Potentiation of Gram-positive specific antibiotics against Gram-negative bacteria through outer membrane disruption

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Chapter 4

Exploring Gram-positive specific antibiotics potentiation by human serum against Gram-negative bacteria

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Abstract

The rise of antibiotic resistance will lead to millions of deaths worldwide if left unchecked. Especially Gram-negative bacteria are inherently harder to target due to the outer membrane that functions as an additional barrier. Recent work has shown the ability of human serum to potentiate Gram-positive specific antimicrobials nisin and vancomycin against Gram-negative bacteria and thus expanding their range. Serum contains proteins of the complement system that upon activation trigger the formation of the membrane attack complex that damages the outer membrane of Gram-negative bacteria. Damage to the outer membrane allows some Gram-positive specific antimicrobial agents to reach their targets. In this study, the potential of human serum for synergy with multiple classes of Gram-positive specific antibiotics was systematically investigated via inner membrane permeability and bacterial viability assays. Three Gram-negative bacteria were selected for screening: *E. coli*, *K. pneumoniae* and *P. aeruginosa*. Inner membrane permeability was observed for most Gram-negative bacteria when treated with a mixture of serum + nisin or serum + vancomycin. For *E. coli* the combination of serum with daptomycin, telavancin, oritavancin, or dalbavancin also resulted in a moderate increase of inner membrane permeability. The viability of *E. coli* was significantly reduced when incubated with serum in combination with nisin, erythromycin, quinupristin & dalfopristin, rifampicin, vancomycin, or dalbavancin compared to serum or the antibiotics alone. By comparison, for *K. pneumoniae* only nisin, rifampicin, and vancomycin displayed a significant synergistic effect when combined with serum. Serum was also found to potentiate nisin, rifampicin, vancomycin, as well as quinupristin & dalfopristin against *P. aeruginosa*. This study reveals that the immune system can sensitize different Gram-negative bacteria toward several Gram-positive specific antibiotics.

1. Introduction

The rise of antimicrobial resistance, combined with the lowered discovery rate of new antibiotic classes, has already led to the inability to treat infections in some patients.[1–4] Most notable is the growing resistance among *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* against the few remaining antibiotic treatments leading these multidrug resistant pathogens to be assessed at the highest threat level by the World Health Organization.[1] Gram-negative bacteria are inherently harder to target by antibiotics, due to the presence of an additional barrier: the outer membrane (OM).[2,5–7] Apart from the impermeable OM itself, the entry of compounds for Gram-negative bacteria is highly restricted by porins and selective uptake, for example, through siderophores.[8–10] In addition, efflux pumps effectively transport the few compounds that do gain entry out of the cell and upregulation of these pumps is often directly related to resistance.[9,10]

Disruption of the OM with adjuvants has the potential to counter this inherent resistance and results in the potentiation of antibiotics normally excluded by the OM.[5,11] Several synergistic molecules have been well-described over the past decades even leading to the first successful Phase I clinical trial with such a synergist (Chapter 1).[5,11] Human serum has also been reported to potentiate antibiotics against Gram-negative bacteria.[12–14] In addition, human serum has been described to form pores in cell membranes through the membrane attack complex (MAC).[15–17] Only recently these two findings were combined and systematically investigated in a report describing the potentiation of antimicrobial compounds nisin and vancomycin by the MAC pores.[18]

The MAC consists of five different proteins ($C5b6789_{18}$) and results from a step-wise activation process called the complement cascade (Figure 1).[19] The recognition of the bacterial surface leads to activation of the classical pathway of the complement system. This results in the deposition of surface-bound convertases.[20–22] Then, cleavage of component C3 into C3a and C3b occurs by the surface-bound convertases.[20] This leads to the covalent linkage by a thioester of C3b to the membrane and the high density of C3b deposition leads to the formation of a C5 convertase.[23,24] The conversion of C5 to C5b was found to be critical and this unstable C5b requires rapid binding of C6 or it tends to aggregate.[25,26] The C5b6 complex recruits the C7 component and subsequently C8 to result in the C5b-8 complex.[27,28] The recruitment of 18 copies of C9 finalizes the transmembrane MAC pore with an inner diameter of 11 nm. In addition to the permeabilization of the outer membrane, human serum at higher concentrations is also able to disrupt the inner membrane leading to bacterial killing.[18,29]

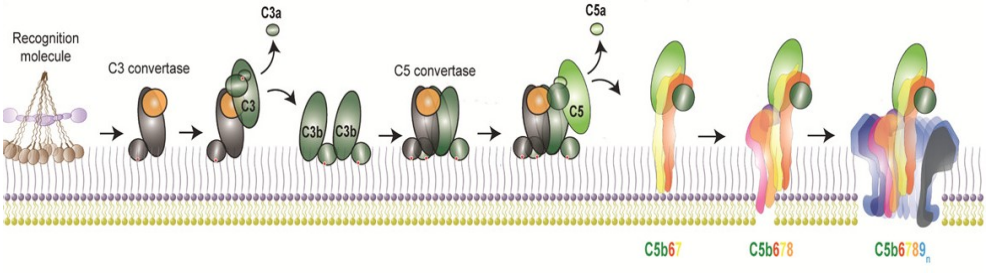


Figure 1. Overview of the step-wise formation of the membrane attack complex (MAC). Image by Doorduijn (2019)[30]

The difference in efficiency between outer and inner membrane permeabilization creates a window in which antimicrobial compounds normally inactive against Gram-negative bacteria such as nisin and vancomycin, can synergize with serum (Figure 2).[18] This leads to faster killing of bacteria and the killing occurs at lower serum or antibiotic concentrations.[18] The potentiation of other Gram-positive specific antibiotics against Gram-negative bacteria has not yet been explored systematically. The potentiation by serum could shine a new light on the range of antibiotic activity in the human body and would contribute to understanding the interactions of human serum with bacteria and antibiotics.

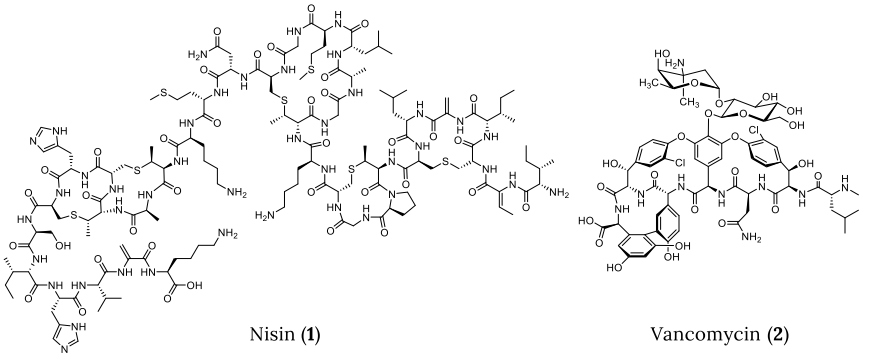
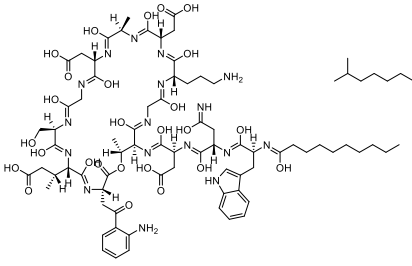


Figure 2. Molecular structures of antimicrobial nisin (**1**) and antibiotic vancomycin (**2**)

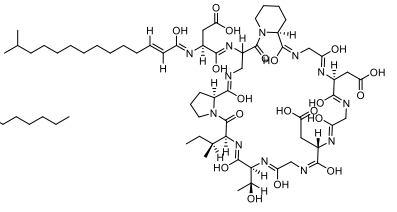
Therefore, in this chapter, the inner membrane permeability and bacterial viability of Gram-negative bacteria was analyzed in order to screen for synergy between serum and selected Gram-positive specific antibiotics (Table 1 and Figure 3). The selection of the specific antibiotics of the classes requires in some cases an additional explanation. Of note is oxacillin, which is part of the first generation of semisynthetic penicillins that do not have broad-spectrum activity (unlike the later generation penicillins).[31] The antimicrobial agent nisin and antibiotic vancomycin serve as controls since their synergy with serum has already been established against *E. coli*, *K. pneumoniae*, and *P. aeruginosa*.[18] Laspartomycin, apart from nisin, is the only antibacterial in our study that is not clinically used, but like vancomycin, nisin, bacitracin and oxacillin it targets the cell wall synthesis and was therefore included (Table 1).[32,33] Lastly, since vancomycin and serum were already reported as a potent synergistic combination, telavancin, oritavancin and dalbavancin were included for a more in-depth structure activity relationship study of glycopeptides with serum (Figure 3).[18]

Table 1. Overview of the antibiotics of each class screened for potentiation by human serum

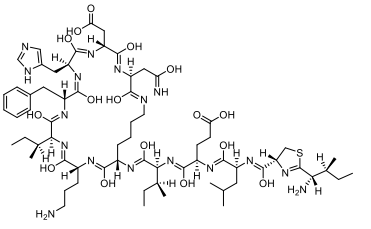
	Antibiotic	Class	Target	Route of administration
1	Nisin	Lantibiotic	Cell wall synthesis; pyrophosphate of Lipid II and pore formation in the membrane	Food preservative[34,35]
2	Vancomycin	Glycopeptide	Cell wall synthesis; D-Ala-D-Ala motif of Lipid II peptide	IV/oral[36,37]
3	Daptomycin	Lipopeptide	Calcium-dependent binding to phosphatidylglycerol, depolarization and permeabilization of the membrane	IV[38–43]
4	Laspartomycin	Lipopeptide	Cell wall synthesis; undecaprenyl phosphate	No clinical trials[32]
5	Bacitracin	Polypeptide	Cell wall synthesis; undecaprenyl pyrophosphate	Topical/intramuscular[44–46]
6	Oxacillin	Penicillins	Cell wall synthesis; DD-transpeptidase	IV/oral/intramuscular[31]
7	Fusidic acid	Steroid antibiotics	Inhibition of protein synthesis; EF-G	IV/oral/topical[47]
8	Lincomycin	Lincosamides	50S	IV/intramuscular[48,49]
9	Linezolid	Oxacolidinone	50S	IV/oral[50]
10	Erythromycin	Macrolide	50S	IV/oral/topical(eye)[51]
11	Quinupristin & dalfopristin	Streptogramins	50S	IV/topical[52,53]
12	Rifampicin	Rifamycins	RNA polymerase	IV/oral[54]



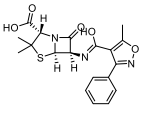
Daptomycin (**3**)



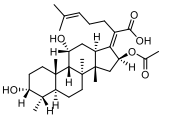
Laspartomycin (**4**)



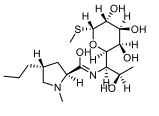
Bacitracin (**5**)



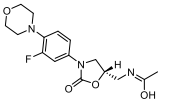
Oxacillin (**6**)



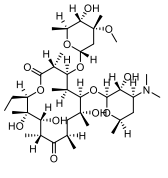
Fusidic acid (**7**)



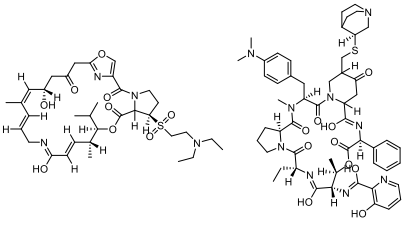
Lincomycin (**8**)



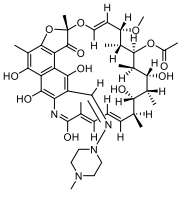
Linezolid (**9**)



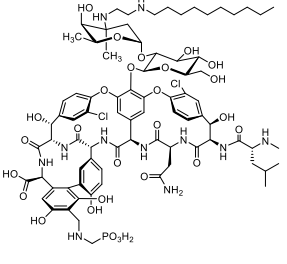
Erythromycin (**10**)



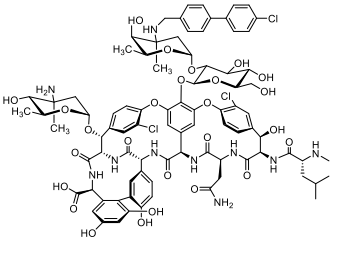
Quinupristin & dalfopristin (**11**)



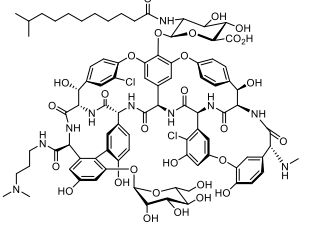
Rifampicin (**12**)



Telavancin (**13**)



Oritavancin (**14**)



Dalbavancin (**15**)

Figure 3. Molecular structures of the Gram-positive specific antibiotics screened in combination with human serum

2. Results

2.1. Inner membrane permeability assay reveals glycopeptides, nisin, and daptomycin as synergists

The inner membrane permeability assay used was based on the previous work that reported the potentiation of nisin and vancomycin by MAC.[18] SYTOX Green functions as the probe for inner membrane permeability, since this nucleic acid stain cannot penetrate intact bacterial cells. Gram-positive specific antibiotics were screened against *E. coli*, *K. pneumoniae*, and *P. aeruginosa* to establish their effectiveness in the presence of serum. The intensity of the SYTOX Green signal was monitored over 2 hours (Supporting information, Figure S1-S15). For the read-out, we selected the 2-hour time point where the expected synergy of nisin and human serum is clearly visible and absent when serum was heat-inactivated (Figure 4).

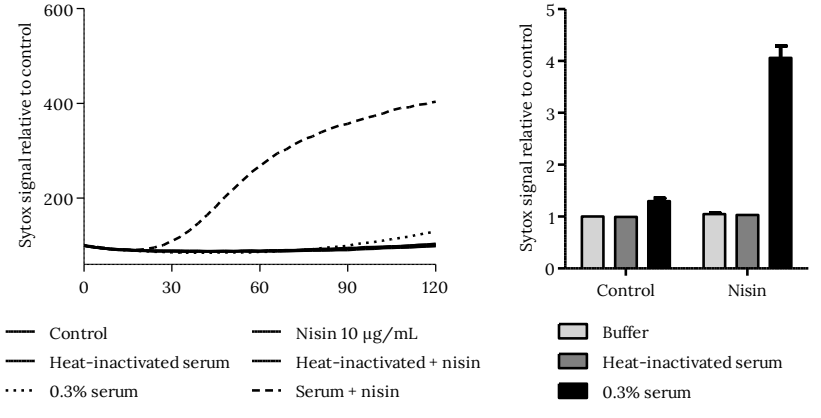


Figure 4. Inner membrane permeability assay using SYTOX Green of *E. coli* following incubation with buffer (control), 0.3% heat-inactivated serum, and 0.3% serum with or without 10 µg/mL nisin at 37 °C. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were depicted as relative values to the control condition and represent mean ± SD of three independent experiments.

