Interaction between a Broad-spectrum Antibiotic and Silver Nanoparticles in a Human Gut Ecosystem

Das P*, Saulnier E*, Carlucci C, Allen-Vercoe E, Shah V and Walker VK*

1Department of Biology, Queen’s University, Kingston, ON K7L 3N6, Canada
2Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON N1G 2W1, Canada
3College of the Sciences and Mathematics, West Chester University, West Chester, PA 19382, USA
4Department of Biomedical and Molecular Sciences, and School of Environmental Studies, Queen’s University, Kingston, ON K7L 3N6, Canada

Abstract

The antimicrobial properties of engineered silver nanoparticles (AgNPs) have led to their wide use in diverse consumer products. Ampicillin too, acts as a broad-spectrum antibiotic and thus is prescribed for the treatment of many common infections, but with the problematic emergence of ampicillin-resistant bacteria. As a consequence, there has been some interest in the combination of these two distinct chemistries prompted by the clinical challenge of resistance. Prior to trials of combination therapy, however, it is important to understand the impact on human microbiomes. Here we investigated the effect of ampicillin and AgNPs, both individually and in a combined therapy on a human intestinal ecosystem known as a defined experimental community (DEC-60). The DEC-60 consortia was co-treated with a concentration of AgNPs (50 mg/L) known to have a minimal impact, and a broad range of the antibiotic up to the clinical dose (6 mg/L). The addition of AgNPs to sub-clinical doses of ampicillin (0.06 and 0.6 mg/L) had a significantly impact (p<0.001-0.05) on gas production (%CO₂ and %N₂) and changed the composition of fatty acid methyl ester signatures so that they were distinct from each individual antimicrobial, as well as un-treated control cultures. DNA sequencing, supported by multidimensional scaling analysis, confirmed the community shift and showed distinct phylogenetic distributions at different concentrations of ampicillin, depending on the presence of AgNPs. Together, these results suggest that the antibacterial efficacy of sub-clinical doses of ampicillin was increased by AgNPs, possibly due to the synergistic effect of damage to the bacterial cell walls. Not only does this analysis provide insight into AgNP toxicity, it offers some promise that combined antimicrobial therapies may have value in a clinical setting.

Keywords: Silver nanoparticles; Ampicillin; Human gut consortium; Sequencing; Fatty acid methyl esters; Anaerobic metabolism

Introduction

The known antimicrobial effects of engineered silver nanoparticles (AgNPs), combined with increasing efficient production methods have contributed significantly to their prevalence in diverse consumer goods ranging from food items and packaging, to sporting equipment, clothing, water purification units and cosmetics [1-3]. Surface charges associated with some AgNPs preparations may contribute to their toxicity [4]. However, multiple studies have highlighted the critical role of nano-size dependent effects compared to ionic silver, with an emphasis on the unique properties of AgNPs [5-7]. Although there are reports of mammalian cell cytotoxicity [8-10], these effects are likely relatively minimal at the concentrations used for antimicrobial activity [11]. Biofilms, which provide a refuge for Gram positive and negative bacteria, appear to be also inhibited by AgNPs [11]. Taken together, these observations have helped foster the use of AgNPs for medical and personal health applications. However, even though these concentrations of AgNPs may not be directly toxic to human tissues, the efficacy of AgNPs against bacteria can result in shifts in bacterial consortia, which in turn could have an impact on host organisms [e.g. 12-14]. Certainly, community changes in response to these NP have been noted in intestinal flora subsequent to oral ingestion in a range of species including fish, insects, and in a human model gut ecosystem [5,7,15].

The human microbiome is a diverse system composed of 100-200 species per individual [16], with the majority (~90%) falling into Bacteroidetes (generally staining Gram negative) and Firmicutes (generally staining Gram positive) phyla. Most of the remaining species belong to the Actinobacteria and Proteobacteria [17,18]. Suiting the intestinal microenvironment, resident human gut microbes are either facultatively or obligately anaerobic [19]. It is difficult to accurately recapitulate fecal ecosystems in a reproducible manner because of variations in the ecosystem abundance profiles as influenced by diet and transient microbial presence. However, one solution to this problem has been to create model fecal communities from diverse strains isolated from single donor fecal samples [20]. One such model community, Defined Experimental Ecosystem Consortium-60 members (DEC-60), has been developed as a proxy for a healthy fecal ecosystem and displays similar diversity at the phylum level to that found generally in human stool samples, and as well specifically includes species that are commonly found in the healthy human gut [21]. A subset of DEC-60 strains has been successfully used as a therapeutic ecosystem in the treatment of clinically-challenging Clostridioides difficile infections as an alternative to antibiotic therapy [21].

