Combination therapy in sepsis and septic shock. Sequence of antibiotics and bacterial clearance in a peritonitis rat model of septic shock

by

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Abstract

Higher microbial load and antibiotic delay are associated with increased morbidity and mortality in septic shock. Antibiotic combination is the basis of empiric treatment for sepsis and septic shock, current recommendations suggest the use of a combination of antibiotics to broaden the initial spectrum of coverage to treat some multi-resistant bacteria. Antibiotic combination therapy leads to more rapid pathogen clearance, which may translate into improved patient outcome.

The systematic review in this thesis based on the available randomized clinical trials, does not support the use of combination therapy as the initial treatment for sepsis and septic shock. Although, this could have been due to study design differences, variable degree of severity and even sequence of antibiotic administration.

While antimicrobial synergy has been established for beta-lactam combinations with aminoglycosides or fluoroquinolones, antimicrobial sequence has only been studied in vitro. These studies suggest that giving the beta-lactam first or at the same time as an aminoglycoside improves bacterial clearance. Herein we sought to confirm this in the first in vivo study of antimicrobial sequence in septic shock.

This thesis confirmed better bacterial clearance using antibiotic combination therapy, but failed to show significant differences in bacterial clearance depending on the sequence of administration of antibiotics in our *E. coli* and *S. pneumoniae* models of septic shock.

This study sheds light on sequence of administration of antibiotics in sepsis, helping to inform further research.

Dedication

I dedicate this thesis to my sons. I am beyond excited to see you grow up and discover the world through your eyes. We love you both, unconditionally.

Acknowledgements

My deepest thanks go to my advisor and mentor, Dr. Anand Kumar, who made possible this ongoing Canadian adventure.

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Special thank you to my parents and my brother. You all have been my role models and have supported me through every stage of this wonderful road. Without you I wouldn't have made it. Thanks for inspiring me, cheering for me and putting up with me.

To my husband, you're my rock, a constant inspiration, you challenge me and complete me. Life is so much better with you. Te quiero… Mucho!!

Co-authorship statement

This thesis contains the following manuscripts that have been published, accepted, submitted or are in preparation for submission:

Chapter 1: This chapter was written by Gloria Vazquez-Grande. It was adapted from a review article entitled “Optimizing antimicrobial therapy of sepsis and septic shock: focus on antibiotic combination therapy“, that was published in Seminars of Respiratory Critical Care Medicine in 2015. This review article was written by Gloria Vazquez-Grande, the co-author is Anand Kumar.

Chapter 2: This chapter represents a manuscript entitled “Antimicrobial combination therapy in sepsis patients: a systematic review and meta-analysis“. This manuscript was written by Gloria Vazquez-Grande for the Systematic Review course. Co-authors are: Colin Graydon, Hal Loewen, Rasheda Rabbani, Ahmed M. Abou-Setta, Ryan Zarychanski and Anand Kumar. Part of this content was presented in the ESICM international conference in 2018 and published in ICM as an abstract communication to the 2018 ESICM international conference.

Chapter 3: Gloria Vazquez Grande is the author of this chapter. Parts of this chapter have been presented in international conferences and have been published as abstract communications to conferences. The statistical analysis was performed by Robert Balshaw.

Chapter 4: This chapter was written by Gloria Vazquez Grande.

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List of Acronyms

APACHE	Acute Physiology and Chronic Health Evaluation
ATCC	American Type Culture Collection
AUC	Area Under the Curve
BA	blood agar
bpm	Beats per minute
C->G	Cefotaxime then gentamicin
Cef	Cefotaxime,
CFU	Colony Forming Units
CI	Confidence Intervals
C_{max}	Peak concentration
C_{max}/MIC	Peak concentration above the MIC
CPIS	Clinical Pulmonary Infection Score
E. coli	*Escherichia coli*
G->C	Gentamicin then cefotaxime
Gent	Gentamicin,
GNB	Gram-negative bacilli
HIV	Human Immunodeficiency Virus
HSSS	Highly Sensitive Search Strategy
ICUs	Intensive Care Units
iv	intravenously
LOS	Length of stay
MAC	*Mycobacterium avium* complex (), (
MAP	Mean Arterial Pressures
MECIR	Methodological Expectations of Cochrane Intervention Reviews
MHA	Mueller-Hinton agar (
MIC	Minimum Inhibitory Concentration
MICU	Medical Intensive Care Unit
mm^3	Cubic millimeter
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NR	Not Reported