Next, *E. coli* was screened with a 0.3% serum concentration in combination with the different antibiotics (Figure 5A). After 2 hours of incubation inner membrane permeability increased when bacteria were treated with serum in combination with nisin or vancomycin compared to serum only (Figure 5A). A moderate increase in inner membrane permeability was observed for daptomycin, oritavancin, and dalbavancin (Figure 5A).

For *K. pneumoniae* and *P. aeruginosa*, the concentration of serum was based on a previous study (10% and 1% serum respectively).[18] The bacteria were screened with medium, serum, and heat-inactivated serum in the absence and presence of antibiotics. At the 2-hour time point, serum alone already caused inner membrane damage for *K. pneumoniae* and *P. aeruginosa* (Supporting information Figure S16). Therefore, a different time point was taken for which the SYTOX Green signal for nisin in combination with serum was higher as the serum control (Supporting information Figure S1). For *K. pneumoniae* this was at 15 minutes and for *P. aeruginosa* at 90 minutes (Figure 5B-C).

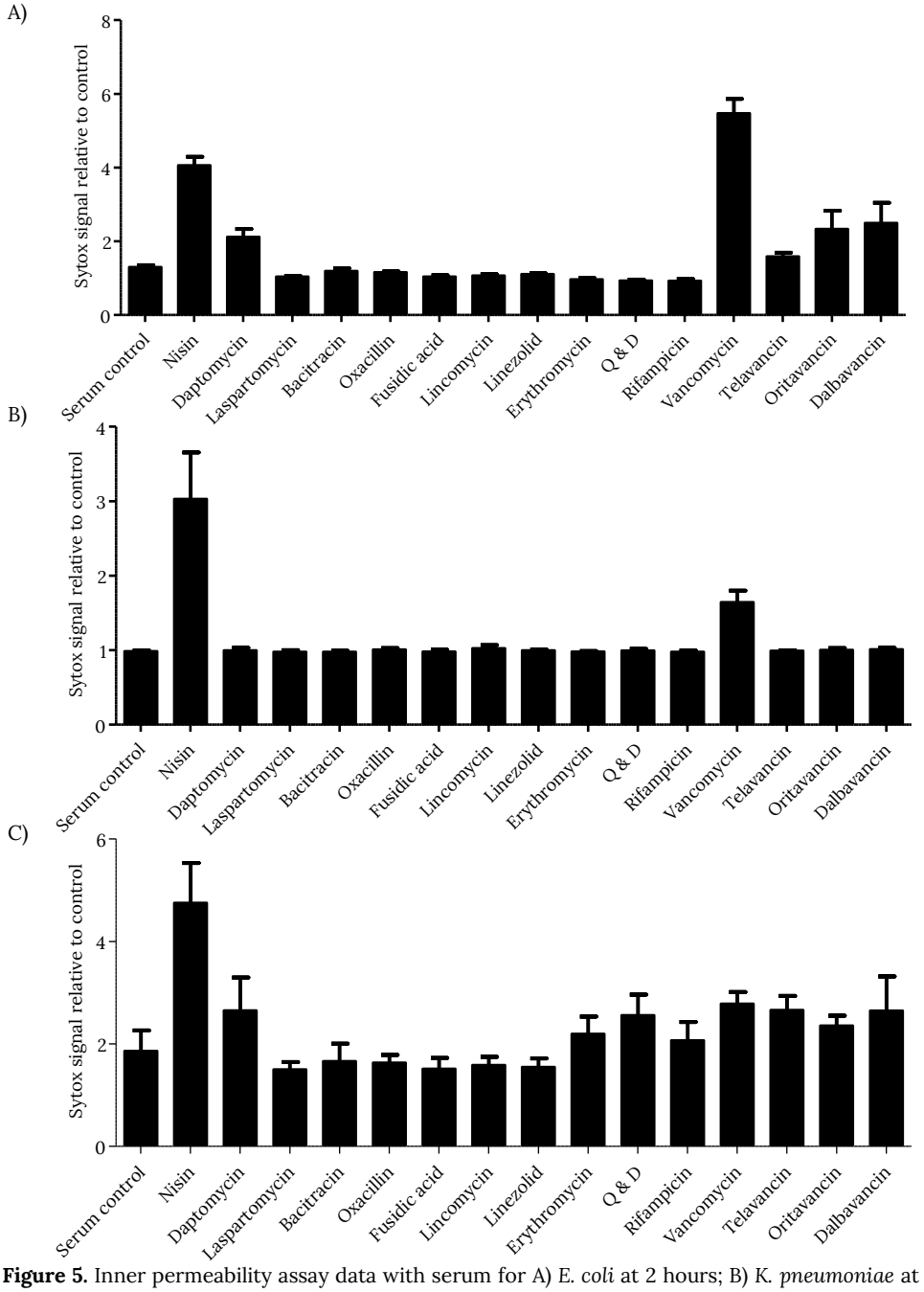


Figure 5. Inner permeability assay data with serum for A) *E. coli* at 2 hours; B) *K. pneumoniae* at 15 minutes; C) *P. aeruginosa* at 90 minutes. The antibiotics (10 μg/mL, except for erythromycin (5, 2.5, 5 μg/mL respectively) and rifampicin (5, 2.5, 2.5 μg/mL respectively) were screened in combination with serum (0.3% for *E. coli*, 10% for *K. pneumoniae*, and 1% for *P. aeruginosa*). Values were depicted as relative values to the buffer control conditions and represent mean ± SD of three independent experiments.

In addition to the nisin and serum combination, only vancomycin in combination with serum resulted in an increase in SYTOX Green signal for *K. pneumoniae* compared to the serum control. Erythromycin, quinupristin & dalfopristin (Q&D), and rifampicin combined with serum display a slight increase in inner membrane permeability compared to the serum control, but this effect falls within the standard deviation (Figure 5C). These screenings thus revealed the combinations of serum with nisin, daptomycin, or the glycopeptides resulted in increased levels of inner membrane damage compared to serum controls in at least one of the three Gram-negative strains.

2.2. Validation of the inner membrane permeability by a bacterial viability assay

The antibiotics that resulted in increased inner membrane permeability when combined with serum were selected for further screening in a bacterial viability assay. This included nisin, daptomycin, vancomycin, telavancin, oritavancin, and dalbavancin. Also, given the unresolved effects on *P. aeruginosa* inner membrane permeability observed for erythromycin, Q & D, and rifampicin when combined with serum, these antibiotics were added to the screen. Bacitracin was also selected as a negative control. While the inner membrane permeability assay used in the previous section provides a very sensitive read-out, the bacterial viability assay here employed allows for the determination of whether the combination of serum with antibiotic has a significant effect on bacterial viability.

Notably, bacterial viability was found to be affected by antibiotic concentration of 10 μg/mL for rifampicin and erythromycin in the absence of serum (Supporting information, Figure S17-18). To validate the effect of serum potentiation, inherent activity of the antibiotics should preferable not be detected. For this reason, serial dilutions of erythromycin and rifampicin were screened against the bacteria (Supporting information, Figures S17-18) and the concentrations of both adjusted accordingly for *E. coli*, *K. pneumoniae*, and *P. aeruginosa*.

Bacterial viability was measured under similar conditions as for the inner membrane permeability assay. The combination of serum with antibiotics showed a significant reduction in bacterial viability for nisin, rifampicin, Q & D, vancomycin, and dalbavancin for *E. coli* (Figure 6A). The combinations of serum with daptomycin, telavancin, and oritavancin, also led to reduced bacterial viability, but not significantly. Bacitracin, which was taken along as an additional negative control, showed similar levels of viability as the control. Additionally, heat-inactivated serum in combination with antibiotics was also screened and these combinations had no effect on viability (Supporting Information, Figure S19, S22, and S23).

For *K. pneumoniae*, a concentration of 10% serum was selected, which was found to reduce bacterial viability by 90% (Figure 6B). However, addition of rifampicin, nisin, and vancomycin further decreased viability significantly, while erythromycin and Q & D resulted in a moderate decrease in viability compared to serum alone (Figure 6B). Heat-inactivated serum in combination with these antibiotics did not display this effect (Supporting information, Figure S20, S22, and S23). The effect of serum with bacitracin, daptomycin, telavancin, oritavancin, and dalbavancin on bacterial viability was found to be negligible.

Similar to *K. pneumoniae*, serum also resulted in more than one log reduction of bacterial viability for *P. aeruginosa* when added at a concentration of 1% (Figure 6C). The

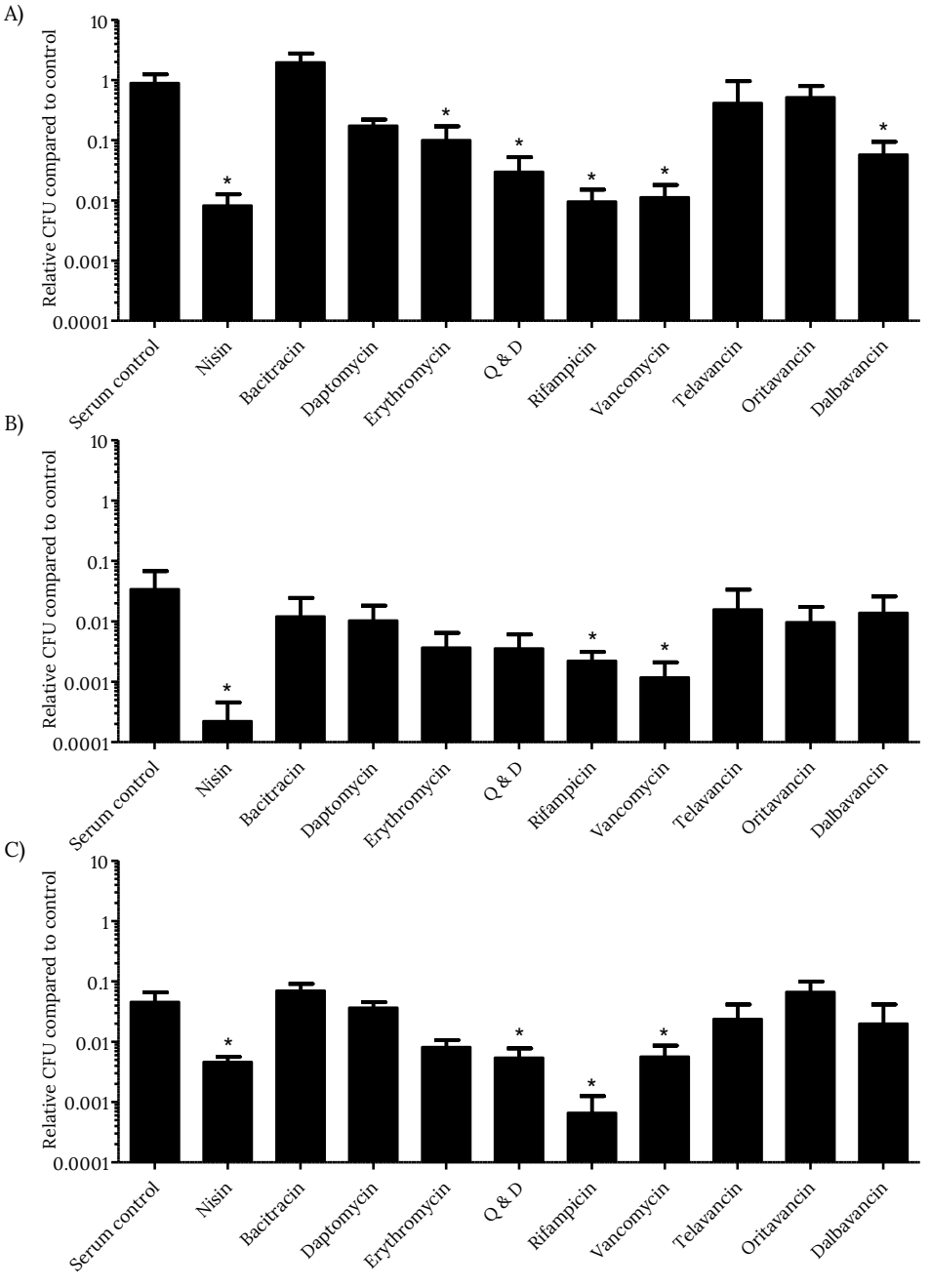


Figure 6. Synergy between serum and antibiotics: viability of A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. 2 hours of incubation with serum (0.3% for *E. coli*, 10% for *K. pneumoniae*, and 1% for *P. aeruginosa*) with 10 μg/mL antibiotics (for erythromycin 5, 2.5, 5 μg/mL and rifampicin 5, 2.5, 2.5 μg/mL respectively) at 37 °C with shaking. CFU counts were normalized to buffer controls. Data represent mean ± SD of three independent experiments and were analyzed by an one-way ANOVA and Dunnett test (*$p < 0.05$) using the serum as control group.

combinations of serum with nisin, vancomycin, rifampicin, or Q & D significantly reduced the bacterial viability compared to the serum control (Figure 6C). Again, this effect was absent in the heat-inactivated serum with antibiotics combinations (Supporting information, Figure S21, S22, and S23). Erythromycin with serum did lower the bacterial viability visibly, but not significantly.

2.3. Potentiation by human serum differs among the glycopeptide antibiotics

In addition to vancomycin, the glycopeptides telavancin, oritavancin, and dalbavancin were selected to investigate the potentiation with serum within a class of antibiotics. For *E. coli*, a large increase in inner membrane permeability was observed for vancomycin, while for oritavancin, dalbavancin, and telavancin only a moderate increase was detected in the presence of serum (Figure 5A). In agreement with these findings, vancomycin combined with serum resulted in a significant decrease in bacterial viability (Figure 7A) while telavancin and oritavancin did not affect the viability. Notable, however, was the finding that dalbavancin also resulted in a significant reduction of bacterial viability (Figure 7A).

The combinations of telavancin, oritavancin, and dalbavancin with serum did not affect the inner membrane permeability of *K. pneumoniae*, nor its bacterial viability (Figure 5B and 7B). Vancomycin + serum resulted in both inner membrane permeability and a significant reduction in bacterial viability (Figure 5B and 7B). For *P. aeruginosa*, the inner membrane permeability was only slightly increased with the different glycopeptides (Figure 5C) with only vancomycin + serum causing a reduction of bacterial viability (Figure 7C).