The persistent and pervasive use of antibiotics both therapeutically in human medicine, and as growth promoters in livestock agriculture has led to the widespread and unsettling emergence of antibiotic resistant bacteria [22]. It is generally agreed that antibiotic resistance is a significant threat to human health and that restriction of their source are credited.
use may help preserve their efficacy [23,24]. AgNP treatment, or a combined use of AgNPs with antibiotics, could reduce dosage thresholds for therapeutic activity and may also be effective against multi-drug resistant bacteria [25-28]. Indeed, this is the reason that AgNPs are currently being tested in a number of experimental point-of-use treatment systems for hospitals and nursing homes [29-31].

Here we have used the DEC-60 model microbiome system to investigate changes induced by both AgNPs and ampicillin treatment, both individually and in a combined therapy. Our goal was to examine if there were any synergistic effects of the combination therapy. There is some urgency to these experiments considering the increasing use of AgNPs in medical settings and the unknown impacts on the human intestinal microbiome. We hypothesized that co-treatment of the model ecosystem with AgNPs and ampicillin could perturb the system in a manner distinct from each individual antimicrobial. Ideally, combinatorial treatment with both amendments would allow a reduction in the dosage of the broad-spectrum antibiotic, ampicillin, in order to decrease the probability of the development of resistance to this drug.

**Materials and Methods**

### Silver nanoparticle and microparticle characterization

Suspended AgNPs, capped with poly(vinyl)pyrrolidone (PVP) were purchased from nanoComposix (San Diego, CA; DAG1964). Capping reduces agglomeration as well as the release of silver ions into the medium. The manufacturer reported diameters of 10.6 ± 1.7 nm (1.0 mg/mL stock concentration). The AgNPs were independently characterized via determination of number–based size distributions, dynamic light scattering (DLS), and zeta potential with a Malvern Instruments Ltd., UK. As well, although nominal stock concentrations for AgNPs were provided by the manufacturer, the actual 107Ag concentration for the stock AgNPs suspension was confirmed by inductively coupled plasma-mass spectrometry (ICP-MS; XSeries III, Thermo Scientific, Germany), optimized using PlasmaLab software, with 5 µg/L stock solutions of silver and indium as internal standards, as described [30]. Suspensions (50 mg/L) of AgNPs with each concentration of ampicillin used experimentally (0.06, 0.6, 3, and 6 mg/L) were further analyzed for size by transmission electron microscopy (TEM; Hitachi H-7000, Japan). To control for silver ions, powders of silver microparticles (7.5 µm; AgMPs) were purchased from MK Nano (MK Impex Canada, Mississauga, Canada). Suspension of these non-functionalized AgMPs were also characterized by TEM as reported elsewhere [7].

### Human gut ecosystem

A defined experimental community prepared with a total of 60 different bacterial isolates, designated as DEC-60, was cultured as described using an Infors Multifors bioreactor system (Infors, Switzerland) converted into a chemostat for continuous culture [21]. Nitrogen was bubbled through the system to maintain anaerobiosis. To mimic conditions of the human distal colonic microenvironment, the temperature was maintained at 37°C, and at a pH of 6.9-7.0. The autoclaved single-stage chemostat media, a mixture of insoluble starches, bile salts, mucin and other microbial growth supporting components was prepared, added to the culture vessel, reduced, and inoculated with freshly-cultured DEC-60 components, which had been each cultured to this drug.

Prior to experimentation, all materials were placed in the anaerobic chamber for several hours to ensure that oxygen was excluded from the cultures [21]. While continually maintaining anaerobic conditions, the subsamples of DEC-60 inocula were seeded into sterile growth medium (1:10). Diluted aliquots (5 mL) were then placed in sterile glass serum bottles (50 mL; Sigma-Aldrich) with treatment-groups established in triplicate, with antibiotic concentration and the presence or absence of nanoparticles as the primary variables. Ampicillin (Sigma Aldrich) was added separately to the serum bottles to a final concentration of 0.06, 0.6, 3, and 6 mg/L, both with and without AgNPs. Ampicillin concentrations were based on the use of 6 mg/L as a concentration approximating the clinical dose used for infections [34], and others at subclinical doses, with the lowest concentration representing two orders of magnitude below the clinical dose. The separate addition of AgNPs to a final concentration of 50 mg/L was chosen based on our previous observations of a minimal AgNP-mediated impact at this concentration [5]. Control treatment-groups consisted of unamended 1:10 DEC-60, Ag NPs (50 mg/L) in 1:10 DEC-60, and a control for the silver treatment consisting of 50 mg/L AgMPs in 1:10 DEC-60. Treated serum bottles were sealed with sterilized 20 mm rubber stoppers and subsequently fitted with WheatonTM aluminum seals (Fisher Scientific). Bottles were then packed in airtight bags with GasPakTM EZ anaerobic gas pouches (Fisher Scientific), and placed in a styrofoam container maintained at ~37°C. They were subsequently incubated in the dark at 37°C for 48 h, while shaken at ~15 rpm (Bibgill Thermolyne shaker; Dubuque, USA).

