Introduction

The start of the "antibiotic era" during the 20th century led to the optimistic belief that many communicable diseases caused by bacteria were a problem of the past [1]. However, more than a century has passed and infections resulting from antibiotic resistant strains are on the rise, predicted to generate a global healthcare burden of $100 trillion, and affect 10 million lives annually by 2050 [2, 3]. The first factor behind antibiotic resistance development comes from the high adaptability of bacteria resulting from their high populations, rapid division cycles, genomic plasticity and an ability to transfer genetic information between bacterial species [4]. This coupled with the historic misuse of antibiotics in healthcare and agriculture, a void in new antibiotic classes and the public belief that these wonder drugs are an inextinguishable resource, have all resulted in the rise of an Antimicrobial Resistance (AMR) crisis [1, 5].

The increasing prevalence of AMR has caused a global call for action led by the World Health Organization (WHO), resulting in the publication of the "Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics" in 2017 [6]. In this document, several bacterial species are listed as critical (*Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*), high (*Enterococcus faecium*, *Staphylococcus aureus*, *Helicobacter pylori*, *Campylobacter*, *Salmonella* and *Neisseria gonorrhoeae*) and medium (*Streptococcus pneumoniae*, *Haemophilus influenza* and *Shigella*) priority to encourage and guide the research and development of antimicrobial therapies. The magnitude and implications of AMR over the years are especially noticeable in the critical priority carbapenem resistant *P. aeruginosa* and high priority methicillin resistant *S. aureus* (MRSA) whose presence in clinical isolates has reached values between 5-50% and 30-50% of all infections caused by *P. aeruginosa* and *S. aureus* in Europe and the USA [7, 8]. Thus, the emergence of AMR is leading to what some refer as the dawn of the post-antibiotic era, which critically calls for the development and implementation of distinctive antimicrobial therapies.

Silver (Ag) is a well-known antimicrobial metal with a wide antibacterial spectrum [9] and low human toxicology [10, 11] firstly used to transport fresh water in ancient times [12, 13]. Before the coming of antibiotics, silver compounds were commonly used to treat ulcers, burn wounds and other ailments [13], but the discovery of these substances rapidly replaced the application of silver in healthcare. The necessity of alternative therapies has reinvigorated the interest in silver-based products with numerous devices and chemicals available in the care market. From 2007 to 2017, the number of patents registered with antimicrobial silver

increased from 200 to nearly 1400, for medical, personal care, domestic, agricultural and industrial applications [14]. In the healthcare sector, these patents include diverse products such as implants, surface coatings, wound dressings, gels or water purifiers, showcasing the need to implement different antimicrobials to tackle AMR and Health-care Associated Infections. This interest is heavily linked to research developed on silver based antimicrobial treatments where ionic silver, salts and nanoparticles are heavily applied on biomaterials, making this metallic element as one of the main niches in antimicrobial research [15, 16]. Nevertheless, as the use of this metal becomes more and more widespread, concerns regarding the development of silver resistant bacteria have arisen in the research community, endangering its future prospects as an alternative therapy [17].

The ability to degrade cell walls, disrupt cellular respiration and metabolic pathways, and damage DNA confer silver a multimodal mode of action responsible for its strong antimicrobial ability against numerous bacteria [9, 18, 19]. Nevertheless, silver resistant *Escherichia coli* [20, 21] and *Salmonella enterica serovar Typhimurium* [9, 22] strains have been isolated from healthcare environments since 1960. Analysis of these resistant bacteria demonstrated that silver resistance was encoded on plasmid pMG101 in the *sil* operon, which is the most-characterized silver resistance mechanism to date [9]. The region responsible for silver resistance comprises nine genes [9, 17, 23] which ensure the binding and efflux of silver ions from the cytoplasmic and periplasmic regions, granting an effective survival mechanism to silver toxicity. Nevertheless, silver resistant bacterial strains with and without these genes have been reported in the literature, revealing our lack of knowledge in the regulation of the sil operon and alternate modes of Ag resistance [24, 25]. The prevalence of this resistance operon coupled with the increasing use of silver as an antimicrobial could further enhance the prevalence of AMR with grave socioeconomic consequences. This danger is further aggravated by our limited understanding of the effect of silver dosage in acquisition of resistance to other antimicrobials with only short-term analysis available in the literature [26-29]. As the use of silver in clinical settings rises, it is crucial to for the biomaterials community to understand how silver resistance is developed and its influence on other antibiotic therapies at risk of AMR.

The management of infected implants with combination antimicrobial therapies has been widely explored for orthopaedic prosthetic infections. The drug combination is targeted at both implant-adherent and locally disseminated microbes. For example, clinical evidence in humans shows significantly enhanced efficacy of rifampicin-ciprofloxacin therapies against staphylococcal orthopaedic implant infections when compared with either antimicrobial in isolation [30-32]. It is unsurprising that attention has focused on inorganic-organic combination therapies with silver. From all available antibiotic therapies, the high activity

spectrum, pharmacokinetics and long established safety and efficacy have made fluoroquinolones one of the most broadly prescribed antibiotics [33]. Ciprofloxacin, a second generation fluoroquinolone approved for oral and IV administration by the FDA since 1987 and 1991, respectively [33] targets DNA gyrase and DNA topoisomerase IV. These are essential initiators for DNA replication and protein binding, for which inhibition blocks normal bacterial DNA synthesis and results in rapid bacterial death [34, 35] although reduced effectiveness has been observed against Gram positive (GP) infections [36]. As a result, ciprofloxacin has been a broadly used antibiotic, which has led to a rapid emergence of bacterial resistance. Reports of ciprofloxacin resistant bacteria date from the early 90s [37-40], with a continued rise since the 2000s (i.e. 4.8% in France, 20.3% in Germany, 30.8% in Spain, 7.3% in Sweden and 15.3% in the UK) [41-43]. This increase in resistance had been countered with a more responsible use of fluoroquinolones in clinical settings, but UTIs and GN species gaining quinolone resistance (i.e. europathogenic *Escherichia coli*) are only one of the multiple species endangering antibiotic therapies [44, 45], further complicated by the emergence of inorganic-organic combination therapies. Although robust clinical data is sparse, the locally delivery of Ag in combination with ciprofloxacin has been shown in-vitro or in animal studies to be synergistic against plantonic and biofilm formations of pseudomonas and streptococcal species [46] and against multiresistant carbapenemase-producing Enterobacteriaceae [47]. Against *Staphylococcus aureus* infections on polyester vascular grafts, silver coating in combination with oral ciprofloxacin and rifampicin resulted in significant increased efficacy in eradicating the implant infection -porcine model of 96% compared with 44% for the silver free control [48]. These promising results may encourage adoption of silver alongside common antibiotic approaches, nevertheless, the limited knowledge of their impact on AMR development poses a critical barrier to silver use in clinical settings.

The main aim of this work is to analyse the likelihood of clinically relevant GP and GN bacteria to acquire silver resistance and to investigate whether exposure to silver influences susceptibility to antibiotics. Due to its broad antimicrobial impact and extended use, ciprofloxacin was selected as an exemplar of AMR development in combinatorial therapies. Three bacteria covered in the WHO call for action were selected (*E. coli*, *S. aureus* and *P. aeruginosa*) as well as a coagulase negative *Staphylococcus* (*Staphylococcus epidermidis*) for their relevance in infections of prostheses. For each species, both a well understood laboratory reference strain and a recent clinical isolate were tested. Development of silver resistance was assessed following serial passages in silver nitrate, successive passage to the antibiotic ciprofloxacin was also run as a control experiment, and in addition the impact of silver treatment on ciprofloxacin susceptibility was also tested. Phenotypic and genotype

analysis of the as received and treated bacterium indicated limited variations in silver susceptibility. In contrast, continued passages in ciprofloxacin showed higher resistance building up after silver treatment than those obtained for the as received strains, suggesting that long exposure to silver and ciprofloxacin can enhance antibiotic resistance. These results indicate that albeit silver is a potent bactericidal agent with limited *de novo* resistance development, it can detrimentally influence the long-term response of other antimicrobial molecules. As silver becomes more widespread as a therapeutic tool in clinical settings, it is critical to guarantee the potency and ability to select for resistant strains. This work indicates that silver is a powerful treatment but used in conjunction with common therapies it could lead to an enhancement of AMR. Thus, it is clear that antimicrobial treatments developed by the biomaterials community should be studied alongside common antibiotic therapies, critical in the AMR crisis.