These results were compared to different parameters of the glycopeptides (Table 2). The inherent activity of vancomycin is the least potent against Gram-positive bacteria, which seems contrary to our data on glycopeptide potentiation against Gram-negative bacteria. The greater lipophilicity and/or serum protein binding of the next generation glycopeptides relative to vancomycin could provide an explanation as to these findings. However, the significant reduction of *E. coli* viability by dalbavancin would seem to argue against this possibility.

Table 2. Inherent activity, (calculated) lipophilicity and protein binding of the glycopeptides

Glycopeptide	MIC_{90} (μg/mL)[55,56]	XLogP3-AA	Protein binding[55,56]
Vancomycin	1	-2.6[57]	30-55%
Telavancin	0.06	-2.1[58]	90%
Oritavancin	0.12	1.5[59]	85%
Dalbavancin	0.03	3.8[60]	93%

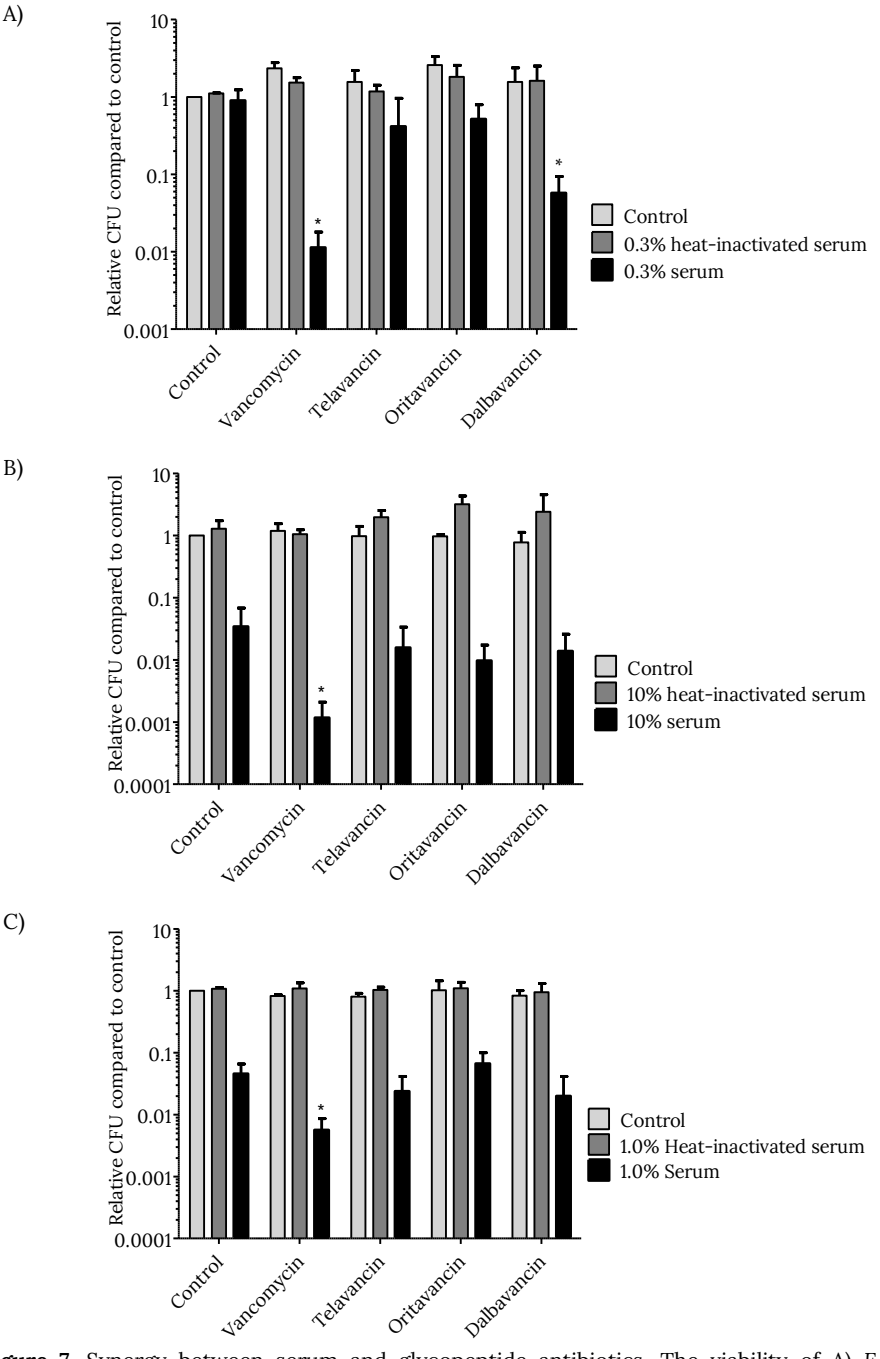


Figure 7. Synergy between serum and glycopeptide antibiotics. The viability of A) *E. coli*; B) *K. pneumoniae*; and C) *P. aeruginosa* was screened after 2 hours of incubation with buffer, heat-inactivated serum or serum with 10 μg/mL antibiotics at 37 °C with shaking. CFU counts were normalized to buffer controls. Data represent mean ± SD of three independent experiments and were analyzed by an one-way ANOVA and Dunnett test (*$p < 0.05$) using the serum as control group.

3. Discussion and conclusion

The potentiation of antibiotics towards Gram-negative bacteria can be achieved by the addition of a chemical synergist capable of disrupting the outer membrane (Chapter 1). Although this field has been widely explored, mainly the discovery of new synergists is reported in addition to the work focused on the (clinical) development of the polymyxin derived synergists (Chapter 1).[5] The previous work by Heesterbeek *et al.* already illustrated that exogenous OM disruptors might not even be required in the presence of serum.[18] Based on the OM disruption mechanism of serum, an expanded screening of antibiotics, such as rifampicin, clindamycin, and erythromycin, often described as the antibiotic partners of the outer membrane disrupting chemical synergists, was suggested in the previous study.[18] In our investigation we therefore systematically explored the potentiation of many more Gram-positive specific antibiotic classes against *E. coli*, *K. pneumoniae*, and *P. aeruginosa.*

The inner membrane permeability assay was employed as a screening tool and clearly revealed the potentiation of nisin against three different Gram-negative bacterial species. For *E. coli* and *K. pneumoniae*, inner membrane damage was enhanced when serum was combined with vancomycin. Of note was the inherent activity of serum as a result of the prolonged incubation time compared to the previous study.[18] This effect was particularly visible for both *K. pneumoniae* and *P. aeruginosa*: at the 2-hour time point the effect of serum overshadowed the results with antibiotics. The inner membrane permeability assay, however, does allow for a dynamic read-out and selection of different time points. Still, ideally a serum concentration should be selected that results in no or a minimal increase in inner membrane permeability.

Other aspects to consider in the selection of the optimal serum concentration is the balanced nature of the choice: since on the one hand you pursue the minimal amount of inner membrane permeability by serum itself, but on the other hand the potentiation of antibiotics should be as potent as possible. Another aspect to consider is the length of incubation, since length of exposure to serum plays a role in the permeabilization of the inner membrane. While longer incubation might positively or negatively influence the gap between the serum and serum in combination with antibiotic signals, the dynamic read-out ensures that this effect can be monitored (Supporting information, Figure S1-S15). Lastly, the mode-of-action of different antibiotics, and therefore the speed of inhibition, should be considered in the selection of an incubation time and consequently the serum concentration. In the case, a different type of assay, such as the bacterial viability assay with an overnight incubation might prove more appropriate.

The effect of the 2-hour incubation with the selected serum concentration also results in a reduction in bacterial viability for *K. pneumoniae* and *P. aeruginosa* in the serum control. A lower serum concentration will reduce this effect. However, it is questionable whether the synergistic effect will be more pronounced: a lower concentration of serum will result in less pores and therefore lower influx of antibiotics.

Apart from screening different classes of antibiotics, several antibiotics of the glycopeptide class (vancomycin, telavancin, oritavancin, and dalbavancin) were selected to investigate their potentiation with serum. The reported synergy of vancomycin with serum against all three strains was clearly visible in the bacterial viability data and in the inner membrane permeability data of *E. coli* and *K. pneumoniae*. Also, the inner membrane permeability data for *E. coli* showed a moderate increase in permeability for

oritavancin, dalbavancin, and telavancin combined with serum (Figure 5A). Interestingly, only the combinations of serum with vancomycin and dalbavancin resulted in a significant reduction in bacterial viability for *E. coli*. When comparing the potentiation of the glycopeptides by serum against Gram-negative bacteria to the inherent activity of the glycopeptides against Gram-positive bacteria, the inherent activity of vancomycin is the least potent, but vancomycin does show the most potent synergy with serum. Characteristics such as lipophilicity and serum protein binding may explain the activity of vancomycin. However, the synergy of dalbavancin with serum against *E. coli* counters this hypothesis. In all assays a very low concentration of serum was employed and equates to low number of pores. The rate of influx could therefore be an important factor in potentiation by serum, since the accumulation of the glycopeptides in the periplasm is key for their activity against Gram-negative bacteria.

In line with the previous study, the combination of nisin and vancomycin showed a significantly reduced bacterial viability compared to the serum control (Figure 7 and Supporting information, Figure S22A).[18] These antimicrobial compounds both target bacterial cell wall synthesis, which resulted in the selection of bacitracin, another inhibitor of this process, as a suitable negative control (Supporting information, Figure S22B).[34–37,61] In addition to nisin and vancomycin, rifampicin also resulted in a significant reduction in viability against all three Gram-negative bacteria screened at concentrations of 5 μg/mL (Supporting information, Figure S23C). Of note, is the small effect of rifampicin itself on the Gram-negative bacteria, which is slightly visible in the *P. aeruginosa* data. Rifampicin, is known as an antibiotic partner to many outer membrane disrupting synergists (Chapter 1) and synergy of rifampicin with serum has been reported against an *E. coli* K-12.[14]

Two more antibiotics were significantly potentiated by serum against at least one strain: erythromycin and Q & D (Supporting information, Figure S23A–B). The macrolide antibiotic erythromycin targets the 50S subunit of the bacterial ribosome and inhibits the protein synthesis.[62] Its activity has been described as bacteriostatic against Gram-positive bacteria.[63] Q & D are two peptides marketed as Synercid, a synergistic combination, that also targets the bacterial protein synthesis.[64,65] Contrarily to erythromycin, Q & D is bactericidal against Gram-positive bacteria.[65] Like rifampicin, erythromycin has been widely described in synergy literature, specifically with outer membrane disruptors (Chapter 1). Interestingly, the study reporting potentiation of rifampicin by serum, also mentions that no synergy was found for erythromycin, contrary to our results.[14] For Q & D, we did not manage to find literature describing potentiation by serum against Gram-negative bacteria. A outer membrane disruptor derived of polymyxin was reported to potentiate Q & D against *E. coli*.[5,66]

Of interest is the lack of inner membrane permeability signal for erythromycin and rifampicin, since in literature for rifampicin an increase in SYTOX Green was reported for *E. coli*[67] and the effect of erythromycin was also screened using SYTOX Green against both Gram-positive and Gram-negative bacteria.[68] Contrarily to erythromycin and rifampicin, serum + daptomycin did display an increase in inner membrane permeability, however, it did not impact bacterial viability significantly (Supporting information, Figure S22C). The mechanisms of action of daptomycin is still being ironed out in literature, the current consensus is the insertion into the membrane after oligomerization results in depolarization leading to the inhibition of growth and division of cells.[44–46] The sensitivity of the SYTOX Green in combination with such a

membrane-targeting mechanism could provide an additional explanation for this difference between the two assays. The sensitivity of the inner membrane permeability assay could be another explanation as there is a one-log reduction in viability for *E. coli*. Daptomycin only seems to have an effect with serum on *E. coli* and it could be speculated that the protein binding of daptomycin, could interfere with its inherent activity since the serum concentration employed is higher for *K. pneumoniae* and *P. aeruginosa* bacterial viability assays.[69] Recently, a study also reported daptomycin tolerance in the Gram-positive bacteria *Staphylococcus aureus* triggered by human serum.[70]

In conclusion, a systematic screening of Gram-positive specific antibiotics in combination with serum against three different Gram-negative bacteria was performed. A clear increase in inner membrane damage and bacterial viability is seen for nisin and vancomycin in the presence of serum. However, for the other tested antibiotics increases in inner membrane damage could not be validated with a significant change in bacterial viability. An exception was the potentiation of human serum with dalbavancin against *E. coli* as the moderate increase in SYTOX Green fluorescence correlated to a significant reduction in bacterial viability. In addition, this study has revealed the need for optimization of the serum concentration and identified the optimal concentrations of rifampicin and erythromycin. Also, in the case of rifampicin, erythromycin, and Q & D, only the bacterial viability assay revealed that these antibiotics were significantly potentiated by serum. An alternative screening method might be more suitable for these antibiotics than the inner membrane permeability assay using SYTOX Green. Most importantly, this study reports new synergistic combinations of serum and Gram-positive specific antibiotics, further revealing how the complement system can work together with Gram-positive specific antibiotics to kill Gram-negative bacteria.

4. Materials and methods

4.1. Antibiotics

All antibiotics employed were of American Chemical Society (ACS) grade or finer and were used without further purification unless otherwise stated. Nisin was HPLC purified as this antibiotic was not obtained in an ACS grade.

4.2. Serum preparation

For normal human serum preparation, blood was drawn from healthy volunteers and allowed to clot for 15 minutes at RT. After centrifugation (10 min, 4000 rpm), serum was collected, pooled and stored at −80 °C. Heat-inactivated serum was obtained by incubating serum for 30 min at 56 °C.