NS	Normal Saline
ºC	Degree Celsius
OD	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*
PD	Pharmacodynamic
PK	Pharmacokinetic
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analysis
RCTs	Randomized Controlled Trials
RR	Risk Ratios
S. aureus	*Staphylococcus aureus*
S. pneumoniae	*Streptococcus pneumoniae*
SAPS	Simplified Acute Physiology Score,
SD	Standard Deviation,
Sim	Simultaneous antibiotic administration
SIRS	Systemic Inflammatory Response Syndrome
SSC	Surviving Sepsis Campaign
T>MIC	Time above the MIC
UTC	Untreated controls
VAP	Ventilator Associated Pneumonia

1 CHAPTER 1: Introduction

Despite modern advances in medicine and the constant improvement of our understanding of sepsis pathophysiology, sepsis and septic shock remain life-threatening diseases with a growing incidence, a high economic burden, and more importantly high mortality across the world.[1-9] Sepsis and septic shock are among the most common causes of potentially preventable death in both the developed and the developing world.[10,11] The World Health Organization (WHO) estimates the annual toll of sepsis cases to be over 49 million, with at least eleven million annual deaths.[11] In 2016, the WHO declared sepsis a global health priority and urged the implementation of measures that improve prevention, recognition, diagnosis and management of sepsis.[12,13]

1.1 Sepsis and septic shock

1.1.1 Definition

Even though the word sepsis has been used in the same context for the last 2700 years,[14] it took until 1992 to get an internationally recognized consensus definition of sepsis and related terms.[15] The concept of the systemic inflammatory response syndrome (SIRS) was introduced as “widespread inflammation (or clinical response to that inflammation) that can occur in patients with such diverse disorders as infection, pancreatitis, ischemia, multiple trauma, hemorrhagic shock and immunologically mediated organ injury”. Sepsis was defined as SIRS due to

infection that could arise in response to multiple sources, highlighting that bacteremia was not a necessary condition.

Table 1 SIRS, Infection and the sepsis syndromes as defined at the 1992 consensus conference.[15]

Infection	History, clinical symptoms, physical examination and laboratory findings suggesting the presence of an infection, which justified the initiation of anti-infective therapy
Systemic Inflammatory Response Syndrome (SIRS)	At least two of the following four criteria: • Fever (>38.3ºC) or hypothermia (<36ºC) • Tachycardia (>90 bpm) • Tachypnea (>20 breaths per minute) or mechanical ventilation • High leucocyte count (>12000/mm^3) or band forms, or leucopenia (<4000/mm^3)
Sepsis	SIRS + probable (clinically documented) or confirmed (microbiologically documented) infection
Severe sepsis	Sepsis + acute organ dysfunction, hypoperfusion abnormality or transient hypotension, independent of other cause than sepsis
Septic shock	Sepsis + hypotension persisting for > 1 hour despite adequate fluid resuscitation

C, centigrade degree; bpm, beats per minute; mm^3, cubic millimeter

In 2003 a second international consensus was held; they refined the definitions by detailing hemodynamic, organ dysfunction and tissue perfusion variables.[16] They also highlighted that signs of a SIRS occur in many infectious and non-infectious conditions and therefore they are not helpful in the differential diagnose of sepsis from other conditions.