2. Experimental section

2.1 Bacterial strains

In this work, two Gram negative (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) bacteria were analysed. For each species, two different strains, one laboratory and one clinical isolate were used. Genome sequence data was used to confirm the correct taxonomic identification of each using Kraken [49] and Multi-Locus Sequence Typing (MLST) provided (Table 1). Clinical isolates were provided by the Birmingham Queen Elizabeth Hospital and laboratory isolates were obtained from the collection held by the Birmingham Dental Hospital and School of Dentistry.

Table 1 Taxonomic identification of all the bacterial species used in the current study

Bacteria	Origin	Best strain match by Kraken	MLST type
Escherichia coli	Laboratory	*E. coli* O44:H18	58
	Clinical	*E. coli* JJ1886	131
Staphylococcus aureus	Laboratory	*S. aureus* Newman	254
	Clinical	*S. aureus* CA-347	46
Staphylococcus epidermidis	Laboratory	*S. epidermidis* ATCC 12228/ RP62A	5
	Clinical	*S. epidermidis* RP62A/ ATCC 12228	2
Pseudomonas aeruginosa	Laboratory	*P. aeruginosa* PAO1	549
	Clinical	*P. aeruginosa* SCV20265/M18/ RP73	254

2.2 Minimum inhibitory and bactericidal concentration assays:

Minimum Inhibitory Concentration (MIC) tests were performed following the Clinical and Laboratory Standards Institute (CLSI) methods described in M07-A9 “Methods for Dilution

Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically" standard [50]. A 1024 µg/mL stock silver nitrate solution, $AgNO_3$ (≥99.0% Sigma Aldrich), or a 64 ug/mL stock ciprofloxacin solution (≥98.0% Sigma Aldrich), was prepared in MH broth for each MIC test. Purity of the inoculum was tested through visual inspection of plated bacteria on Mueller Hinton agar (MHA). Plates were cultivated for 16 h in a shaking orbital incubator at 37 ° and the MIC was measured as the lowest $AgNO_3$ concentration that inhibited visible bacterial growth. All MIC measurements are the result of three independent measurements, average and standard deviation reported. For each MIC cycle, Minimum Bactericidal Concentration (MBC) tests were done following CLSI standards [51]. Briefly, 10 µL of bacterial culture from the 96 well plate were pipetted onto pure MHA, cultivated overnight at 37°C and visually inspected. All MBC measurements are the result of three independent measurements, average and standard deviation reported.

2.3 Repeated passage experiments:

After the initial MICs were determined, for the next dosage cycle, the cultivated well displaying growth at the highest antimicrobial concentration was diluted to ~$5*10^5$ CFU/mL and used as inoculum for the next cycle. This process and the selected concentrations were carried out similarly to the method described by Randal et al. [24] for up to 50 cycles. Every three cycles, Gram staining was performed and samples stored at -80°C. The subsequent cycle was carried out with colonies from the resuscitated vials in pure MHB to prevent silver build-up through cycles. For ciprofloxacin dosing of silver treated samples, antibiotic treatment was started immediately after the last Ag cycle was completed. To study the stability of ciprofloxacin resistance developed on both original and silver treated strains, MIC and MBC tests were carried out on bacterial cultures passaged in base MH media. These assays were conducted with samples from stored bacteria corresponding to the initial and last stages of the study.

2.4 Anti-biofilm assays

Influence of antimicrobial dosage on biofilm production in all Ag and ciprofloxacin treated samples was analysed using a modification of the method proposed by Christensen et al. [52]. Briefly, an overnight culture was diluted in MHB to a density of ~10^3 CFU/mL and 200 µL inoculated onto a 96 well plate. After 24 h, the old media was discarded and 200 µL of fresh MHB inoculated per well. Biomass quantification was performed after 48 h through a modification of the protocol described by O'Toole [53]. Each sample was moved to a new suspension plate, washed three times Phosphate Buffered Saline (PBS, Sigma Aldrich) and fixed with 4% Paraformaldehyde in PBS for 1 h. 200 µL of a 0.5%crystal violet solution was inoculated for 5 min, and excess staining removed by washing in PBS three times. Then,

samples were dried in an incubator at 37 °C for 2 h and staining recovered by immersion in 200 µL of methylated spirit for 2 h. The resulting solution was serially diluted and absorbance measured using a TECAN Spark plate reader (Tecan Trading AG, Switzerland) at 590 nm wavelength. Results were the average of three independent measurements.

2.5 Scanning Electron Microscopy:

To study the influence of adaptation on the morphology of all bacterial strains in this study, samples were prepared for Scanning Electron Microscopy (SEM). 50 µL of bacterial culture from samples cultivated in the initial and last cycle analysed were pipetted on a plastic coverslip placed inside a 24 well plate. After 30 minutes, samples were fixed with 1mL of 2.5% Glutaraldehyde in cacodylate buffer for 1 h, dehydrated through a series of ethanol and deionised water dilutions (20, 30, 40, 50, 60, 70, 90, 95 and 100 %, for 10 min in each), treated with hexamethyldisilzane, and dried overnight. The coverslips were then mounted, gold sputtered and SEM images taken using a Zeiss EVO M10 microscope (Carl Zeiss GmbH, Germany) with an acceleration voltage of 10 kV. Length and width were measured with ImageJ (NIH, USA) from 100 bacterial cells except for the *E. coli* strains subjected to ciprofloxacin, where 50 cells were evaluated. For volume quantification, Gram negative (GN) strains were considered as cylinders with spherical ends, while Gram positive (GP) were treated as spheres.

2.6 Sequencing and genomic analysis:

Sequencing was performed by Microbes NG. DNA was isolated from bacteria stored on Protect beads, three beads were washed with extraction buffer containing lysozyme (or lysostaphin for *Staphylococcus* sp.) and RNase A, incubated for 25 min at 37°C. Proteinase K and RNaseA were added and incubated for 5 min at 65°C. Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer. DNA was quantified in triplicates with the Quantit dsDNA HS assay in an Ependorf AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: two nanograms of DNA instead of one were used as input, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on an Illumina instrument using a 250bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [54].

De novo assembly was performed on samples using SPAdes version 3.7 [55], and contigs were annotated using Prokka 1.11 [56]. Species were confirmed using Kraken and Kraken-translate (version 1.3.1) [49]. ABRicate (version 1.0.1) was used to screen all assemblies for the presence of antimicrobial resistance genes using the resfinder database (containing 3077 sequences – 2019-Sep-10), with settings of 80% minimum identity and an 80% minimum DNA coverage [57, 58]. To determine if the sequenced genomes contained known silver resistance genes Blast was used (version 2.10.0+) to search for alignments against the silver resistance genes in plasmid pMG101 [59, 60]. SNPs were identified for each strain after 50 passages in silver to the using Snippy (version 4.1) and the corresponding progenitor strain as comparison. Larger scale genomic rearrangements were investigated by comparing adapted strains to parental strains using the progressive Mauve method with default parameters by Mauve (v.2.4.0) [61, 62]. Loss of genes was analysed using the "Operate on genomic intervals" and "Join intervals" tools on usegalaxy.org to identify differences in gene content between pairs of strains (adapted versus parental) as predicted by (company tools) [63].