### Gas chromatography

After incubation, head-space gas samples were collected from each serum bottle. Luer-Lok™ Tip BD syringes (10 mL) equipped with 22G Precision Needle guides (Becton Dickinson & Co., USA) were used to collect gas samples (5 mL). The samples were immediately injected into an Agilent Technologies 7890B Gas Chromatograph (GC; USA) using a split-less mode, equipped with a stainless steel column (50 m x 0.53 mm internal diameter, 10 µm film thickness). Gas chromatography was conducted with the following operational controls: helium (He) gas carrier was set at a rate of 15 mL/min, the thermal conductivity detector (TCD) was at 250°C, and oven temperature was maintained at 32°C for 1 min. To correspond with the gas signatures of the consortium bacteria [35], peak areas for both N2 and CO2 were analyzed to determine head-space composition using ChemStation Integration Software (Agilent Technologies; USA).

### Fatty acid methyl ester profile analysis

After incubation, aliquots (three 1 mL samples) of liquid culture were removed from each of the three replicates for all treatment groups. These aliquots were centrifuged at 2,000 x g for 10 min. The supernatant was aspirated and pellets were preserved at -80°C until fatty acid methyl ester analysis was conducted (Keystone Labs, Canada). MIDI Sherlock Microbial Identification System (Microbial ID Inc., USA) was used to
extract the fatty acids from bacterial phospholipids in the pellets [36]. Briefly, the pellets were saponified using 1 mL NaOH in methanol (15% v/v) at 100°C for 30 min. Samples were then methylated through the addition of 2 mL HCl in methanol (54% v/v) at 80°C for 10 min. Fatty acid methyl esters (FAME) were then extracted by phase separation with the addition of 1.25 mL hexane/methyl tert-butyl ether (50% v/v) for 10 min. After discarding the aqueous phase, the organic phase was washed with 3 mL of 0.3 M NaOH with a few drops of saturated NaCl for 5 min. Fatty acid species composition was assessed using an Ultra 2 column with flame ionization detector equipped GC (Agilent).

Chromatographic peaks of each sample were then converted to reflect the percentage of each fatty acid species against the total methylated fatty acids.

DNA isolation and sequencing

After incubation, aliquots (200 μL) of the liquid cultures were sampled from each treatment group and DNA was extracted using a QIAamp DNA stool mini kit (Qiagen Sciences, USA), according to the manufacturer’s protocol. DNA purity and concentration was assessed initially with an Ultraspec™ 1100 pro UV/visible Spectrophotometer (Amersham Biosciences, USA), and confirmed with a NanoDrop-1000 (Thermo Scientific, USA). Bacterial DNA was further assessed by agarose gel electrophoresis and DGGE-PCR exactly as described previously [5]. These two analyses allowed a second estimate of quantity and qualitatively indicated the presence of multiple bacterial species, respectively. Individual DNA samples were inadequate in that they were not sufficiently pure, would not amplify, or showed evidence of problems after visualization in agarose or did not show multiple bands after DGGE-PCR, the DNA purification was repeated.