Since then, another iteration of the consensus definition of sepsis committee has changed the sepsis definition to the one we use today: “sepsis is life-threatening organ dysfunction caused by a dysregulated host response to infection”.[17]

1.1.2 Epidemiology

Historically, the mortality associated with sepsis and septic shock has been approximately 50-75%.[18] It decreased after the development of modern antimicrobial therapies, starting with penicillin in the early 1940s. Since then, sepsis-associated mortality has fallen to the 25-50% range worldwide[3,7-10] and remains the most common cause of death in intensive care units (ICUs) of developed countries.[1,3-9]

Sepsis incidence has gradually increased over the last decades,[1,19] in part due to improved diagnosis as a result of increased awareness and surveillance;[3] but also as a consequence of the rising aging population and the increased survival of immunosuppressed patients thanks to modern therapies. Still, sepsis incidence is thought to be underestimated worldwide.

Respiratory tract infections are the major source of sepsis, accounting for about half of all cases, followed by intraabdominal infections, infections of the urinary tract and skin/soft tissue infections.[20] Microbiological documentation of infection can be obtained in 60-70% of the cases, meaning that in up to a third of cases cultures from all sites, including blood, are negative. Only one third of the patients have positive blood cultures.[20]

The microbial etiology of sepsis has shifted over the past decades, with gram-negative bacilli and gram-positive cocci dominance at different points. Fungal infections have been increasing constantly. In the international study from Vincent et al., involving 14000 ICU patients with severe sepsis, gram-negative bacteria were isolated in 62% of patients, gram-positive bacteria in 47% and fungi

in 19%.[20] Overall, most common gram-positive isolates are *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pneumoniae (S. pneumoniae)*. *Escherichia coli (E. coli)*, *Klebsiella species* and *Pseudomonas aeruginosa (P. aeruginosa)* stand among gram-negative isolates.[20] Since the development of modern antimicrobials, bacterial pathogens have continuously evolved under their selective pressure, developing resistance and becoming a major concern.

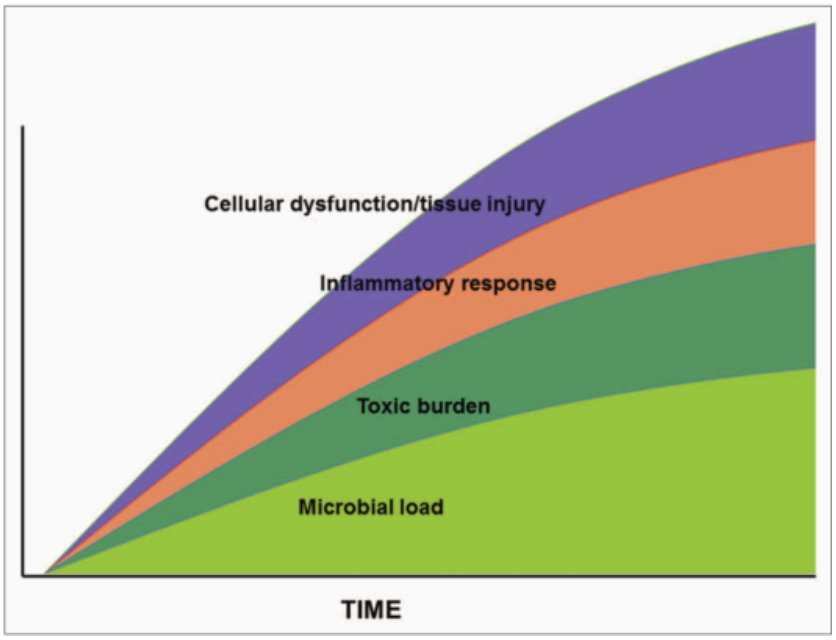
1.1.3 History of sepsis pathophysiology

Derived from the word septicaemia, the initial *germ theory paradigm* considered that the key element of sepsis was the microbe in the bloodstream. But this paradigm did not fully explain sepsis pathogenesis; since patients died despite the successful eradication of the septic source from the blood or in the absence of bacteremia.[21]