2.7. Statistical analysis:

Statistical analysis was performed using SPSS (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0). To limit the influence of invalid assumptions on mean comparison the similarity of variances was assessed with Levene's test. If this assumption was not violated, ANOVA-I was performed followed by Turkey's post-hoc. In contrast, for the case where similarity of variances could not be assumed mean comparison was assessed through Welch's test and Games-Howell's post-hoc as suggested by Field et al. [64]. In all tests an alpha level of 0.05 was selected as the statistical significance threshold.

3. Results and discussion

3.1 Phenotypic effects of silver and ciprofloxacin dosage

To assess the influence of long-term exposure to silver, a panel of pathogens were repeatedly passaged in the presence of controlled dosages of silver nitrate, $AgNO_3$ (Figure 1 and Tables S1 and S2). Testing of the strains showed similar levels of susceptibility between laboratory and clinical strains, the initial mean MIC of $AgNO_3$ against all strains ranged between 8 and 43 µg/mL (Table S1), below the level which has been suggested to reflect susceptibility[24] with initial higher MBCs between 53 and 512 µg/mL (Table S2). These initial MIC values were slightly higher than those commonly reported in the literature for *E. coli* (3 - 14 µg/mL), *S. aureus* (6 - 16 µg/mL), *S. epidermidis* (7 - 16 µg/mL) and *P. aeruginosa* (2 - 16 µg/mL)[47, 65-68]. Comparison between published works is complicated

by differences between methods. Specifically, the bioavailability of ionic silver is a major determinant of its efficacy and is greatly affected by the microbiological medium used during susceptibility testing, as salts and thiol-containing proteinaceous components can act as silver inactivators [24, 69]. At the same time, the MBC of silver containing compounds can be significantly higher than the MIC with Jung et al. [70] recently demonstrated that silver ions can induce an “active but nonculturable” state (ABNC), which contributes to a larger difference between MBC and MIC.

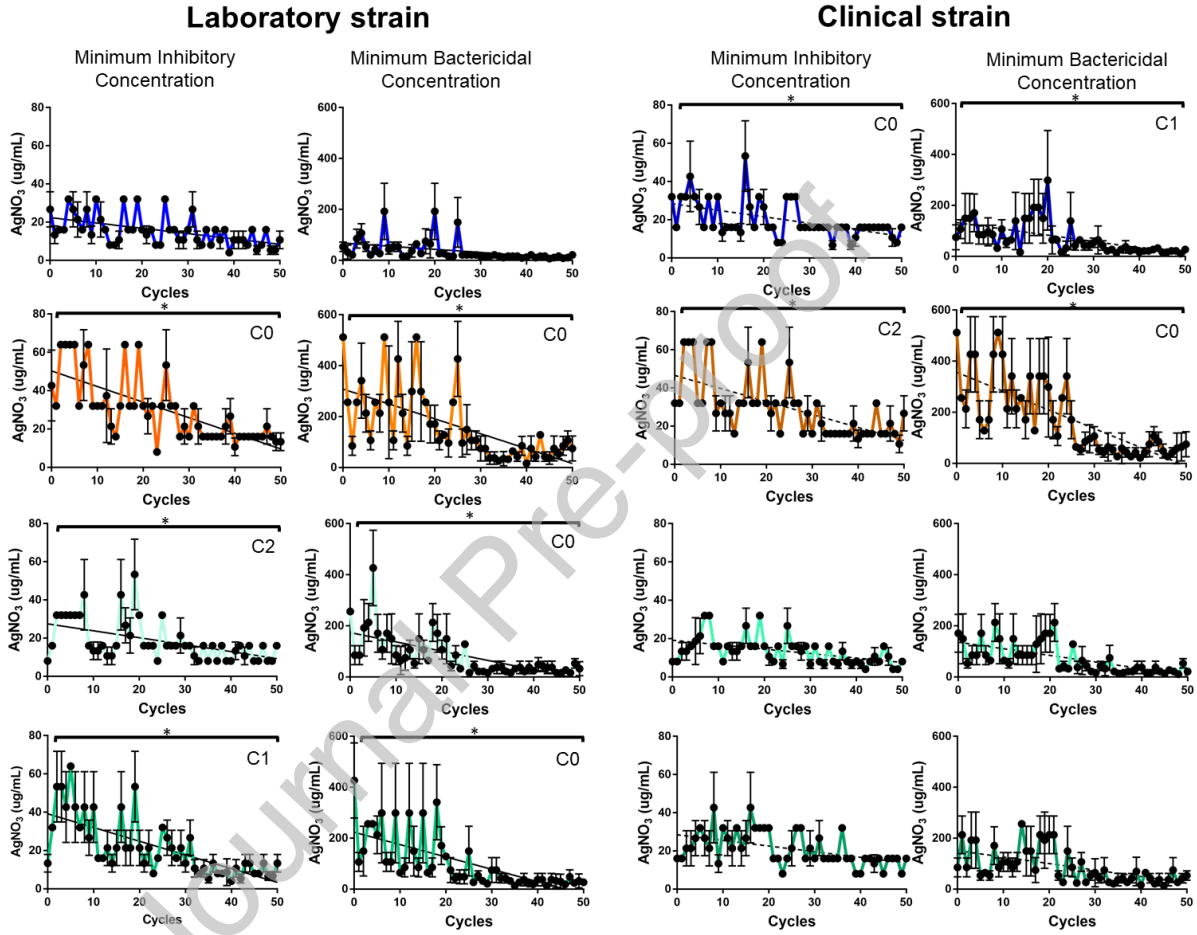

Figure 1 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of $AgNO_3$ determined for Gram positive and Gram negative bacteria during 50 dosage cycles (n = 3 per cycle) where * signifies p value <0.05 between the indicated cycle and the last dosage treatment.

Analysis of silver resistance development showed an initial increase in MIC values for all bacterial strains during the first 5 silver dosage cycles, this was maintained in all groups over the next 15 cycles. After the 25th cycle however, a decrease in silver tolerance was consistently observed in all lineages, which stabilized over the next five dosages and was then maintained until the last cycle. Statistical analysis between the MICs observed during the first five cycles and the last cycle, revealed significant differences ($p < 0.05$) for the

laboratory strains of *S. aureus*, *S. epidermidis* and *P. aeruginosa* and the clinical strain of *E. coli* (Figure 1 where the first group to show statistical difference is included as an inset). These trends are consistent with the MBC results that showed a similar pattern with lineages initially demonstrating decreased susceptibility to $AgNO_3$ before increased susceptibility emerged and was maintained till the end of the exposure.

Previous studies have attempted to identify or select for silver resistance, with Randal et al. [24, 25], Graves et al. [71], Sutterling [19], Elkrewi et al. [72], Panáček et al. [47] and more recently Massani et al. [73] being the main contributors in the field. A variety of methods and forms of silver have been used in these works. Nevertheless, a common theme has been the observation that *de novo* resistance to silver appears, in general, to be difficult to select with relatively few isolates reported to develop decreased susceptibility. Where decreased susceptibility to silver has been reported, it has appeared to occur more readily in GN species than GP, with some GN species developing rapid responses after 5 [24] or 16 [73] cycles. In contrast, GP bacteria have shown a modest response with no changes observable after 42 days [25]. Similar results had been obtained for GN bacteria subjected to silver nanoparticles, showing rapid development of resistance in *E. coli* strains [47, 71], however, comparisons of results from silver ions and nanoparticles should be made with care. ROS production, decoupling of the respiratory chain, envelope degradation and lysis, nucleic acid fragmentation or enzymatic disruption are some of the antimicrobial effects observed in cells subjected to silver compounds, nevertheless, the main mode of action is heavily dependent on the silver species used [74, 75]. For instance, Kędzioraet al. [76] reported a decrease in antimicrobial effect of silver nanoparticles in anaerobic conditions suggesting that oxidation and ROS production is more prominent in their bactericidal effect than for ionic silver. At the same time, nanoparticle morphology, size or synthesis methods can result in other mechanisms (e.g. inhibition of fatty acid biosynthesis) being the main driver of the observed antimicrobial effect [77]. This suggests that resistance mechanisms may vary depending on how the silver is presented. In this regard, it is necessary to clarify that in the present study and except otherwise stated, all reference to antimicrobial silver refers to its ionic form. The results obtained in this study are initially similar to previous reports stating an early decrease in silver susceptibility. Nevertheless, this phenomenon was not constant and higher silver susceptibility was then observed after longer exposure times. A recent article by Massani et al. [73] describes selection of a highly resistant *E. coli* strain (MIC ~ 9000 µg/mL), however, this resistance was highly unstable and was lost after two overnight passages in silver-free media. This instability may reflect the lower fitness often seen in silver resistant strains [19].