4.3. Bacterial inner membrane permeabilization assay using SYTOX Green

The assay was performed based on a protocol described in literature.[18] Bacteria were grown an OD600nm of 0.5 in Lysogeny Broth (LB) medium, pelleted by centrifugation and resuspended to OD600nm of 1.0 in RPMI 1640 (ThermoFisher) supplemented with 0.05% HSA. The bacteria were diluted by a ten-fold, final concentration of OD600nm ~ 0.05. For *E. coli* the bacteria were incubated with 0 or 0.3% serum or 0.3% heat-inactivated serum. For *P. aeruginosa* the selected concentration was 1% and for *K. pneumoniae* 10%. Incubations were done in the presence of 1 μM SYTOX Green Nucleic Acid stain (Thermofisher). Fluorescence was measured in a microplate reader (CLARIOstar, Labtech) at 37 °C under shaking conditions for 2 hours. Synergy experiments were performed by incubating bacteria with the antibiotics in a 10 μg/mL final concentration except for rifampicin and erythromycin: erythromycin 5, 2.5, 5 μg/mL and rifampicin 5, 2.5, 2.5 μg/mL for *E. coli*, *K. pneumoniae* and *P. aeruginosa* respectively

4.4. Bacterial viability assay

The assay was performed based on a protocol described in literature.[18] Bacteria were prepared as described above and incubated with buffer, serum or blood (drawn from healthy volunteers) in the presence or absence of antibiotics (10 μg/ml, except for rifampicin and erythromycin, as described above). For CFU/ml determination, serial dilutions were made in PBS and bacteria were plated onto agar plates followed by colony enumeration after overnight incubation. Relative viability was calculated as the number of CFU/ml relative to the buffer control.

4.5. Statistical testing

Statistical analysis was performed using a one-way ANOVA and Dunnett test (*$p < 0.05$) using the serum as control group in Graphpad. The tests and n-values used to calculate p-values are also mentioned in the figure captions.

4.6. Ethics statement

Human blood was isolated after informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the UMC Utrecht, The Netherlands.

Supporting information

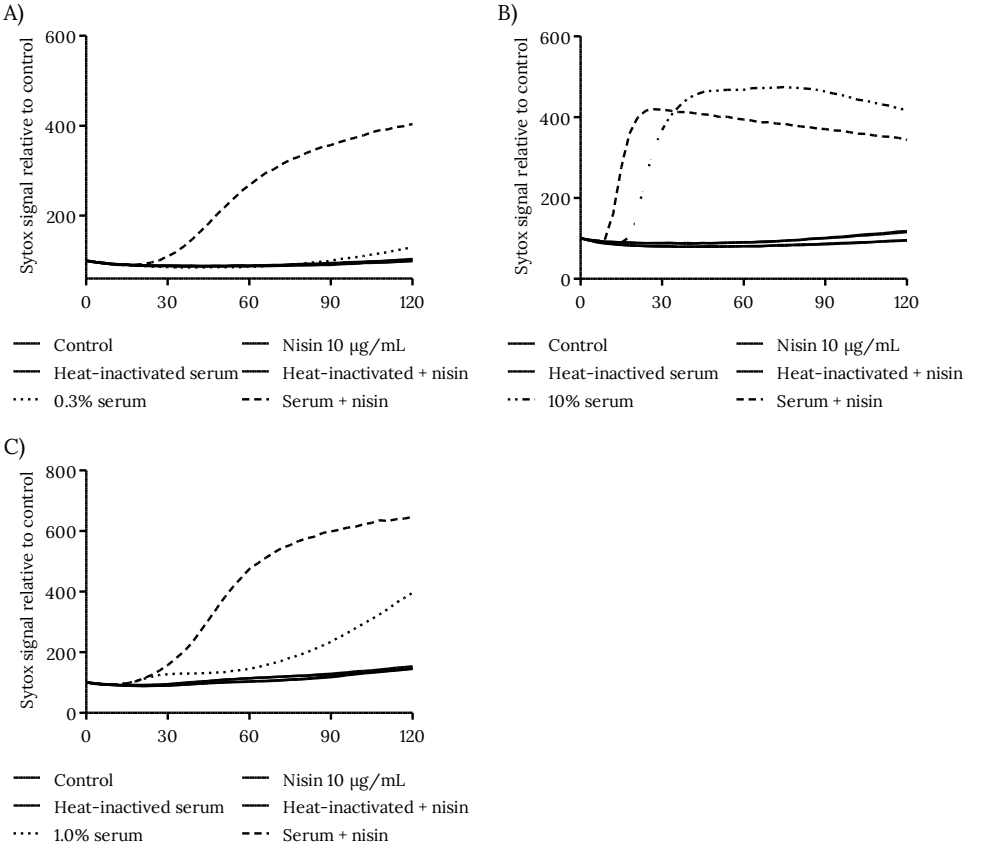


Figure S1. Dynamic read-out of the inner permeability assays for nisin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL nisin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

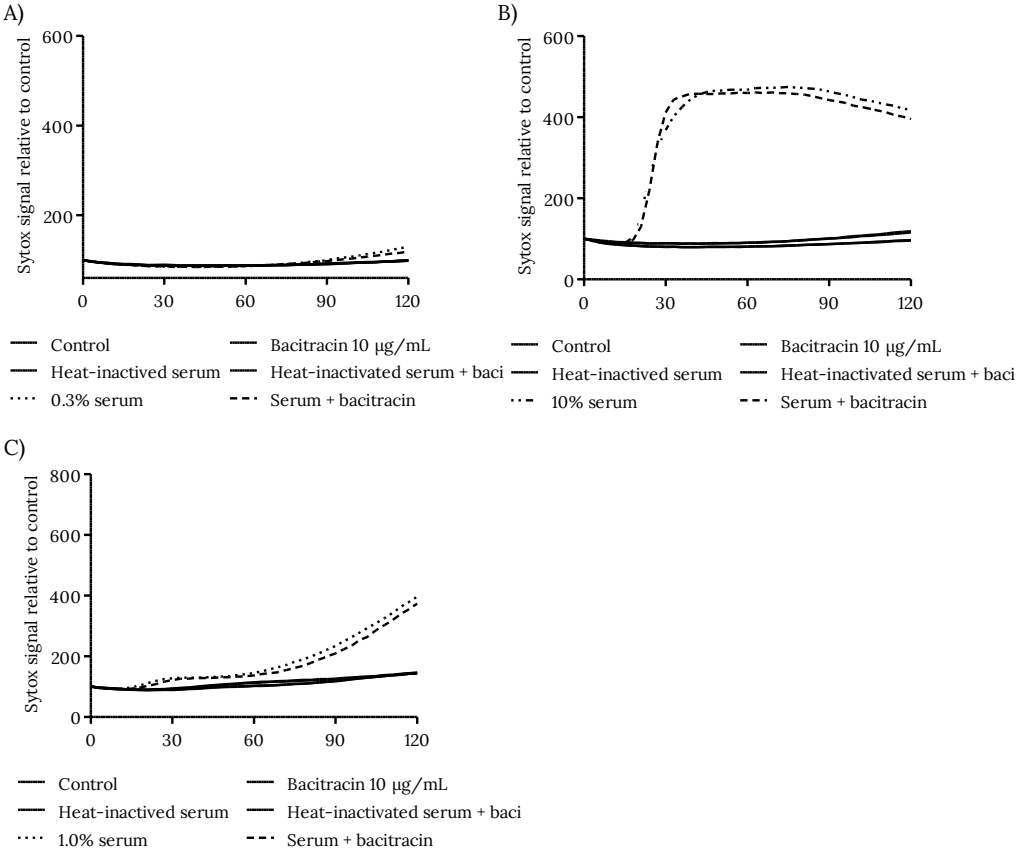


Figure S2. Dynamic read-out of the inner permeability assays for bacitracin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL bacitracin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

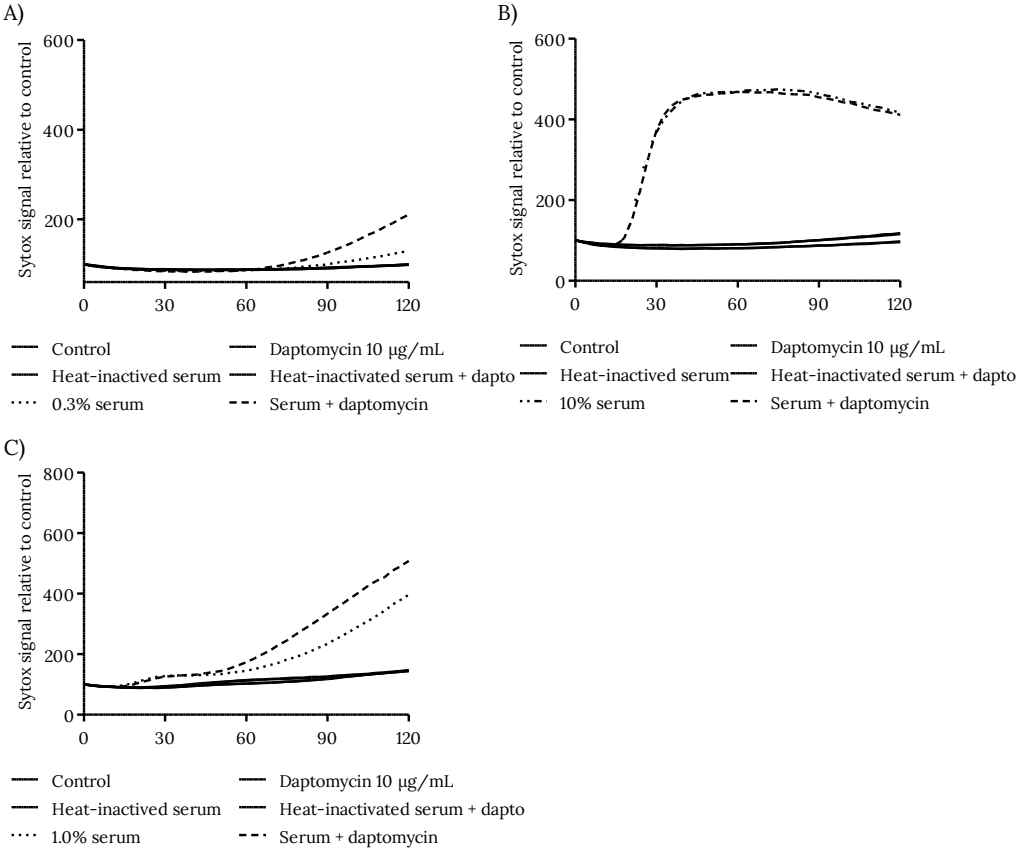


Figure S3. Dynamic read-out of the inner permeability assays for daptomycin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL daptomycin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

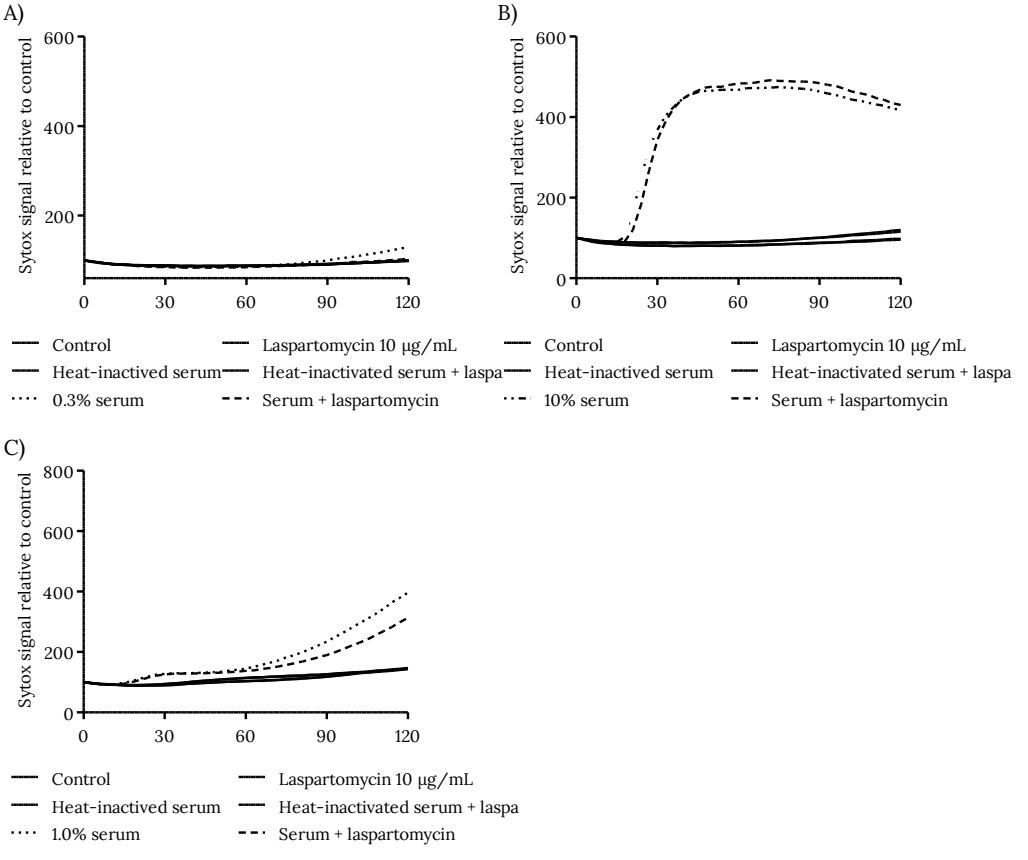


Figure S4. Dynamic read-out of the inner permeability assays for laspartomycin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL laspartomycin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