Bacterial DNA samples, which were pure and sufficient for subsequent analysis, were subjected to PCR using forward primer, Gray 28F (5’-TTGATCCTTGTTACGACTCA-3’), and reverse primer, Gray 519r (5’-TACGCCAGCGGTGTGCTG-3’), resulting in the amplification of the V1-V3 hypervariable regions of the 16S rRNA (~500 bp). PCR was performed with a 50 μL reaction mixture containing 4 μL of DNA template including 2.5 μL of each primer (10 μM), 5 μL of 10X ViiBuffer A (Vivantis), 0.5 μL of dNTP mix (10 mM; Thermo Scientific), 2.5 μL of bovine serum albumin (2 mg/mL), 0.4 μL of recombinant Taq DNA polymerase (10 mM; Vivantis) and 32.6 μL sterile water. PCR conditions were as follows: 5 min at 94°C denaturation, followed by a total of 30 cycles 94°C for 1 min, 64°C for 1 min, 72°C for 3 min, followed with a final extension at 72°C for 7 min. Amplified products (using triplicate samples for each treatment group) were subjected to 454 pyrosequencing according to standard protocols (MR DNA, USA). Briefly, products were purified with Agencourt Ampure beads (Agencourt Bioscience, USA) prior to sequencing. Sequencing was conducted using a Genome Sequences FLX System (Roche, USA) with titanium reagents as described [37]. The raw data was then filtered to exclude primers, as well as failed and low quality reads. Chimeras were eliminated using Black Box Chimera Check software (B2C2) [38,39]. After de-noising, sequences were clustered using a distributed MegaBLAST.NET algorithm [40], and subsequently compared to published reads in the National Center of Biotechnology Information (NCBI) [41]. Bacterial species designation was determined according to recovered reads in comparison with published database sequences and known identity of members of the DEC-60 consortium.

Statistics

Statistical analysis of culture gas composition, FAME profiles, and pyrosequencing were conducted by two-way analysis of variance (2F-ANOVA) as well as independent 2-factor t-tests where appropriate, using the statistics packages R [42] and SPSS (SPSS Inc., USA). Significant differences between pairs of means were evaluated by Tukey’s honest significant differences - post-hoc tests. Multidimensional scaling (MDS) of the relative percent composition of 16S rRNA sequence was used to reduce the bacterial composition data set and compare multivariate responses to treatments with different concentrations of ampicillin and AgNPs using SAS Ver 9.4 (SAS Institute, USA).

Results

Electron microscopy of nanoparticles

Purchased PVP-capped AgNPs were used since as indicated, the capping agent helps prevent agglomeration and previous experiments had shown that no toxicity was attributable to PVP [4]. The AgNPs were analyzed for zeta potential and diameter using DLS and the concentration assessed using plasma-mass spectrometry. The manufacturer’s characterization was confirmed with the diameter of the stock AgNPs suspension at 10.9 ± 1.2 nm by number-based hydrodynamic size distribution (vs. the supplier’s value of 10.6 ± 1.7 nm). As well, TEM confirmed that the capped particles remained well dispersed in the experimental media containing 0.06, 0.6, 3, and 6 mg/L ampicillin (Figure 1A-1D); the particles did not agglomerate and remained within the anticipated size range.

Head-space gas analysis

The head-space gas volume after 48 h could not be determined, but the proportion of each gas was achieved GC analysis. Control (no AgNPs) treatment replicates showed no significant differences in the % CO2 fraction as compared to AgNPs (Figure 2) and AgMP-treatment groups (data not shown). Similar results were also observed for % N2 fraction in controls as compared to AgNPs and AgMPs (Figure 3). Thus the presence of Ag particles alone appeared to be insufficient to significantly impact bacterial anaerobic respiration (Figure 2).

For ampicillin experimental treatments, at a clinical dose (6 mg/L),
Carbon dioxide (CO\textsubscript{2}) was evident at the highest concentration. The percent CO\textsubscript{2} and N\textsubscript{2} composition were elevated relative to that seen at the sub-clinical threshold concentration of 3 mg/L ampicillin, which showed the highest variation (values of ~0.4% and ~0.6% CO\textsubscript{2} in the presence and absence of AgNPs, respectively).

**Fatty acid profiles**

Examination of FAME profiles revealed a total of 28 unique signatures. Some were present at low levels in all samples and thus these were pooled into a single 'Others' category and therefore only 18 dominant signatures (≥ 0.15 mol%) were considered for subsequent analyses (Figure 4). Differences in total FAME signatures were significant in low concentration (0.06 mg/L) ampicillin amendment groups, supplemented with and without AgNPs. They were also significantly different when the 0.6 mg/L ampicillin and the clinical dose (6 mg/L) pairs were compared. The p-values for the differences between treatments at both control (no ampicillin) and the clinical threshold concentration (3 mg/L ampicillin) were both below the significance limit.

Comparisons of mol% composition of FAME signatures in AgNP and AgMP control treatments with untreated controls (0 AgNPs and 0 ampicillin) revealed no significant differences for any of the dominant fatty acids assessed (Figure 4). Conversely, comparison of samples treated with the combination treatment of AgNPs and ampicillin vs. AgNP-alone controls (AgCtr) revealed significant differences in percent composition for several fatty acids (Figure 4). For example, in the low antibiotic dose treatment groups, the 17:0 3 OH signal was absent compared to a mean abundance of ~0.7% across AgCtr. The differences between treatments at both control (no ampicillin) and the clinical threshold concentration (3 mg/L ampicillin) were both below the significance limit.