To analyse the influence of adaptation to silver exposure on the morphology of bacteria, SEM images of strains from the final silver dosage were analysed both when subjected to the MIC of silver, or in drug-free conditions (Figure 2). Exposure to the MIC resulted in major

damage to most of the GN lineages with cells suffering a complete collapse of outer membrane integrity, micrographs showing only remnants of bacterial envelopes for most strains. Interestingly, some undamaged bacteria could be found for the laboratory strain of *P. aeruginosa,* which correspond to the highest MIC value after 50 silver dosage cycles (16 µg/mL). In contrast to the extended damage seen in the envelope of GN cells, most of the GP bacteria analysed were able to retain cellular integrity in the presence of silver with the laboratory isolate of *S. epidermidis* being the only GP bacterial strain displaying significant damage to the membrane (Figure 2 red arrows). These differences in silver tolerance could be the consequence of conformational differences between bacterial strains and the various mechanisms by which silver ions affect the envelope of both GP and GN bacteria . Silver ions can react with sulfhydryl and thiol groups present on the bacterial surface, leading to uncoupling of the respiratory chain, loss of the proton motive-force and cell membrane degradation [9, 24, 76]. Structural analysis of silver treated bacteria has observed pitting and degradation of the bacterial envelope followed by complete loss of integrity of the cell membrane [70, 78-81]. The larger susceptibility to cell membrane damage observed on Figure 2 for the *E. coli* and *P. aeruginosa* isolates may be explained by limited protection offered by the thinner peptidoglycan layer of GN bacteria.

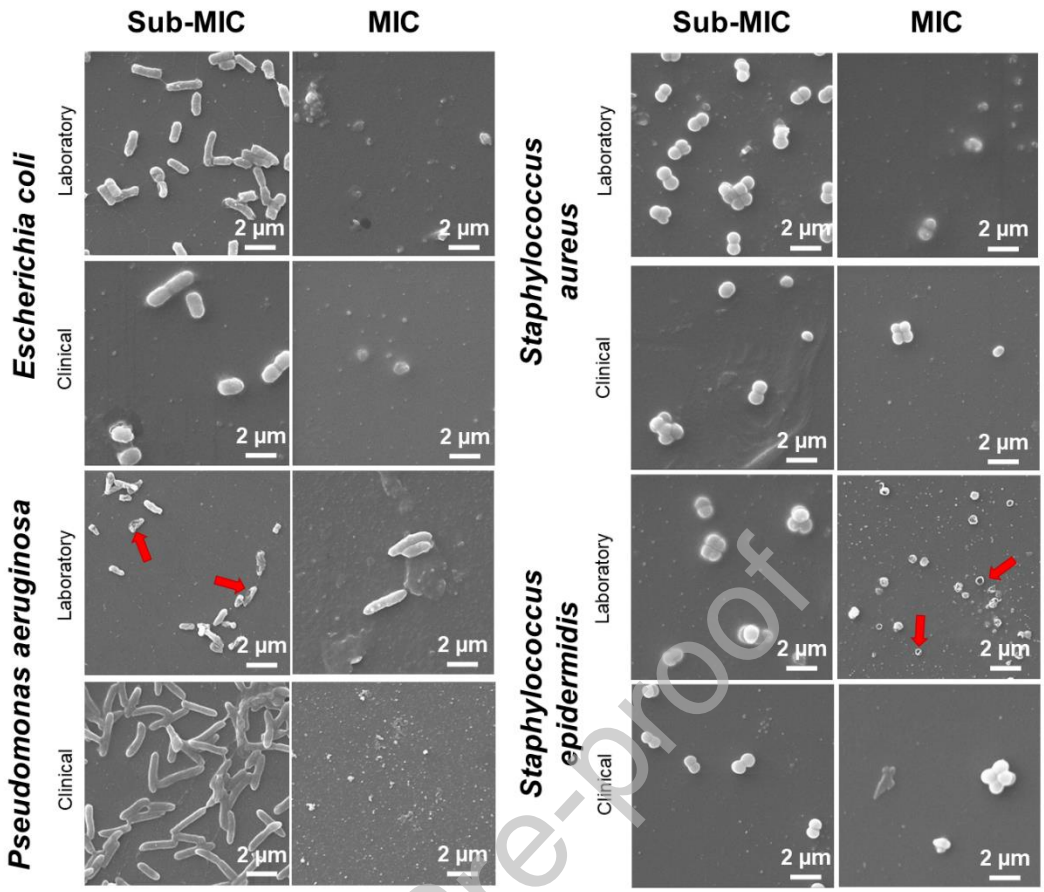

Figure 2 SEM images of $AgNO_3$ dosage effect on the outer membrane of both Gram positive and Gram negative isolates after 50 dosage cycles with samples taken from the last well displaying bacterial growth sub-MIC, and the first without visible bacterial proliferation, MIC. Red arrows indicate damage to the outer membrane of the cells in the form of rupture, pitting or degradation.

The previous results indicate that silver is a promising antimicrobial with no resistance observed after long term treatments, however, the potential use of these therapies with conventional antibiotics is necessary to limit AMR development. To analyse the potential of silver adaptation to influence selection of resistance to other agents was tested using ciprofloxacin. Both silver naïve and silver treated strains were exposed to ciprofloxacin (Figure 3 and Tables S3 to S6). Initial ciprofloxacin MIC values revealed that all base *E. coli*, *S. aureus* and *P. aeruginosa* isolates were susceptible to this antibiotic, MIC≤ 0.25, ≤ 1 and ≤ 0.5 µg/mL respectively [82] while, on the other hand, the clinical isolate of *S. epidermidis* was resistant, MIC ≥ 4 µg/mL. Continued exposure to ciprofloxacin resulted in rapid selection of loss of susceptibility. The laboratory strain of *E. coli* showed a minimal increase in MIC (from 0.004 µg/mL up to 0.016 µg/mL) compared to the larger changes displayed by the clinical isolate (0.016 up to 0.833 µg/mL; considered as intermediate and near resistant

through CLSI standards) [82]. Similarly, resistance development was different between the laboratory and clinical isolates of *S. epidermidis* where the already resistant clinical isolate displayed a more pronounced lowering in susceptibility from 3.33 ± 0.94 µg/mL to a final MIC value of 10.67 ± 3.77 µg/mL. Ciprofloxacin exposure resulted in development of resistance in *S. aureus* and *P. aeruginosa* (MIC ≥ 4 µg/mL and ≥ 1 µg/mL respectively), regardless on the nature of the isolate and consiste nt with the MBC results.