1.1.3.1 The microbiologic model of sepsis

The *microbiologic model* considered the key driving element of sepsis the microbial load and its toxic burden. The septic process starts with a focus of infection, the organisms replicate over time, releasing endo and exotoxins, which will stimulate the inflammatory cascade, **Figure 1**.[21] It is the microbial load that drives the downstream responses. Thus, controlling the septic source should significantly reduce the inflammatory cascade and limit tissue injury and organ dysfunction. This model forms the basis of standard antimicrobial therapy in sepsis and septic shock.

Figure 1. Microbiologic view of sepsis[21]



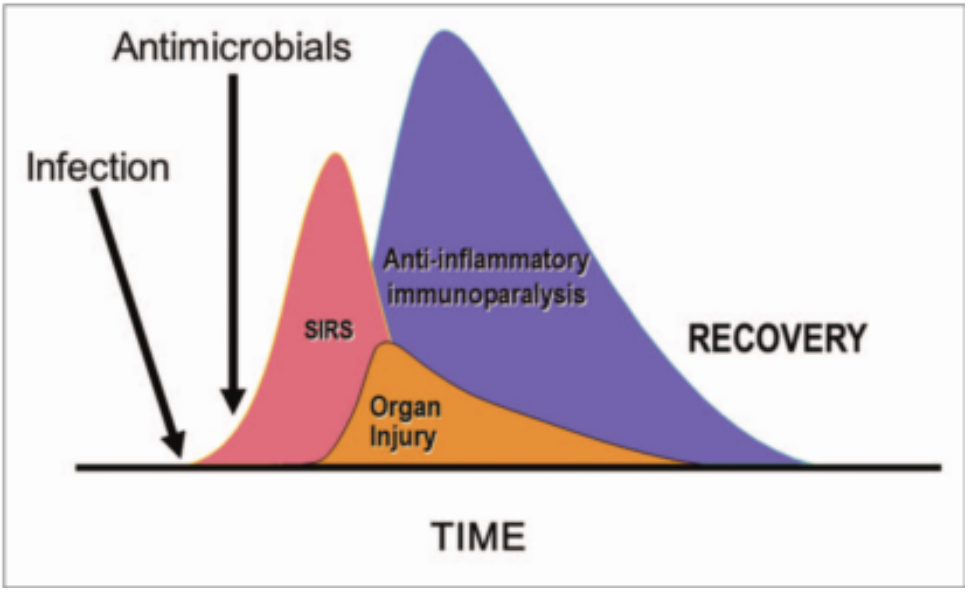
However, this model fails to recognize the occurrence of shock and that it can only be tolerated for a short period of time or it will irreversibly lead to death. This was originally described by Wiggers in 1950,[22] where he introduced the idea of the "golden hour", which was first demonstrated in the context of hemorrhagic shock but is applicable to all shock states. If the condition driving shock is not addressed promptly, shock will irreversibly progress to death. Thus, mortality will not improve without early and definitive control of the source of shock.

1.1.3.2 The immunologic model of sepsis

It was then theorized that it should be the host response what drives the pathogenesis of sepsis, leading to the currently accepted *immunologic paradigm* of sepsis.[21,23,24] This theory suggests that sepsis is a result of an overly exuberant pro-inflammatory host response triggered by infection, with a subsequent

compensatory anti-inflammatory response, that propagates independently of the underlying infectious trigger and despite pathogen clearance.[15,16,25] Under the immunological model, the rapid elimination of the pathogens with antibiotic therapy will not modify the inflammatory and counter-inflammatory response that drive cellular dysfunction.[23-25]

Figure 2. Immunologic view of sepsis[21]



SIRS, systemic inflammatory response. Adapted from reference.