The percent CO\textsubscript{2} was just above the starting gas composition of 10%, suggesting that DEC-60 viability and/or metabolism was severely limited, as expected for the broad-spectrum antibiotic. At sub-clinical ampicillin doses, respiration was elevated relative to that seen at the highest concentration. The percent CO\textsubscript{2} and N\textsubscript{2} composition were significantly different (p<0.01) in both 0.06 and 0.6 mg/L ampicillin treatment groups when compared between treatments, with and without AgNPs (Figures 2 and 3). Summary statistics from 2F-ANOVA revealed significant effects of both AgNPs (p<0.01) and ampicillin (p<0.001) on culture head-space percent CO\textsubscript{2} and N\textsubscript{2} composition, as well as a significant interaction between the two (p<0.001). Finally, replicates within individual treatments were highly consistent with respect to percent CO\textsubscript{2} composition; standard deviations in non-control treatments were all <0.15, with the exception of treatments at a sub-clinical threshold concentration of 3 mg/L ampicillin, which showed the highest variation (values of ~0.4% and ~0.6% CO\textsubscript{2} in the presence and absence of AgNPs, respectively).
when compared to AgCtr treatment groups: 18:1 ω9c, a Gram-positive associated fatty acid, was increased from 11.8% to 13.8% in the presence of 6 mg/L ampicillin (p<0.05), 14:0 3OH/16:1 i o1, an ‘unnamed’ species was eliminated in the presence of high doses of antibiotic from 0.8% (p<0.05), and another fatty acid, 18:2 ω6,9c/18:0 ante, was increased from 3.6% to 4.6% (p<0.01).

AgNPs and ampicillin treatment effects on community composition

After assigning the 16S rRNA V1-V3 hypervariable region sequences to the bacterial species in DEC-60, those with low sequence abundance in all treatment groups (≤1%) were pooled into a single ‘Others’ category. Treatments were then compared for both total sequence reads, a proxy for total bacterial abundance, as well as the relative abundance of each species normalized to the total fatty acids recovered from each experimental treatment, as previously discussed [5] (Figure 5). Significant differences in total response were observed between treatments (± AgNPs) at all concentrations of ampicillin (except for control treatments) with p<0.01, p<0.01, p<0.05, and p<0.05 for 0.06, 0.6, 3, and 6 mg/L ampicillin treatments, respectively. Large-scale changes in relative species abundance were seen across treatments, with the percentage composition of Bacteroides fragilis decreasing with higher ampicillin doses, and with a corresponding increase in Bacteroides ovatus. Furthermore, MDS analysis of the sequencing results highlighted distinct phylogenetic distributions at each given ampicillin treatment depending on the presence of AgNPs (Figure 6). The tight clustering of replicates by MDS and clear shifts with the changing treatment conditions reflected the dose-dependent bacterial community modulation mediated by the combination AgNP and ampicillin treatment. No significant differences were observed between the AgMTPs treatment groups compared to the control without AgNPs (Figure 5).

Discussion

As a broad-spectrum low cost antibiotic, ampicillin is used orally to treat common infections and because of its utility, it has been placed on the list of essential medicines by the World Health Organization (http://www.who.int/medicines/events/is/en/). It is a β-lactam, which interferes with the assembly of the bacterial cell wall. Not only do these properties make this a valuable antimicrobial but it is also vulnerable to inactivation in resistant bacteria by the production of β-lactamases, which hydrolyze the β-lactam ring [45]. Thus, resistance can sometimes be alleviated by the co-administration of drugs that inhibit the hydrolyzing enzymes. Although the antimicrobial mechanism of AgNPs is not known, a popular explanation suggests that interactions between AgNPs and bacteria under aerobic conditions can lead to the production of reactive oxygen species (ROS), which in turn can damage DNA, RNA and proteins [46]. Although we conducted our experiments anaerobically, it is interesting in this regard that our sequencing results showed that facultative anaerobes including Veillonella dispar and Collinsella aerofaciens without ROS-scavenging pathways, either increased in relative abundance or were not different in comparison with treatment groups that lacked AgNPs, respectively (Figure 5). There is also evidence that AgNPs can disrupt bacterial membranes [47]. This mechanism would suggest the potential for a synergistic interaction with the combination of ampicillin and AgNPs, as was observed in our experiments. It is important to note that such interactions may depend critically on the size of the nanoparticle. For example, Habash et al. [48] reported a combination effect of 10 nm and 20 nm sized AgNPs when added to the antibiotic aztreonam and tested against Pseudomonas aeruginosa, whereas no interaction was observed with 40, 60 and 100 nm sized AgNPs. We only tested 10 nm AgNPs since dose-response data was available for these NPs, and thus it is unknown if larger particles would be less effective; certainly we saw no significant effect with AgMTPs.