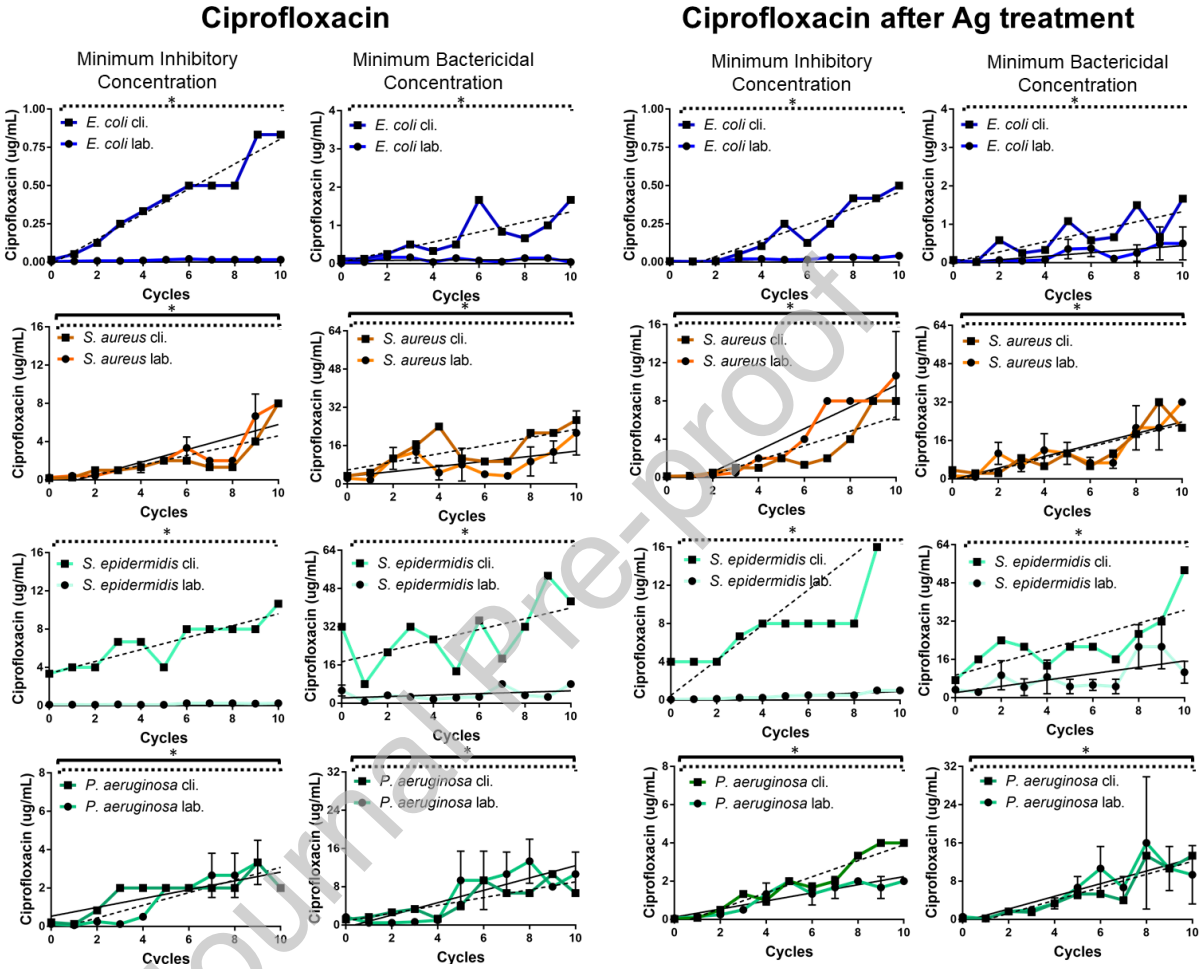

Figure 3 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of ciprofloxacin determined for Gram positive and Gram negative bacteria for the as received and silver treated bacterial strains (n = 3 per cycle), where * signifies p value <0.05 between the first and the last dosage cycle.

Ciprofloxacin MIC determination showed that those which had been pre-adapted to silver were generally more susceptible (Cycle 0 in Table S5) when compared to the parental strains (Cycle 0 in Table S2). This initial increase in susceptibility did not, however, significantly alter the trajectory of resistance development for strains when exposed to ciprofloxacin with ciprofloxacin dosage resulting in rapid resistance development. Interestingly, the MIC values observed in the silver treated bacteria after 10 dosage cycles were either similar or higher than those seen in the parental strains after 10 cycles (Table S3). This increase in ciprofloxacin resistance appears to be generally stable in all treatments

considered with main differences observed between bacterial strains (Figure 4). During the first two cycles of the stability study, both parental and silver treated strains seem to display a slight increase in resistance (Tables S7 to S10) with limited statistical significance observed in the as received bacteria. Some of these strains appear to reveal a continued decrease in ciprofloxacin resistance, nonetheless, the variability in MIC and MBC shown by these strains may indicate that resistance is similarly maintained over time. In the case of ciprofloxacin resistance developed after silver treatment, comparable responses can be observer to the parental strains. It is of notice the increase in resistance displayed by the *S. epidermidis* laboratory strain which becomes significant after silver treatment, alongside the punctual decrease in susceptibility shown by both *P. aeruginosa* strains after five cycles in non-antibiotic containing media. From Figure 4, it is clear that ciprofloxacin resistance has not increased the fitness cost, resulting in a stable mutation maintained after antibiotic withdrawal regardless of any complementary antimicrobial treatment. Ciprofloxacin resistance has been shown to be stable in both GP and GN bacterium [83-85], which coupled with the long term decrease in susceptibility revealed by silver treated strains further supports the necessity to understand long term interactions of inorganic-organic combinations.