A key deficiency to the immunologic paradigm is ignoring the septic source and its toxic burden. Despite the use of appropriate antimicrobials, it will take time to fully eliminate the microbial load, and the remaining pathogen load will maintain an inflammatory potential.[21,26]

1.1.3.3 Composite model

If we consider sepsis under a different composite perspective and integrate the immunological model of sepsis with the microbiological one and we add the shock threshold, we get this composite model (**Figure 3**).[21] In this model, the presence of shock becomes the key driver of irreversible organ injury and the source of shock is the microbial load. The faster you reduce the microbial load after the onset of hypotension, the higher the survival. Thus, faster bacterial kill will reduce mortality.

Figure 3. Composite view of sepsis[21]

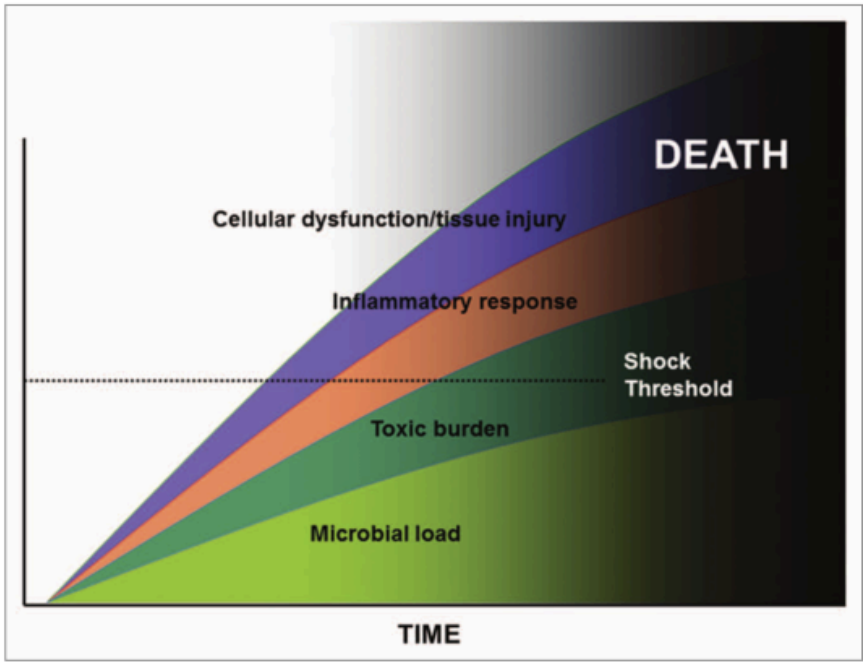
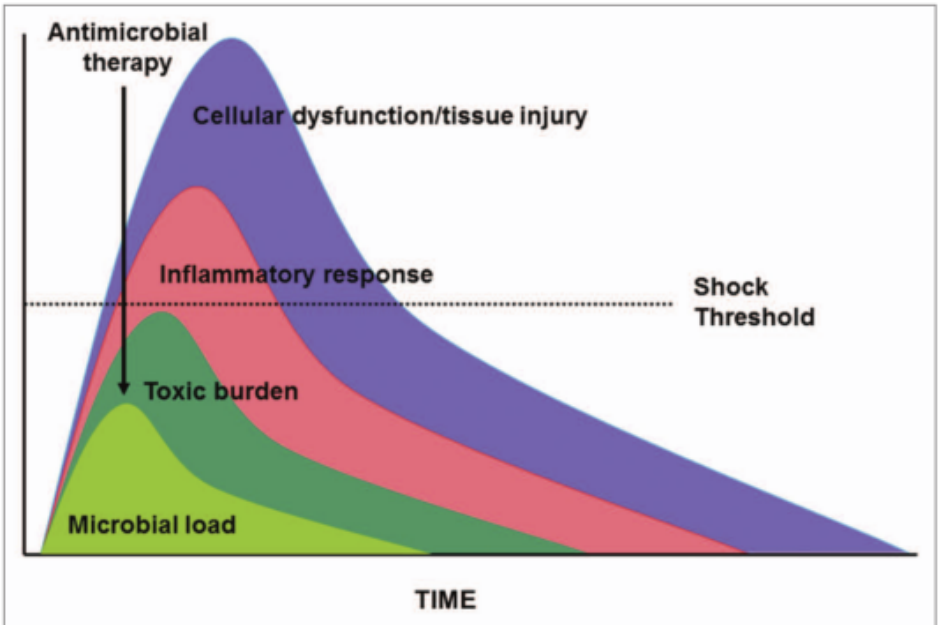