Over a 48 h incubation period, the strong antimicrobial effect of ampicillin used at the 6 mg/L clinical dose was evidenced by negligible gas production likely due to lower metabolic processes or cell death. This is in contrast to the results obtained with the lowest antibiotic doses (0.6 and 0.06 mg/L) that showed no difference from unamended controls (Figures 2 and 3). Strikingly, when AgNPs were co-incubated with ampicillin at these same low concentrations there was a significant reduction in produced CO2 (Figure 2). Together, this suggests that co-treatment with ampicillin and AgNP results in a synergistic reduction in targeted bacterial metabolic activity or viability. Furthermore, there was a strong agreement between the total fatty acid responses of each treatment and CO2 production; the increase in respiration was positively correlated (r=0.64) with a shift in the total fatty acid methyl ester profile. Thus, the reduction in total FAME mol% from the culture substrate, as well as decreased CO2 fermentation suggests an overall reduction in bacterial mass and viability when samples were treated with AgNPs and the higher ampicillin concentrations. Similarly, Birla et al. [49] observed a substantial reduction in metabolic activity when the antibiotic gentamicin was combined with AgNPs and tested against Escherichia coli and Staphylococcus aureus.

While detection of FAME shifts in treated DEC-60 communities revealed unique microbiome profiles for the treatment groups, as indicated, the identification of fatty acids ‘signatures’ should be treated with caution. Cellular stress, potentially in the form of ampicillin or AgNPs-mediated toxicity, could result in changes to bacterial lipids [50], reducing the reliability of signature benchmarks, but would not affect FAME recovery. Sequencing results, however, also revealed dramatic shifts in the abundance of several key species, suggesting that FAME identifications could be a useful and efficient screen for combinatorial effects.
Thus, the concentration of AgNPs used here was deliberately chosen to have no impact on the gut ecosystem by itself. Nevertheless, our results indicate that the addition of AgNPs to ampicillin treatments have the potential to increase antibiotic efficacy and reduce collateral effects on beneficial intestinal communities. We further suggest that the observed synergistic antibacterial efficacy of AgNPs is as a keystone species for overall gut microbial competition.

Consequently, the sustained abundance of this species when treated with AgNPs suggests that co-treatment with NPs and ampicillin while therapeutic, might reduce the inadvertent incidence of gut dysbiosis. In further support of this interpretation, *B. ovatus*, one of the primary constituents of the DEC-60 culture (~20% abundance in control conditions) increased in relative abundance to ~53% when treated with 6 mg/L ampicillin (thus was not affected by the antibiotic), but this change was abrogated by co-treatment with AgNPs (~18% abundance). Other key commensal species such as *Roseburia intestinalis* and *Coprooccus cocmes* (immune regulation through butyrate secretion [51]), and *C. aerofaciens* (contributes to host health) maintained or increased in relative abundance when amended with AgNPs.

Notably, the concentration of AgNPs used here was deliberately chosen to have no impact on the gut ecosystem by itself. Nevertheless, our results indicate that the addition of AgNPs to ampicillin treatments have the potential to increase antibiotic efficacy and reduce collateral effects on beneficial intestinal communities. We further suggest that the analysis of synergistic effects may also provide insight into the mechanism of AgNP antimicrobial action. Our results indicate that the observed synergistic antibacterial efficacy of AgNPs combined with ampicillin at different concentrations was nano-size dependent, as also evidenced from the non-significant effect by AgMPs. In addition, we suggest that AgNPs increased the efficacy of sub-clinical doses of 0.06 and 0.6 mg/L ampicillin at the cell wall by fostering disruption, inhibition of cell-wall synthesis and/or direct damage of it by cross linking of the cell wall peptidoglycan. These experiments thus show promise for combinatory therapeutic treatment with higher concentrations of AgNPs and sub-clinical doses of ampicillin resulting in an overall reduction in the use of this antibiotic.

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**References**