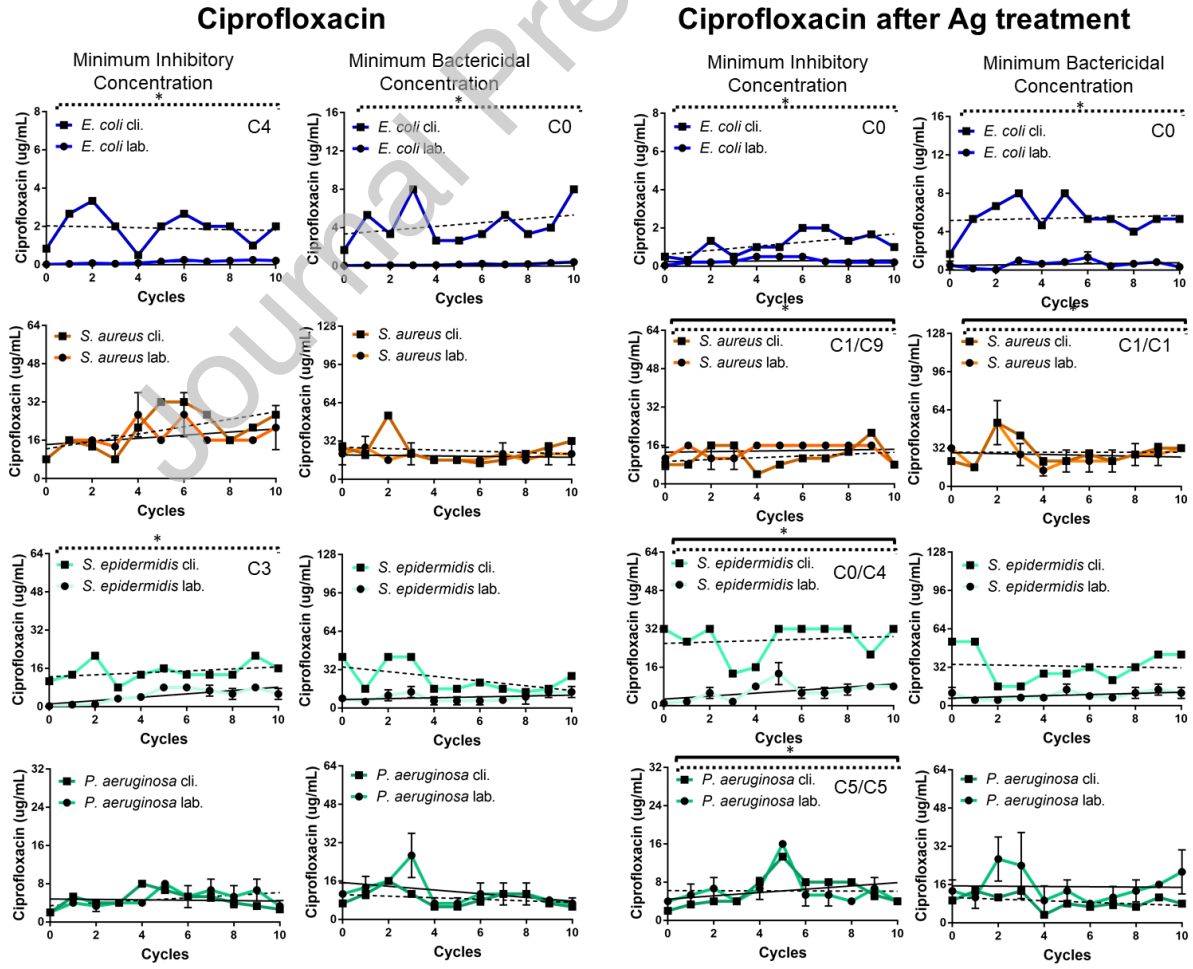

Figure 4 Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of ciprofloxacin determined for Gram positive and Gram negative bacteria showcasing resistance stability (n = 3 per cycle), where * signifies p value <0.05 between the indicated cycle and the last dosage treatment.

From the previous sections it is evident that that silver and ciprofloxacin dosage have long lasting influence in the phenotypes of all treated bacteria, nevertheless, it is not clear which mechanisms could be responsible for ese effects. To further analyse these differences, biofilm production and morphological studies of all strains subjected to different dosing regimens of silver and ciprofloxacin were conducted (Figure 5 and Tables S11 to S13). Biofilm formation assays demonstrated distinct responses to silver and ciprofloxacin treatments with clinical isolates for both *E. coli* and *S. aureus* not significantly influenced by single or complementary antimicrobial treatments ($p > 0.05$). In contrast, their laboratory ciprofloxacin treated counterparts displayed an increase in biomass, contrasting with their decrease after silver and subsequent ciprofloxacin treatments. Interestingly, silver treatment negatively impacted biofilm formation after silver dosing on the aforementioned GP bacteria while enhanced biomass was found in the GN bacteria. This seems to indicate that the combination of both treatments resulted in biofilm impairment for *E. coli*, while silver seemed to be the main contributor to this effect in *S. aureus*. For the *S. epidermidis* and *P. aeruginosa* laboratory strains analysed, silver and further ciprofloxacin treatment resulted in synergic interaction, raising biofilm production significantly higher than dosing in either antimicrobial. In contrast, silver did not significantly influence biofilm production for the clinical isolate of *S. epidermidis* indicating that ciprofloxacin is the main cause behind the enhanced biomass measured. Similarly, ciprofloxacin resulted in enhanced biofilm production for the clinical isolate of P. aeruginosa, although it must be noticed the significant reduction observed after silver passaging.

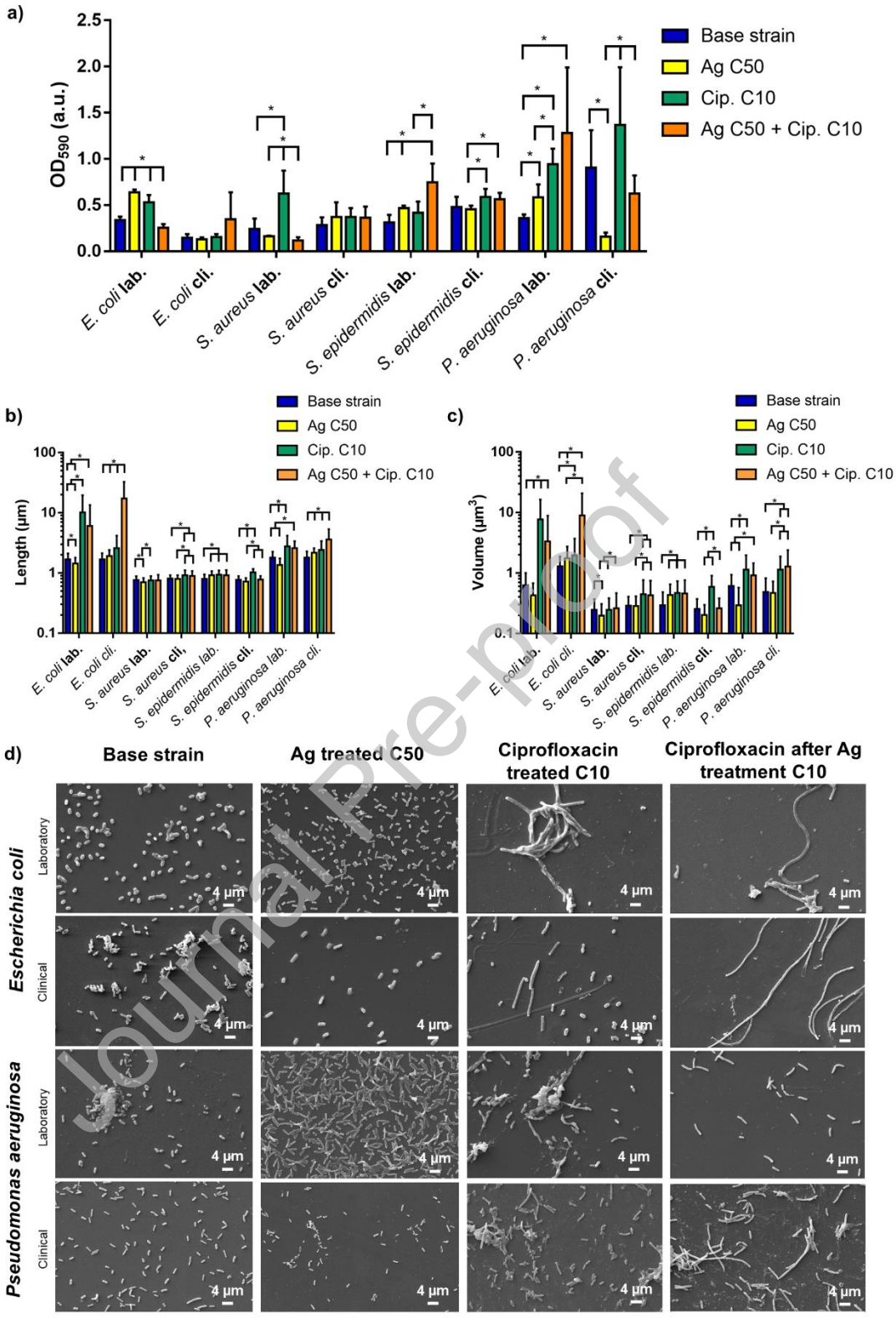

Figure 5 a) Biofilm and morphological analysis of all bacterial strains including b) length, c) volume measurements and d) SEM details of the Gram negative strains (*E. coli* and *P. aeruginosa*) as received, Ag treated, ciprofloxacin treated and ciprofloxacin after silver treatment where * signifies $p<0.05$. Biofilm results were obtained from 3 independent measurements (n = 3) while morphological analysis are the average of 100 independent cell measurements (n = 100).