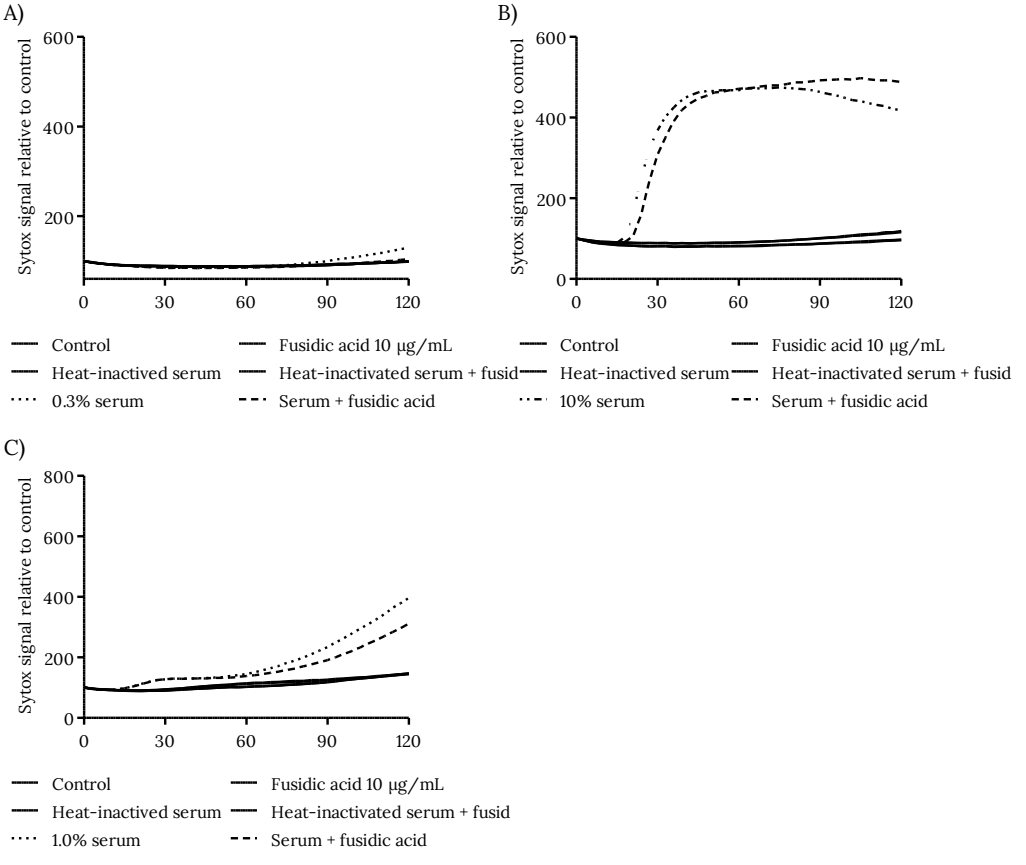


Figure S5. Dynamic read-out of the inner permeability assays for fusidic acid with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL fusidic acid at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

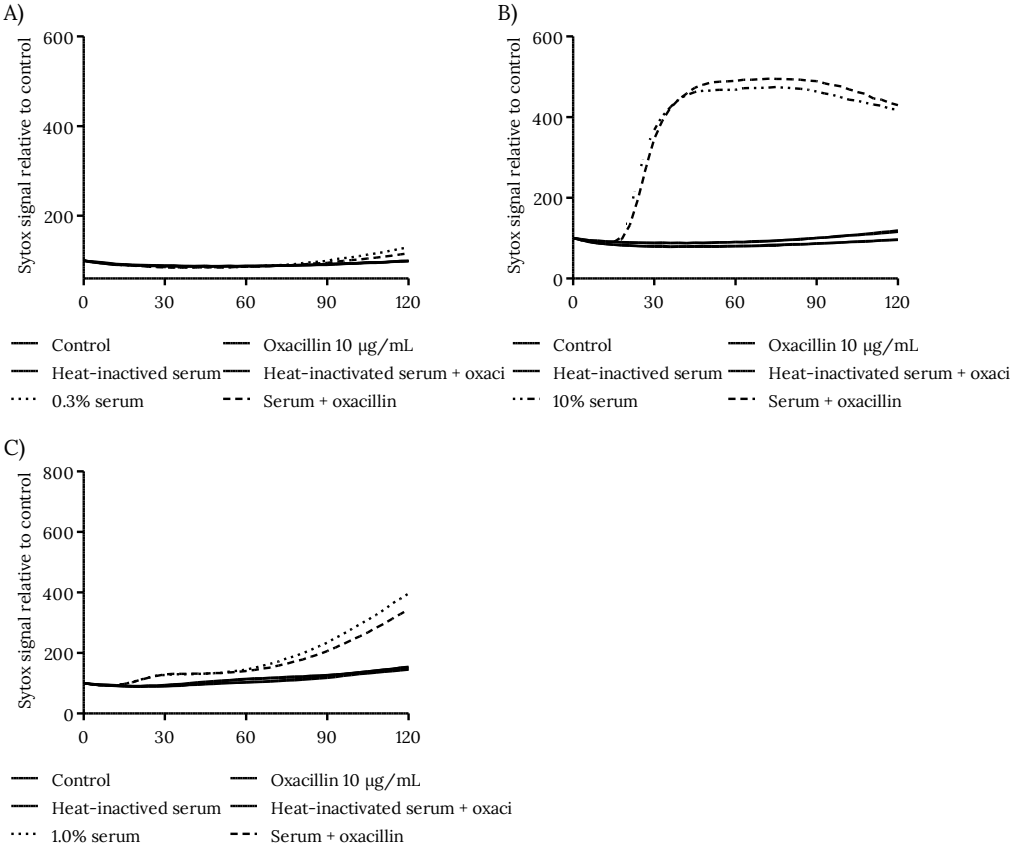


Figure S6. Dynamic read-out of the inner permeability assays for oxacillin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL oxacillin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

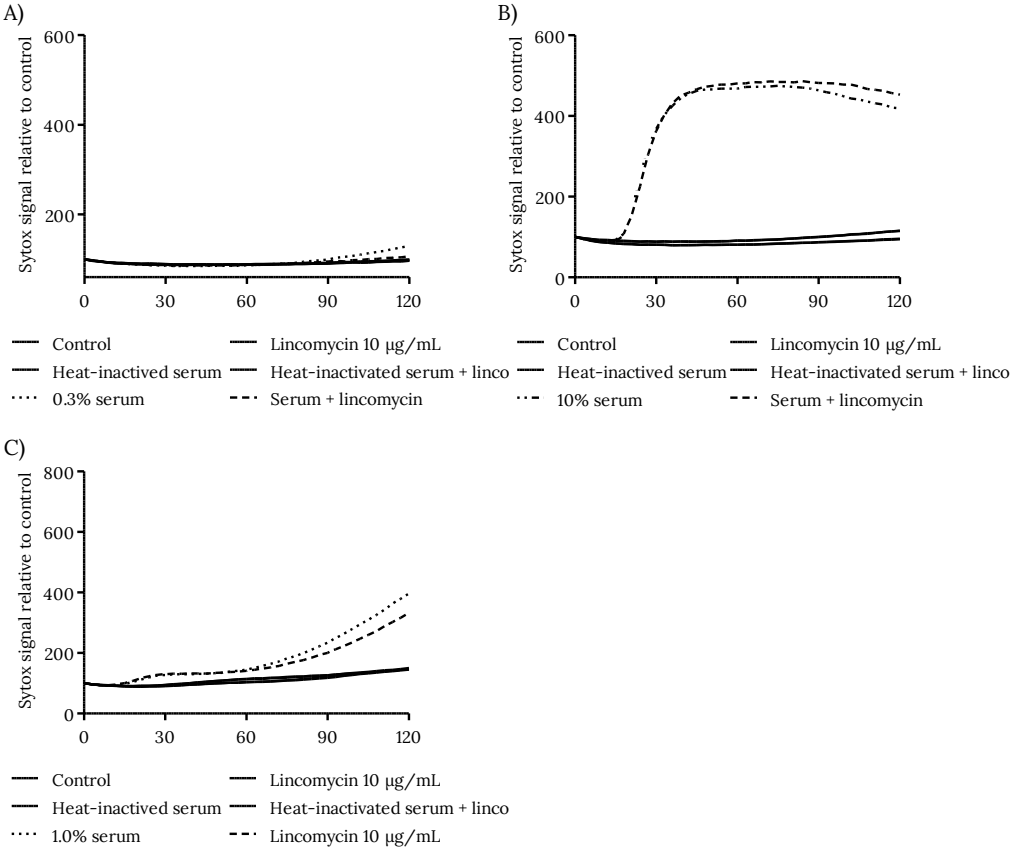


Figure S7. Dynamic read-out of the inner permeability assays for lincomycin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL lincomycin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

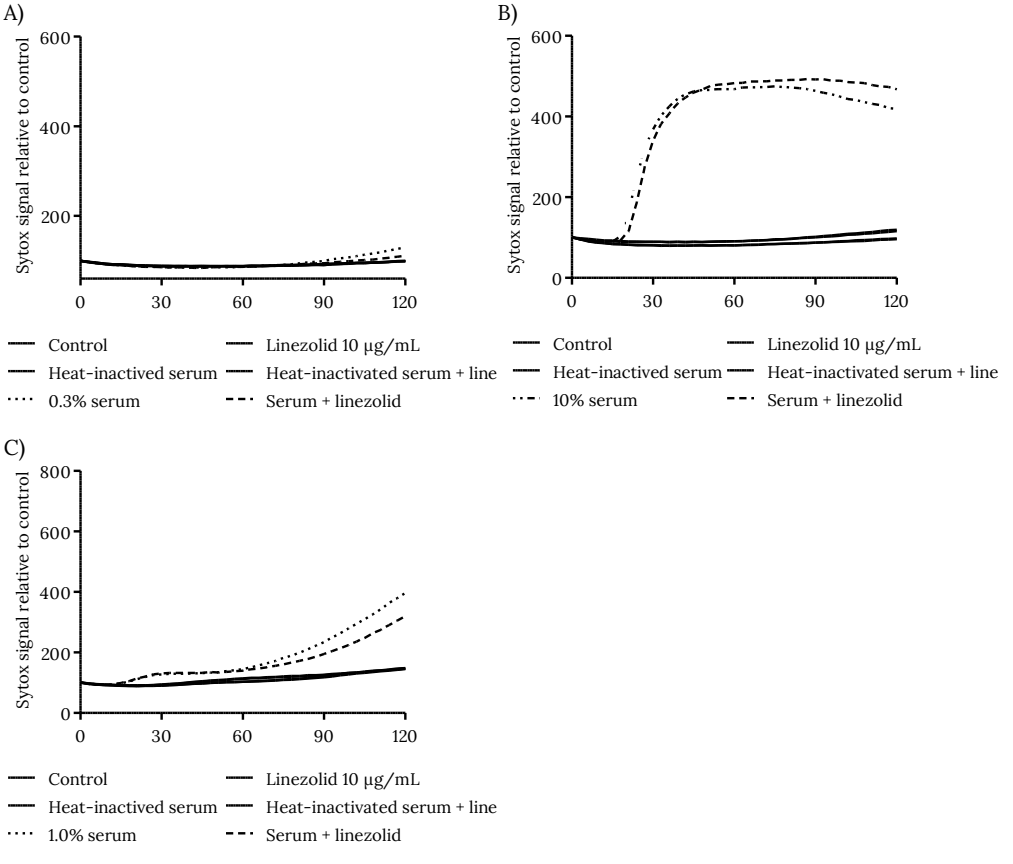


Figure S8. Dynamic read-out of the inner permeability assays for linezolid with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactivate serum, and serum with or without 10 µg/mL linezolid at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

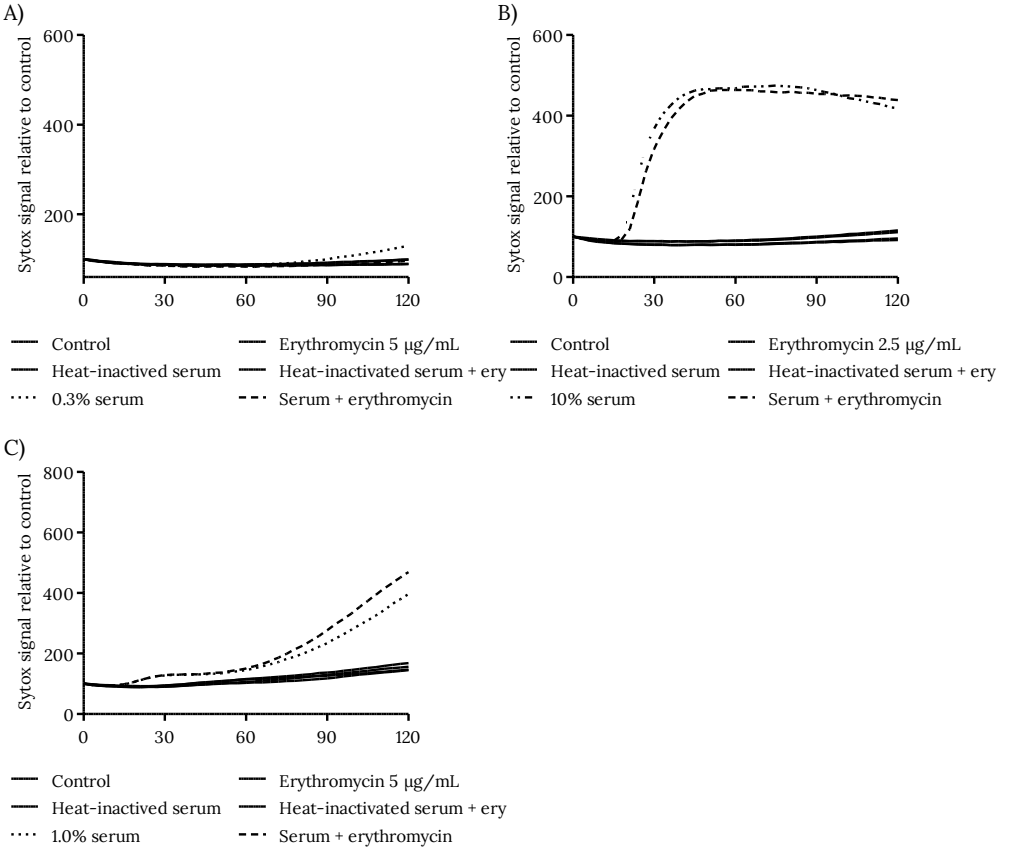


Figure S9. Dynamic read-out of the inner permeability assays for erythromycin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 5, 2.5, and 5 µg/mL erythromycin respectively at 37 °C. The concentrations of serum were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