Figure 4. Impact of appropriate antimicrobial therapy in sepsis and septic shock.[21]



This model has a major pathophysiologic implication that has previously been described in patients and animal models: delays in appropriate antimicrobial therapy will be associated with a higher microbial load[27-29] and with increased morbi-mortality.[30-36] Hence, early appropriate antimicrobial therapy will accelerate pathogen clearance and should be associated with increased survival.[37,38] See **Figure 4.**

1.2 Sepsis treatment

Multiple editions of clinical practice guidelines have been developed over the years to help clinicians with sepsis management. The Surviving Sepsis Campaign (SSC) has been the international organism responsible for developing the sepsis clinical practice guidelines.[2,39-41] The goal of these guidelines is to standardise care, improve outcomes, limit variability and dangerous practices and facilitate future research.

Although these guidelines are based on the best clinical evidence available, their recommendations are often based on low-quality of evidence.[42-44] Of the 87 therapeutic recommendations given in the last SSC guidelines,[2] 42% were based on low-quality of evidence and only 10% were supported by randomized controlled trials (RCTs).[42] Although ten of the 17 recommendations involving antimicrobials and source control were characterized as strong, none were based on high quality of evidence.[42] Nevertheless, we should take into consideration the value of expert opinion in specific topics where there is a paucity of randomized trials, like in antimicrobial stewardship, source control and rapid administration of antimicrobials.[45-47]

The SSC divides sepsis treatments in five major categories: 1. Resuscitation, 2. Antimicrobial and source control, 3. Management and adjuvant therapy, 4. Mechanical ventilation, and 5. Nutrition. Of these five pillars, only antimicrobials and source control can modify pathogen clearance, which as explained in **section 1.1.3.3** is the key determinant of outcome in sepsis and septic shock.

Going forward I will focus on antimicrobial optimization principles that improve pathogen clearance in clinical practice. **Table 2** provides a list of the antimicrobial determinants of pathogen clearance of serious infections.

Table 2. Antimicrobial determinants of pathogen clearance in sepsis.

1. Early antimicrobial therapy	• Appropriate therapy
	• Avoiding delays
	• Loading doses
2. Antimicrobial Potency	• Ensure antimicrobial cidality
	• Optimize pharmacodynamics
	• Synergy: Combination therapy

1.2.1 Early antimicrobial therapy

In order to achieve early effective antimicrobial therapy, we must achieve three goals: 1. Use of appropriate empiric therapy, 2. Avoid administration delays, and 3. Achieve therapeutic levels as soon as possible (ideally after the initial dose). Or in the words of Paul Ehrlich in 1913: “frapper fort et frapper vite” (i.e. “hit hard and hit fast”).[48]

1.2.1.1 Appropriateness of empiric antimicrobial therapy

Failure to administer adequate initial therapy in septic shock is associated with increased mortality,[2,49-58] although data in sepsis without shock are inconsistent.[59-61] Microbially inadequate empirical therapy is started frequently in both conditions (15-35%), increasing the mortality risk.[49,57]

In order to guarantee appropriate empiric antimicrobials in sepsis, and especially in septic shock, we require an empiric broad spectrum of coverage. International guidelines recommend the use of multidrug therapy for sepsis empiric treatment for this purpose.[2]

However, this empiric multidrug therapy must be deescalated as soon as possible and adjusted to a narrower regimen to minimize selective pressure towards resistant organisms, ideally within the first 72h.[2] De-escalation of antimicrobials is actually associated with improved outcomes.[62-65]

The optimal selection of antimicrobials will depend on the local resistance epidemiology, as well as individual risk factors for resistance, including recent antibiotic use, hospitalization, and previous colonization or infection with resistant pathogens.