Length and width measurements of cells were obtained from SEM images and volumes calculated by considering spherical cells or cylinders with cupped ends for the GP and GN bacteria respectively (Figures 5b and 5c) [86, 87]. Results revealed a limited influence of silver dosage on the morphology of GP bacteria with only the *S. epidermidis* laboratory strain demonstrating a noticeable change in volume between parental and silver-adapted cells (0.29 ± 0.19 μm^3 to 0.43 ± 0.22 μm^3, respectively). Ciprofloxacin dosage before and after silver treatment caused a more prominent change in morphology, with a generalised swelling significant in all groups except in the *S. aureus* laboratory strain. GN bacteria were found to be more susceptible to filamentation with both *E. coli* and *P. aeruginosa* strains subjected to 10 cycles of ciprofloxacin being two to ten times longer than untreated cells. The increase in ciprofloxacin driven morphological changes in the silver-adapted strains suggests the silver treatment may have increased permeability to this antibiotic and this could explain the initially higher ciprofloxacin susceptibility of strains which had been adapted to silver. These morphological changes observed throughout the ciprofloxacin dosage are consistent with previous reports and the known mechanisms of action and responses to this drug. Ciprofloxacin treatment results in the arrest of DNA duplication and triggers the SOS response and filamentation [88, 89]. The SOS response also induces error prone DNA repair and may help explain both the initial increased susceptibility of silver-adapted strains (due to cellular damage and permeability), but also greater capacity to develop ciprofloxacin resistance (due to a higher frequency of mutation from induction of the SOS response).

The limited pipeline for new classes of antibiotics is pushing forward the use of different antimicrobial agents, an important aspect that needs study is how these treatments may impact other antimicrobial therapies. We examined this by addressing long term silver exposure affects ciprofloxacin resistance development. The presented data revealed that whilst silver adaptation seemed to impose some cost to ciprofloxacin susceptibility, subsequent exposure to ciprofloxacin itself resulted in quicker development of ciprofloxacin resistance after silver dosage for *E. coli* and *S. epidermidis*. Similar experiments developed by Kędziora et al. [90] using different silver nanoformulations have shown a comparable increase in antibiotic resistance to that presented in this manuscript. However, this contrasts with the lack of increase to antibiotic susceptibility reported by other groups, as presented by a recent review from the same authors [76], concluding that silver exert selective pressure depending on the individual features of bacterial strains and the physicochemical properties of the silver formulations. Thus, it is clear that the effects of silver are still poorly understood and fundamental studies are still needed to unravel its influence in AMR development. It must be said that the increase in silver susceptibility seen over time in Figure 1 was unexpected, potential sources of artefacts were tested; measurements of the efficacy of the

$AgNO_3$ stocks used confirmed it remained equally active against the parental strains over time and there has been no unexpected loss of activity of the silver. The parallel set of control experiments developed with the antibiotic ciprofloxacin demonstrate the model system could select for resistance. Together these data suggest there was no inherent problem with the experimental set up and that the selection for decreased susceptibility seen in multiple, independent lineages was a reliable observation.

3.2 Genetic changes identified after silver exposure

To further explore the effect of silver dosing and elucidate genetic mechanisms behind the phenotypic changes observed, parental and silver-adapted lineages were sequenced and compared. Initial analysis of the strain panel used did not detect a gene that could be correlated with the *sil* operon, a known determinant of silver resistance in GN organisms [24, 25] in any strains. Screening of all the strains for other antimicrobial resistance genes identified the presence of protein sequences associated with antibiotic resistance in most bacterial strains (Table S14). Nevertheless, silver treatment had no impact on carriage of most of these genes at the end of the experiments. Only the *P. aeruginosa* clinical isolate was discovered to possess mobilizable quinolone resistance genes in the form of a multidrug efflux pump (*oqxB*) and a ciprofloxacin-modifying enzyme (*crpP*). Interestingly, the *oqxB* gene was lost after the silver passage, this resistance mechanism is usually plasmid encoded, which suggests plasmid loss during the silver exposure. The ciprofloxacin passage experiments (Figure 3) indicated that both GP bacteria exhibited higher propensity to develop antibiotic resistance than the GN counterparts. This is likely to be due to selection of target site mutations in the chromosome in these strains.

Analysis of Single-nucleotide polymorphism (SNPs) was used to identify mutations selected by silver exposure, this showed a limited number of SNPs (between 0 and 36) identified in the exposed strains when compared to the parental strain (Table 2). The laboratory strain of *E. coli* carried SNPs within, *dnaX*, and *mrcA* although both were synonymous and unlikely to impair the function of the corresponding proteins. Similarly, the clinical strain of *E. coli* also carried two SNPs; a synonymous variant within putative protein YnfD [91] and a missense change within *pabB,* which alongside *pabA* is responsible for 4-aminobenzoate synthesis.

Table 2 Single-nucleotide polymorphism (SNPs) obtained for all the GP and GN bacteria before and after the silver treatment through Snippy [92]

Species	Strain	Gene	Effect	Annotated product
E. coli	Laboratory	*dnaX*	Synonymous variant	DNA polymerase III subunit tau
		mrcA	Synonymous variant	Penicillin-binding protein 1A
	Clinical	*pabB*	Missense variant	Aminodeoxychorismate synthase component 1
		ynfD	Synonymous variant	putative protein YnfD
S. aureus	Laboratory	*bceB*	Frameshift variant	Bacitracin export permease protein BceB
		rluB	Missense variant	Ribosomal large subunit pseudouridine synthase B
		gsiC	Missense variant	Glutathione transport system permease protein GsiC
		ulaA	Missense variant	Ascorbate-specific PTS system EIIC component
		treP	Synonymous variant	PTS system trehalose-specific EIIBC component
		xerC	Synonymous variant	Tyrosine recombinase XerC
	Clinical	*cysK*	Stop gained	Cysteine synthase
		apt	Missense variant	Adenine phosphoribosyltransferase
		prfA	Synonymous variant	Peptide chain release factor 1

Table 2 (Continuation) Single-nucleotide polymorphism (SNPs) obtained for all the GP and GN bacteria before and after silver treatment through Snippy [92]

Species	Strain	Gene	Effect	Annotated product
S. epidermidis	Laboratory	*ackA*	Missense variant	Acetate kinase
	Clinical	-	-	
P. aeruginosa	Laboratory	*ccoN1*	Missense variant Synonymous variant	Cbb3-type cytochrome c oxidase subunit CcoN1
		mexT	Missense variant	Efflux regulator
		dltA	Missense variant	D-alanine-poly(phosphoribitol) ligase subunit 1
		tuf1	Synonymous variant	Elongation factor Tu
	Clinical	*napA*	Missense variant	Nitrate reductase
		cusS	Missense variant	Sensor kinase CusS
		rhtC	Missense variant	Threonine efflux protein
		mdtK	Start lost	Multidrug resistance protein MdtK
		ccoN1	Synonymous variant Missense variant	Cbb3-type cytochrome c oxidase subunit CcoN1
		mdeA	Missense variant	L-methionine gamma-lyase
		nicP	Stop gained	Porin-like protein NicP
		tuf1	Synonymous variant	Elongation factor Tu
		hcaB	Synonymous variant	3-phenylpropionate-dihydrodiol/cinnamic acid-dihydrodiol dehydrogenase
		fliY	Missense variant	L-cystine-binding protein FliY
		ftsN	Synonymous variant	Cell division protein FtsN
		kefC	Missense variant	Glutathione-regulated potassium-efflux system protein KefC
		htpX	Missense variant Synonymous variant	Protease HtpX
		trxC	Missense variant Synonymous variant	Thioredoxin 2