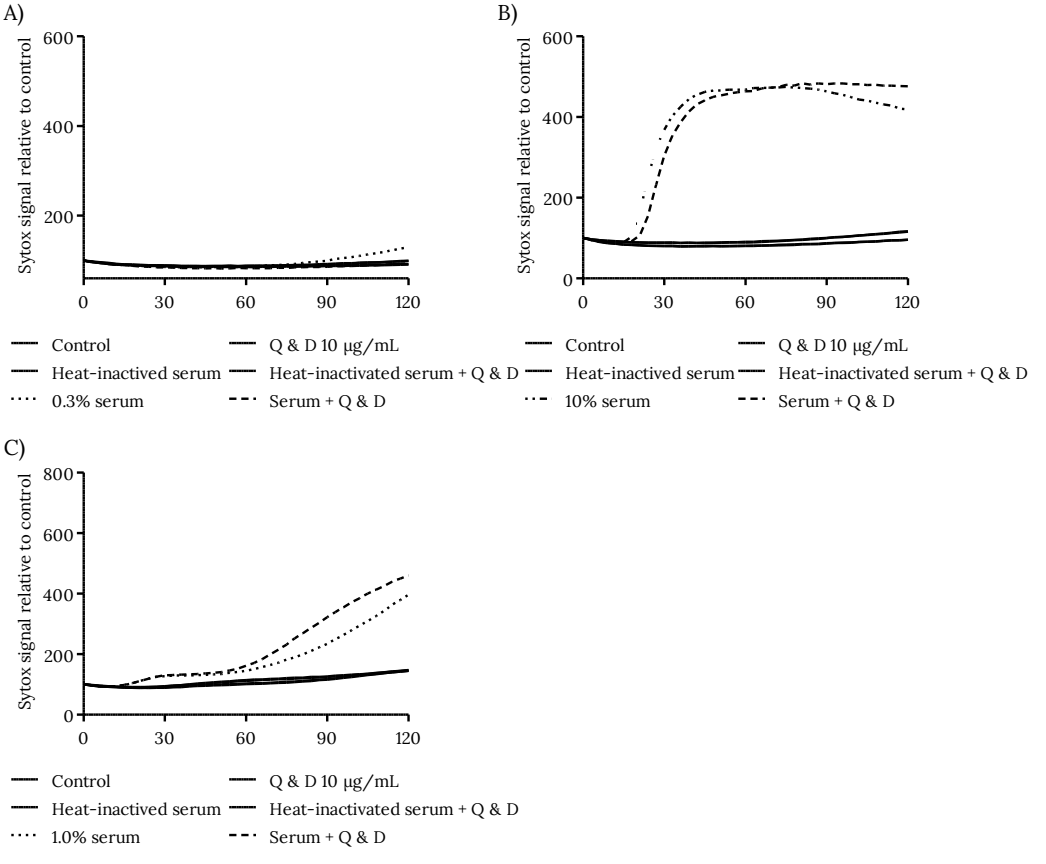


Figure S10. Dynamic read-out of the inner permeability assays for quinupristin & dalfopristin (Q & D) with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL Q & D at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

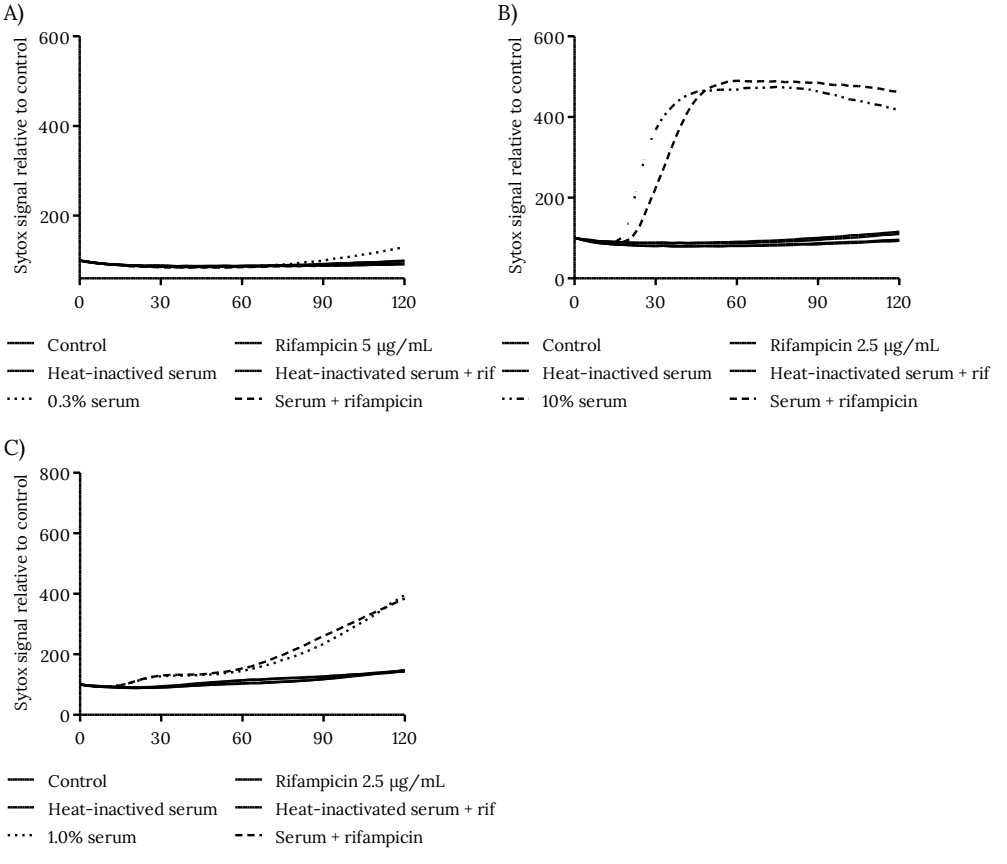


Figure S11. Dynamic read-out of the inner permeability assays for rifampicin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 5, 2.5, and 2.5 µg/mL rifampicin respectively at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

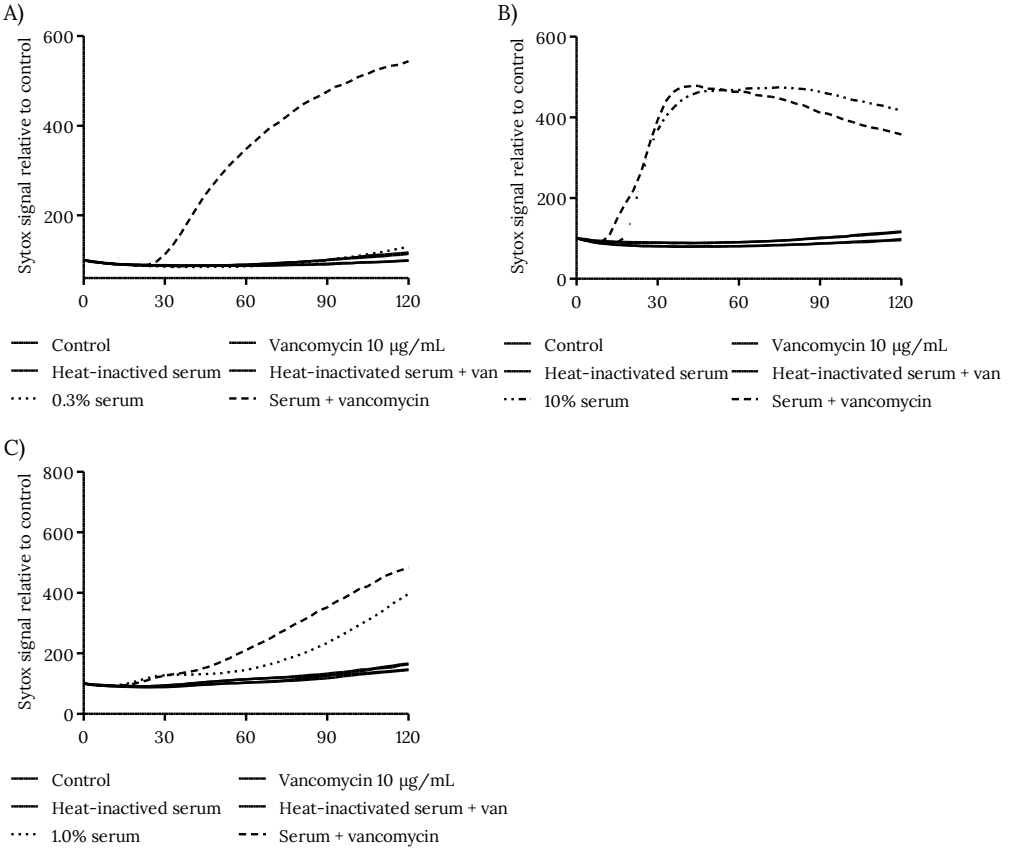


Figure S12. Dynamic read-out of the inner permeability assays for vancomycin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL vancomycin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments

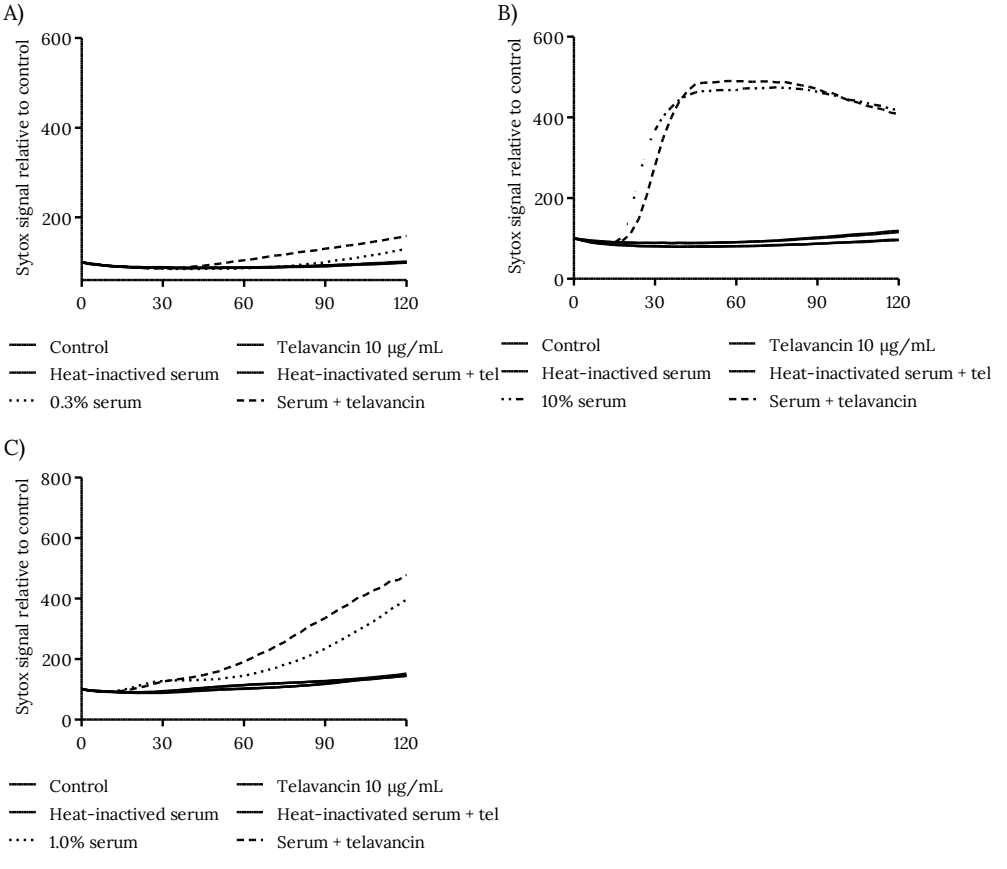


Figure S13. Dynamic read-out of the inner permeability assays for telavancin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL telavancin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments.

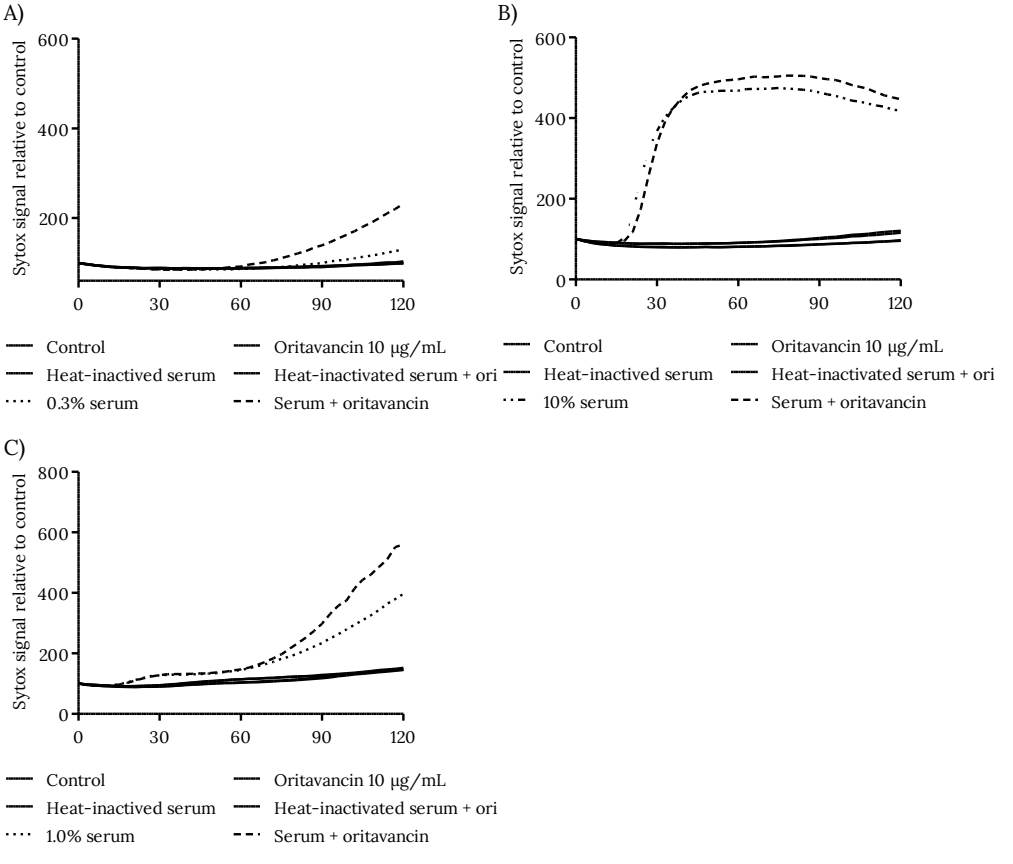


Figure S14. Dynamic read-out of the inner permeability assays for oritavancin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL oritavancin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments

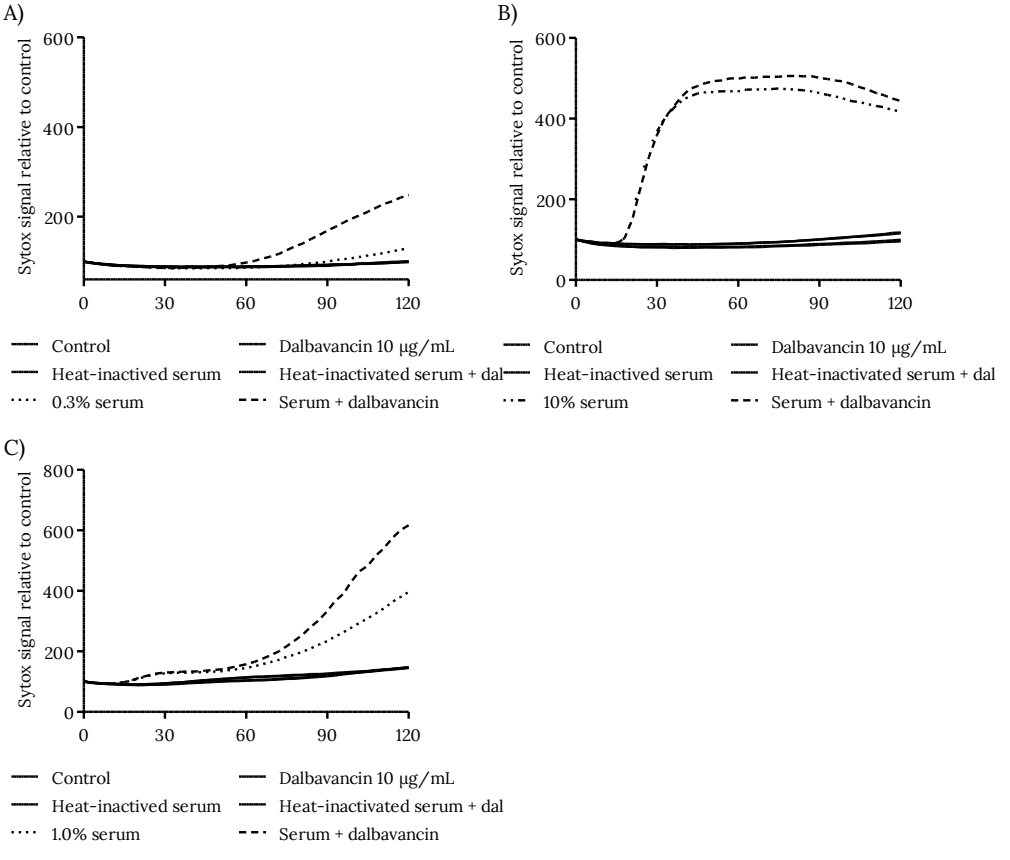


Figure S15. Dynamic read-out of the inner permeability assays for dalbavancin with A) *E. coli*; B) *K. pneumoniae*; C) *P. aeruginosa*. The bacteria were incubated with buffer (control), heat-inactive serum, and serum with or without 10 µg/mL dalbavancin at 37 °C. The concentrations of serum used were 0.3%, 10%, and 1.0% respectively. SYTOX Green intensity was measured every 3 minutes for 120 minutes in a microplate fluorometer. Values were corrected and represent mean ± SD of three independent experiments

SYTOX read-out of *K. pneumoniae* **and** *P. aeruginosa* **at 2 hours**

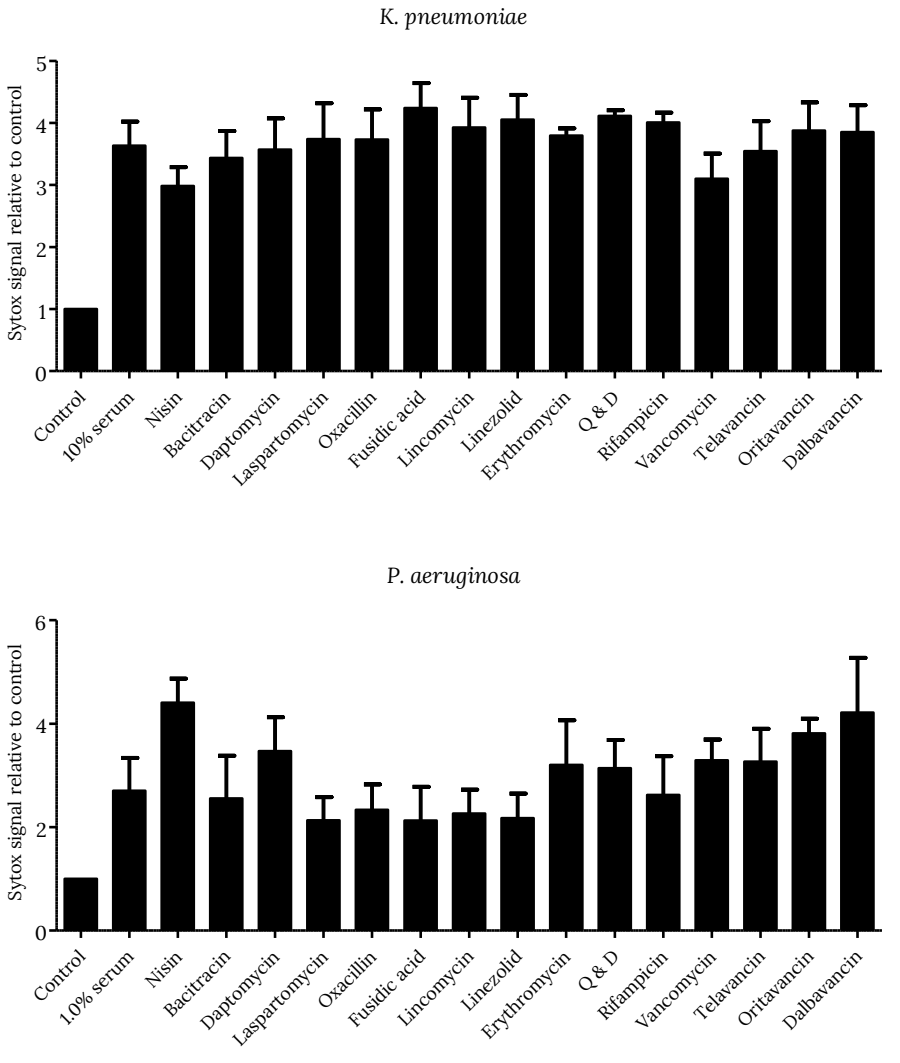


Figure S16. Inner permeability assay data of *K. pneumoniae* and *P. aeruginosa* at 2 hours. The antibiotics (10 μg/mL, except for erythromycin (2.5, 5 μg/mL respectively) and rifampicin (2.5, 2.5 μg/mL respectively) were screened in combination with serum (10% for *K. pneumoniae*, and 1% for *P. aeruginosa*). Values were depicted as relative values to the control condition and represent mean ± SD of three independent experiments.

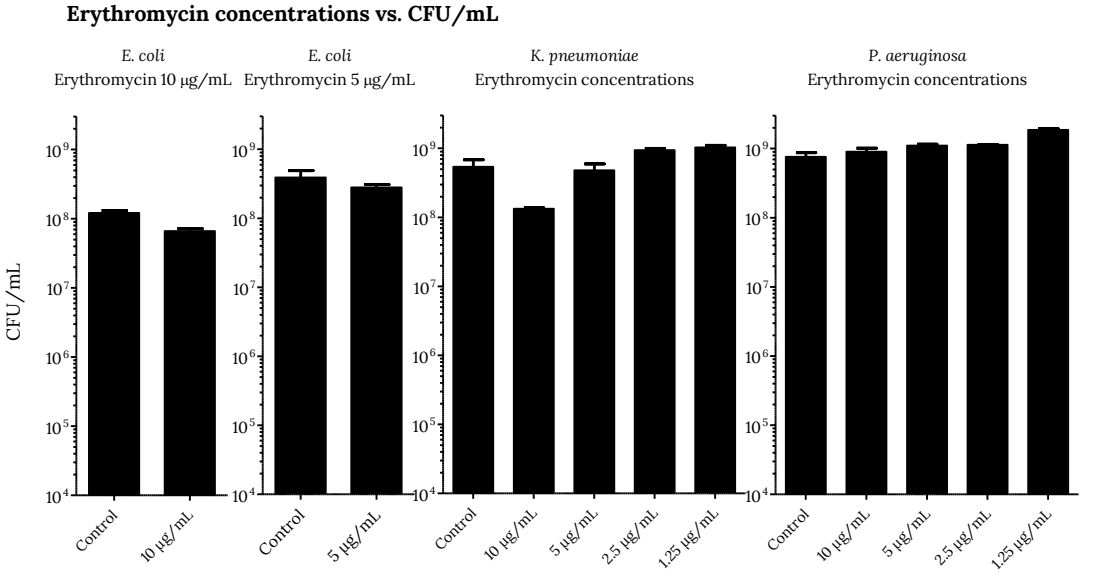


Figure S17. Bacterial viability assay for establishing inherent activity of erythromycin. The viability of the bacteria was screened after 2 hours of incubation with buffer with concentrations of 10, 5 or 10 to 1.25 µg/mL at 37 °C with shaking. Data represent mean ± SD of 2 technical replicates from a single experiment.

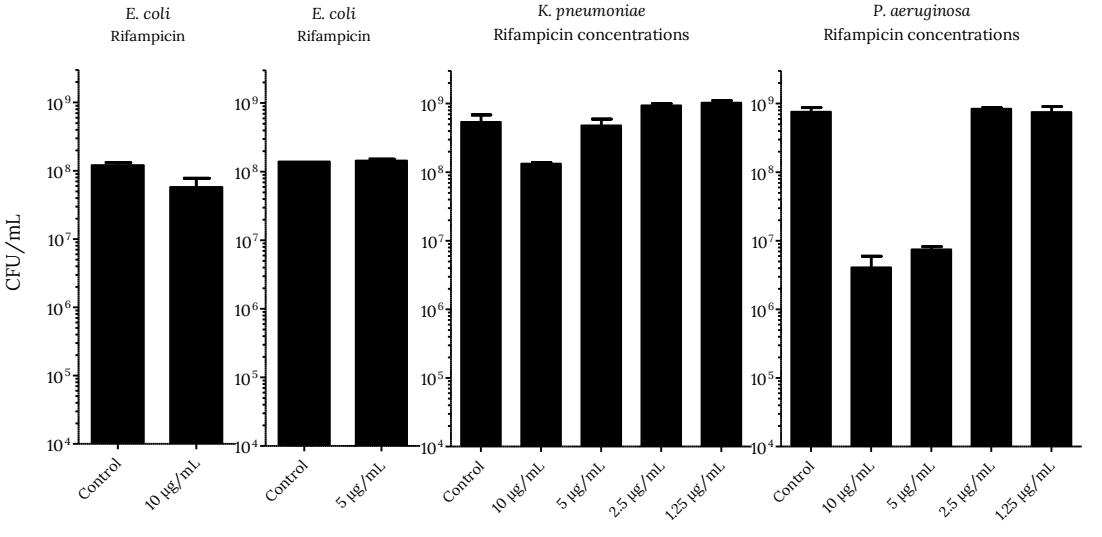


Figure S18. Bacterial viability assay for establishing inherent activity of rifampicin. The viability of the bacteria was screened after 2 hours of incubation with buffer with concentrations of 10, 5, or 10 to 1.25 µg/mL at 37 °C with shaking. Data represent mean ± SD of 2 technical replicates from a single experiment.

Overview results of bacterial viability assay

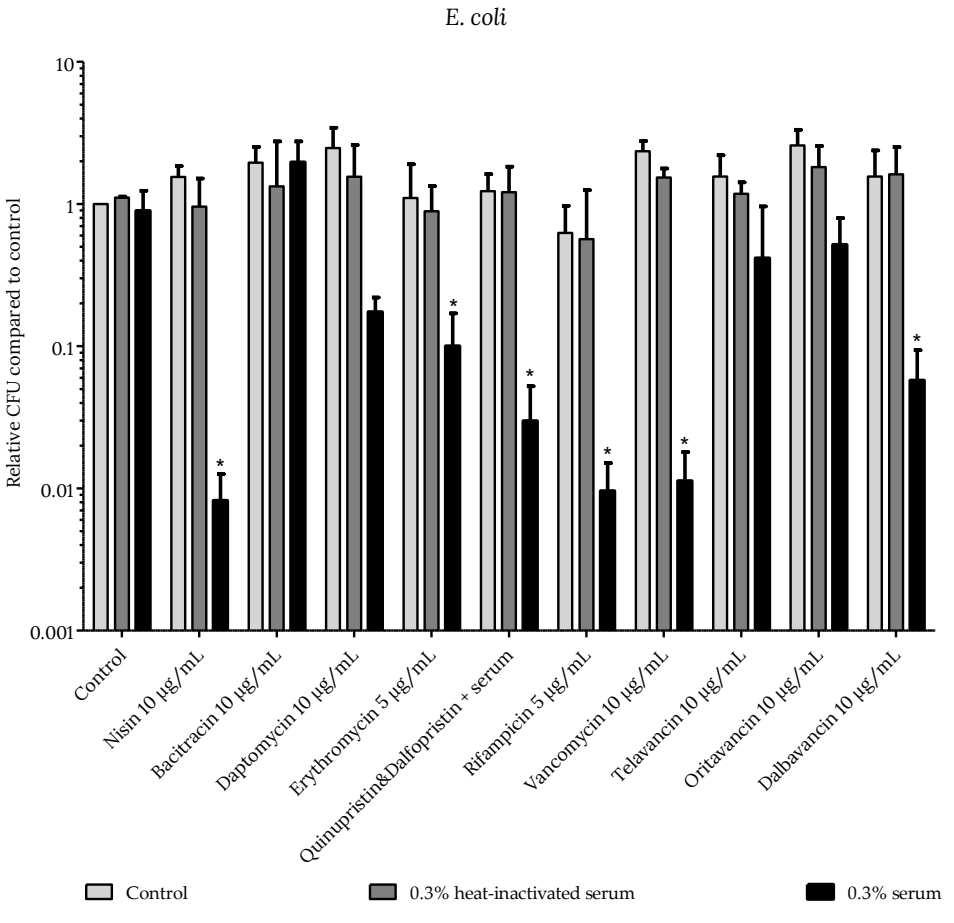


Figure S19. Synergy between serum and antibiotics in the bacterial viability assay. The viability of *E. coli* was screened after 2 hours of incubation with buffer, 0.3% heat-inactivated serum or 0.3% serum with 10 µg/mL antibiotics at 37 °C with shaking. CFU counts were normalized to buffer controls. Dashed line represent detection limit. Data represent mean ± SD of three independent.