A larger number of SNPs were identified in both *S. aureus* strains, including a frameshift variant in bacitracin permease *bceB.* The BceAB transporter consists of an ATPase (BceA) and a permease (BceB) with 10 transmembrane helices which has been correlated with resistance to bacitracin, mersacidin, and actagardine in *Bacillus subtilis* [93-95]. Albeit not known to have a role in silver sensitivity, modifications to a resistance transporter could be relevant to the increased susceptibility to silver seen in the adapted strain. At the same time, missense variants were found in genes responsible for pseudouridine synthase catalysis, rluB, an alternative carbohydrate uptake system, ulaA, and a glutacione transport complex,

gsiC. From these, gsiC is a glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH) importer vital in thiol-dependent redox system (TDRS) which reduce Ribonucleotide reductases (RNRs) required for DNA synthesis and repair [96, 97]. In the clinical strain of the same species, two potentially significant changes were seen; an early stop within cysteine synthase, cysK and a missense change within apt. Cysteine is essential in the catalytic activity and structure of many proteins some of which perform major roles in preserving an intracellular reducing environment and protecting against oxidative stresses [98], reducing the viability of *S. aureus* cells. A missense variant was observed in the adenine phosphoribosyltransferase apt that enables nucleotide salvage reactions, this gene is involved in the stress response to starvation [99]. Fewer changes were seen in *S. epidermidis* with only the laboratory strain carrying a missense variant in the acetate kinase catalysis ackA. This gene plays a role in the conversion of acetyl phosphate from acetate and ATP while enabling the reverse reaction. This transformation is a vital ATP generating pathway in GP bacteria for which disruption has been shown to significantly reduce growth rate and viability [100].

The laboratory strain of *P. aeruginosa* carried missense variations in Cbb3-type cytochrome c oxidase subunit, ccoN1, nodulation protein D 2, NodD2, and D-alanine--poly(phosphoribitol) ligase subunit 1, dltA. ccoN1 is part of gene cluster ccoN1O1Q1P1, one of the five terminal oxidases for aerobic respiration present in *P. aeruginosa* and active under high oxygen conditions [101]. MexT is a LysR-type transcriptional regulator of the MexEF-OprN efflux system while tuf1 is responsible for binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis. DltA is part of the dltABCD operon which is in charge of incorporating d-alanine to teichoic acids. Mutations on this gene can lead to a lack of d-alanine in these highly charged cell wall polymers, resulting in a stronger negative charge on the cell surface and affecting resistance to cationic antimicrobial peptides, vancomycin, autolysins activity and a reduction in biofilm formation [102-105]. This, coupled with the variant observed on MexT, which can confer quinolone resistance [106], may have influenced the response of this *P. aeruginosa* strain to both antimicrobials studied, although it must be said that the ligands and mechanism of activation of MexT are still poorly understood [107].

A larger number of SNPs were identified in the clinical isolate of *P. aeruginosa* than the others (Table 2). These included missense changes within ccoN1 in the same site as seen in the laboratory strain of *P. aeruginosa*. This parallel selection of substitutions in the same protein in independent mutants suggests that this gene is under strong selective pressure from silver exposure. Additional changes were seen in the multidrug resistance protein mdtK (loss of a start codon) and the introduction of a premature stop codon in nicP (a porin like protein). A lack of expression in mdtK alongside a possible change in permeability caused by variations in a porin like protein may be relevant to the reduced silver MIC displayed by this

strain during the first dosage cycles (0.21 ± 0.06 μg/mL to 0.06 μg/mL, before and after silver treatment respectively). Further missense changes were observed in genes that influence the proton motive force; napA, [108] efflux systems, cusS, rhtC, mdeA and kefC, [109-112] chemosensory system, fliY [113], and stress responses, htpX and trxC [114, 115].

Herein it has been shown that silver dosage can lead to increased susceptibility of this antimicrobial metal in ionic form. Nevertheless, further analysis of ciprofloxacin dosage on previously silver treated GP and GN strains revealed their ability to generally select for ciprofloxacin resistance more rapidly. The SNP analysis reveal changes affecting genes encoding central physiological processes of the species analysed, however, no specific silver locus was found, lacking a clear basis to explain the increased silver susceptibility observed. As the use of antimicrobial metals to tackle antibiotic resistant bacteria becomes more widespread in clinical settings, it is critical to ensure the long-term ability of these materials to maintain their potency and limit selection of resistant strains. The results provided in this manuscript clearly suggest that although silver is a potent agent against relevant bacterial strains in clinical settings, its use could lead to unexpected long term antibiotic resistance enhancement. Thus, it will be critical to deepen our understanding of these mechanisms in the long-term such that the biomaterials field may appropriately incorporate and deliver these alternative antimicrobial molecules.

4. Conclusions

In this study, the impact of silver exposure on a panel of laboratory and clinical strains of healthcare relevant GP and GN bacteria was assessed and analysed phenotypically and genotypically. Several unexpected observations were made, drawing the following conclusions:

- Silver dosage initially selects for decreased susceptibility albeit to a low level. However, this pattern is unstable, and susceptibility then consistently increases during prolonged exposure. Selection of *de novo* silver resistance therefore appears unlikely in these organisms, even after prolonged silver exposure.
- Exposure of organisms to silver led to an initial increase in susceptibility to the antibiotic ciprofloxacin but did not prevent selection of ciprofloxacin resistance for emerging, in fact this occurred somewhat faster in silver adapted strains.
- Ciprofloxacin resistance did not increase the fitness cost in any of the antimicrobial treatment analysed, resulting in a stable mutation conserved after antibiotic withdrawal.
- No clear genetic basis to explain the increased silver susceptibility observed was seen. Sequencing of base and Ag treated strains identified SNPs in many genes

encoding centrally important parts of bacterial physiology but only changes in cytochromes were selected in parallel lineages (for *P. aeruginosa*).

The results obtained in this manuscript suggest that clinical use of silver is unlikely to select for silver resistance, however, silver adaptation may influence the rate of development of resistance to other antimicrobial agents. Although more work is needed to understand the genetic basis for silver susceptibility, linkage between mutations and resistance development and other their correlation with other antibiotic molecules, it is clear from the present study that silver therapies can influence more conventional antibiotic approaches. Thus, it is recommended that the potential benefits of silver in clinical practice should be studied alongside other common antimicrobial therapies, to ensure that detrimental interactions are prevented from quickening microbial resistance.

5. Data availability

The present study can be found under BioProject number PRJNA692851, and BioSample numbers: SAMN17372948, SAMN17372949, SAMN17372950, SAMN17372951, SAMN17372952, SAMN17372953, SAMN17372954, SAMN17372955, SAMN17372956, SAMN17372957, SAMN17372958, SAMN17372959, SAMN17372960, SAMN17372961, SAMN17372962 and SAMN17372963. Raw sequencing reads were deposited to the Sequence Read Archive under accession number SUB8904177.

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7. Conflict of interest

The authors declare no conflict of interests.

8. Author contribution

Phenotypic assays and data analysis was performed by V.M.V. while genomes where studies by M.A.W., D. L. B. and V. M. V. The manuscript was written by V.M.V., D.L.B.,

M.A.W., T.H., M.L., O.A., S.A.K., L.M.G. and S.C.C. The project was supervised by L.M.G. and S.C.C. All authors have given approval to the final version of the manuscript.

Statement of significance

The adaptability of microbial life continuously calls for the development of novel antibiotic molecules, however, the cost and risk associated with their discovery have led to a drying up in the pipeline, causing antimicrobial resistance (AMR) to be a major threat to healthcare. From all available strategies, antimicrobial metals and, more specifically, silver showcase large bactericidal spectrum and limited toxic effect which coupled with a large range of processes available for their delivery made these materials as a clear candidate to tackle AMR. Previous reports have shown the ability of this metal to enact a synergistic effect with other antimicrobial therapies, nevertheless, the discovery of Ag resistance mechanisms since the early 70s and limited knowledge on the long term influence of silver on AMR poses a threat to their applicability. The present study provides quantitative data on the influence of silver based therapies on AMR development for a panel of reference and clinical strains of major nosocomial pathogens, revealing that prolonged silver exposure may detrimentally impact sensitivity to antibiotics.

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