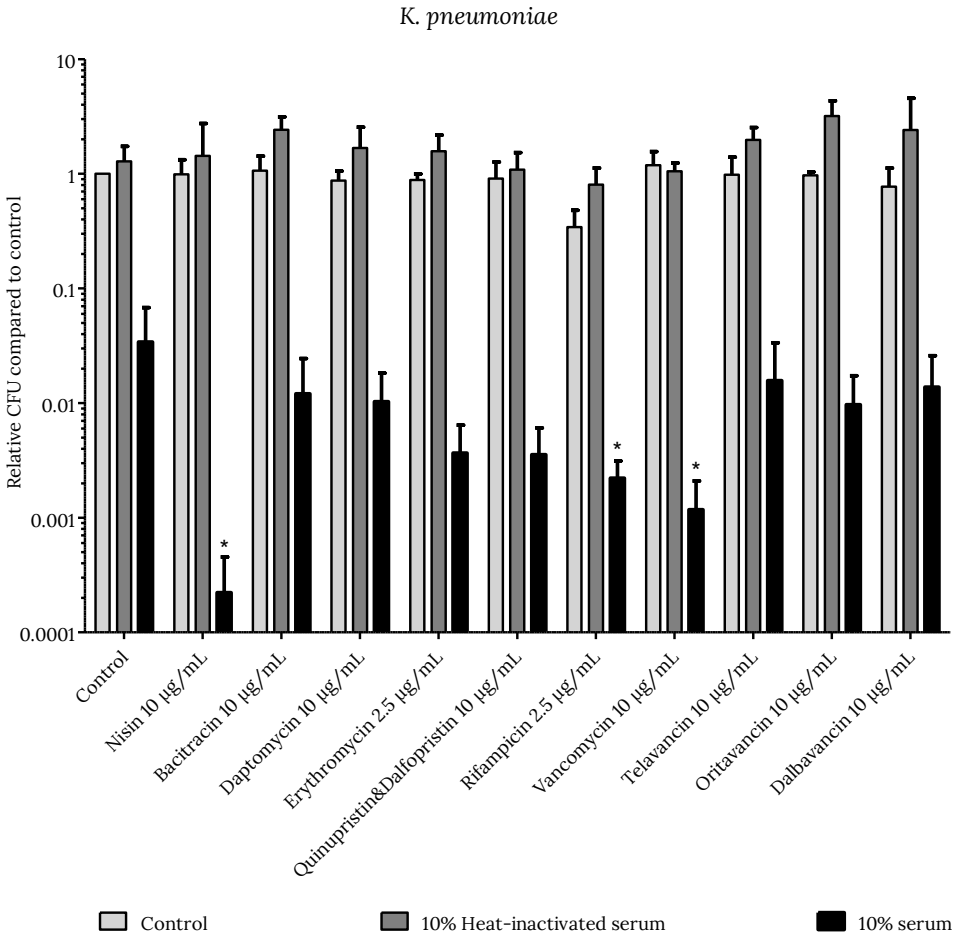


Figure S20. Synergy between serum and antibiotics in the bacterial viability assay. The viability of *K. pneumoniae* was screened after 2 hours of incubation with buffer, 10% heat-inactivated serum or 10% serum with 10 µg/mL antibiotics at 37 °C with shaking. CFU counts were normalized to buffer controls. Dashed line represent detection limit. Data represent mean ± SD of three independent.

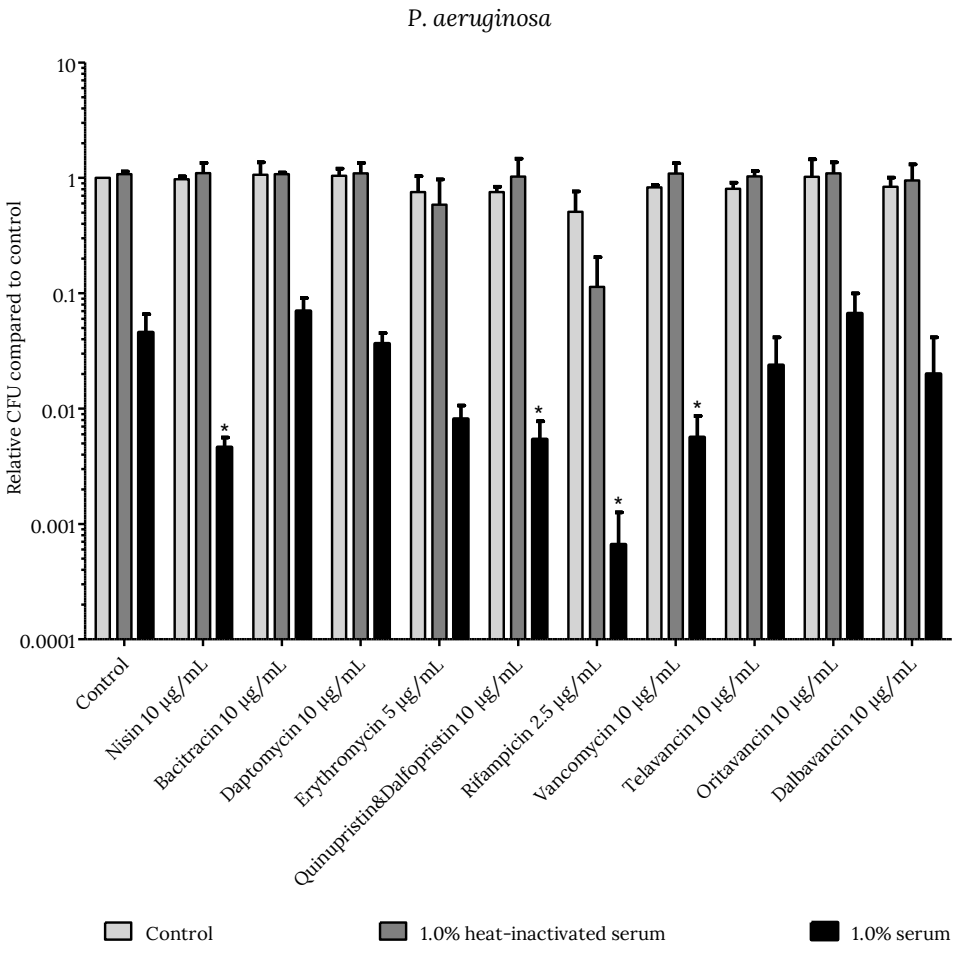


Figure S21. Synergy between serum and antibiotics in the bacterial viability assay. The viability of *P. aeruginosa* was screened after 2 hours of incubation with buffer, 1.0% heat-inactivated serum or 1.0% serum with 10 µg/mL antibiotics at 37 °C with shaking. CFU counts were normalized to buffer controls. Dashed line represent detection limit. Data represent mean ± SD of three independent.

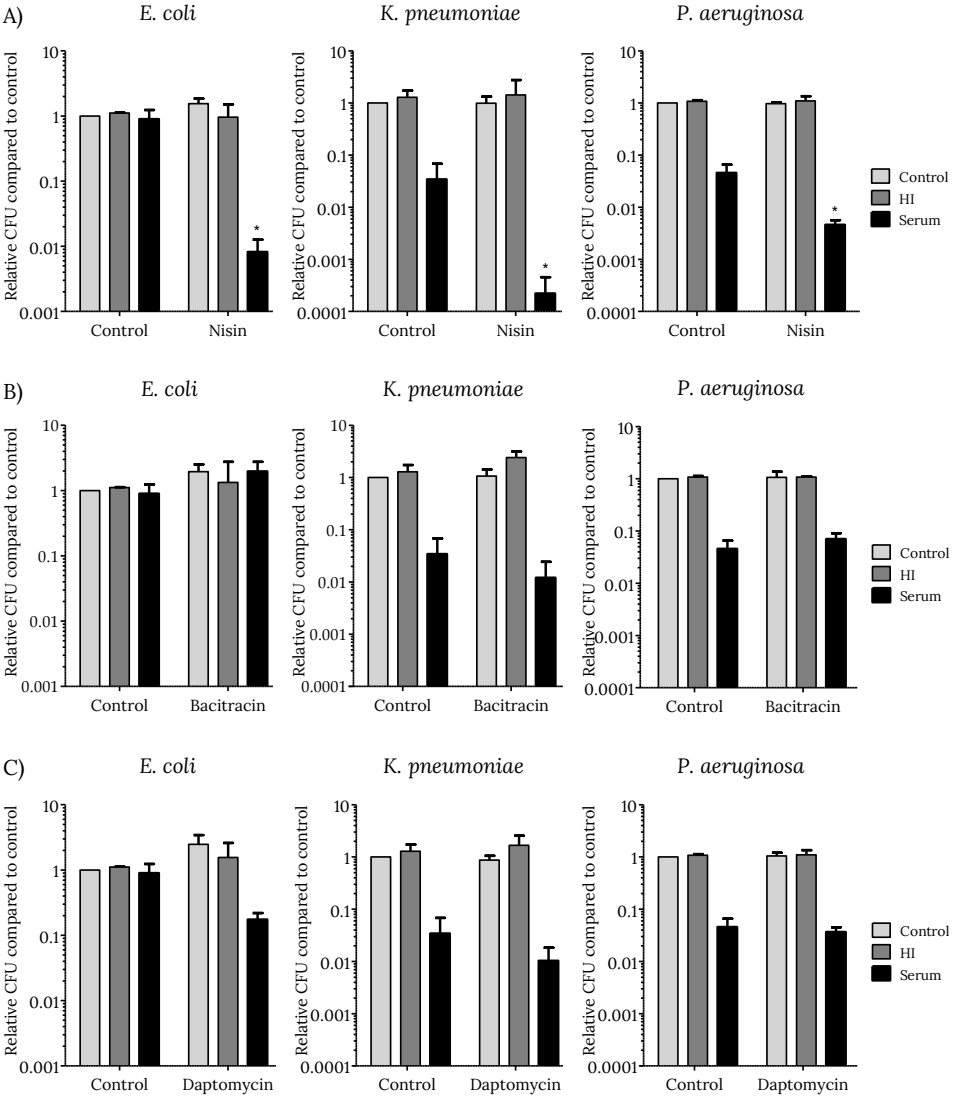


Figure S22. Synergy between serum and antibiotics A) nisin; B) daptomycin; C) bacitracin. The viability of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* was screened after 2 hours of incubation with buffer, heat-inactivated serum (HI) or serum with 10 µg/mL antibiotics at 37 °C with shaking. Concentrations of serum differed per pathogen and were 0.3%, 10% and 1.0% respectively. CFU counts were normalized to buffer controls. Data represent mean ± SD of three independent experiments and were analyzed by an one-way ANOVA and Dunnett test (*$p < 0.05$) using the serum as control group.

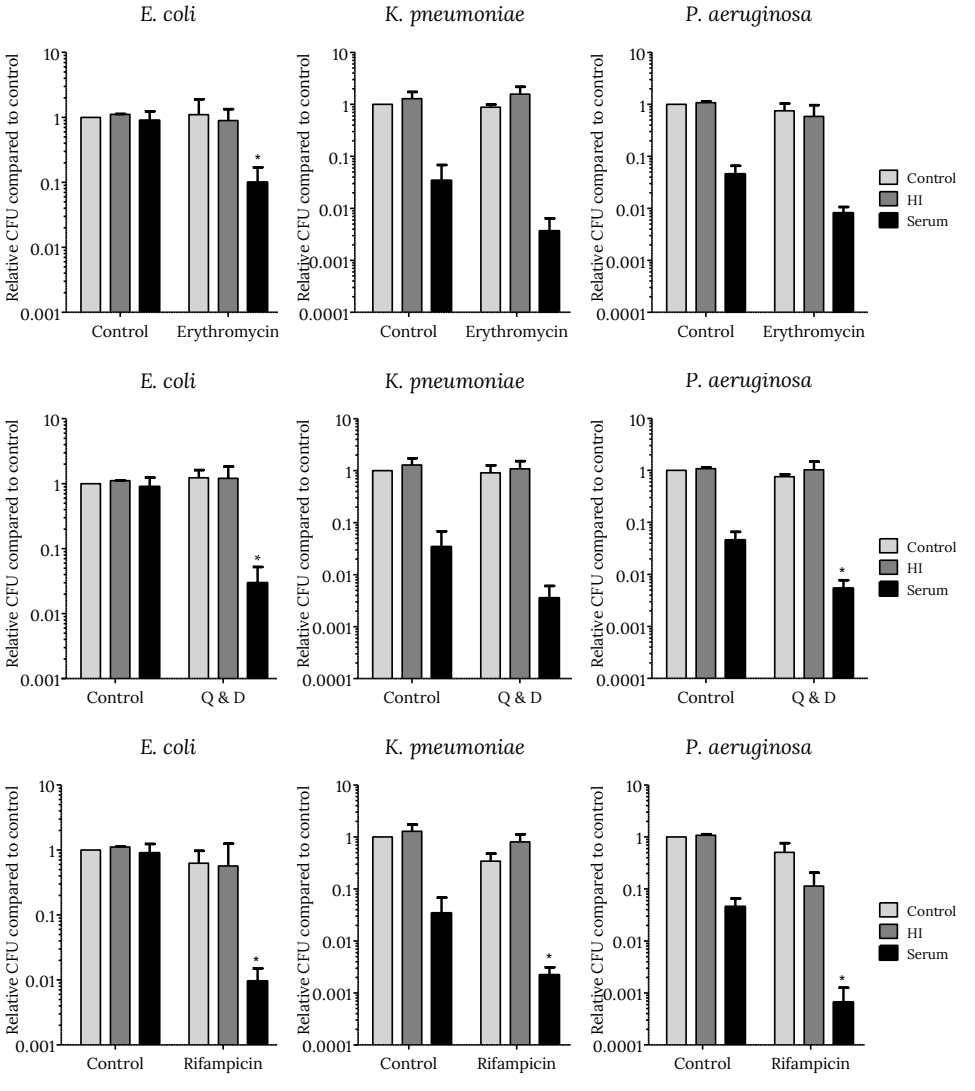


Figure S23. Synergy between serum and antibiotics A) erythromycin; B) quinupristin & dalfopristin; C) rifampicin. The viability of *E. coli*, K. *pneumoniae*, and *P. aeruginosa* was screened after 2 hours of incubation with buffer, heat-inactivated serum or serum with antibiotics at 37 °C with shaking. Concentrations of serum differed per pathogen and were 0.3%, 10% and 1.0% respectively. Erythromycin and rifampicin concentrations had to be lowered due to their inherent activity at the 10 µg/mL. Erythromycin was dosed at 5, 2.5, and 5 µg/mL for the pathogens respectively and rifampicin concentrations were 5, 2.5, and 2.5 µg/mL respectively. CFU counts were normalized to buffer controls. Data represent mean ± SD of three independent experiments and were analyzed by an one-way ANOVA and Dunnett test (*$p < 0.05$) using the serum as control group.

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