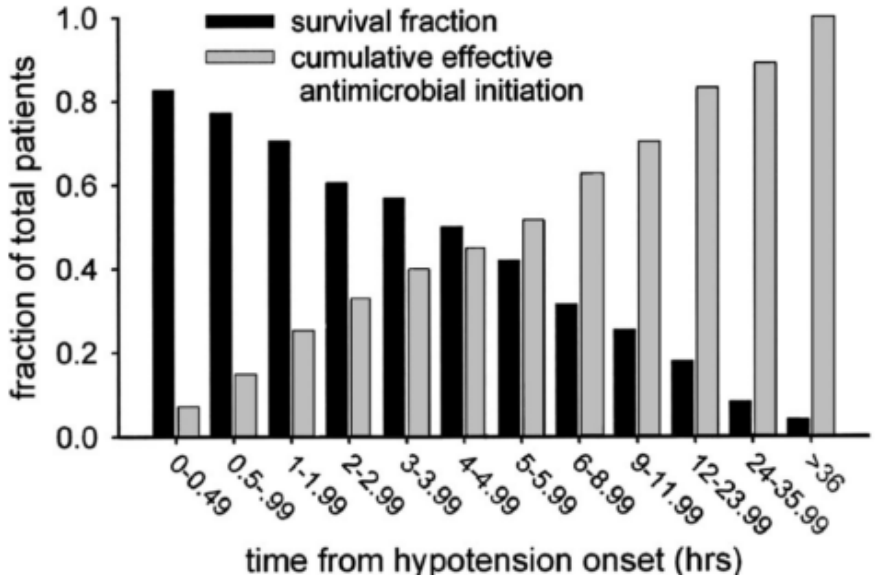
1.2.1.2 Avoiding delays

The lag in starting appropriate antimicrobial therapy has a strong association with increased mortality in septic shock,[51,66-71] while delays are inconsistently associated with mortality in sepsis without shock and bacteremia/candidemia.[72-76]

One famous retrospective study suggested that delays in the delivery of the initial dose of appropriate antimicrobials in septic shock patients is the single and strongest predictor of survival.[30] It showed a 7.6% decrease in survival of septic shock patients for every hour of delay in the delivery of adequate antimicrobials

after hypotension is documented (**Figure 5**).[30] In this particular study, the median time to delivery of adequate antimicrobials in septic shock patients was six hours.

Figure 5. Cumulative effective antimicrobial initiation following onset of septic shock-associated hypotension and associated survival.[30]



Since this study was published 15 years ago, there has been a better recognition of sepsis with the implementation of the SSC guidelines, with a downward trend on sepsis mortality.[77] Over this time, there have been inconsistent published results regarding timing to antibiotics and mortality.[53,78-81] The overall existing evidence on time to antibiotics and septic shock outcomes demonstrates an improved survival with earlier adequate antimicrobial therapy,[53,54,56,78] including a recent prospective multicenter observational study with a propensity score analysis.[82]

The impact on mortality of time to appropriate empiric antibiotics in comparison with other sepsis therapies, like fluid resuscitation, suggests that there

is a greater potential for improvement of patient outcomes associated with the speed of treatment.[2,30,83] In view of these data, intravenous delivery of appropriate broad-spectrum antimicrobials should be instituted as soon as possible when there is clinical suspicion of septic shock. In other words, in septic shock time is life.

1.2.1.3 Loading doses

Optimizing antimicrobial dosing should be a priority in the management of critically ill patients. Pathogen clearance will not begin until therapeutic levels of antimicrobials reach the source of infection.

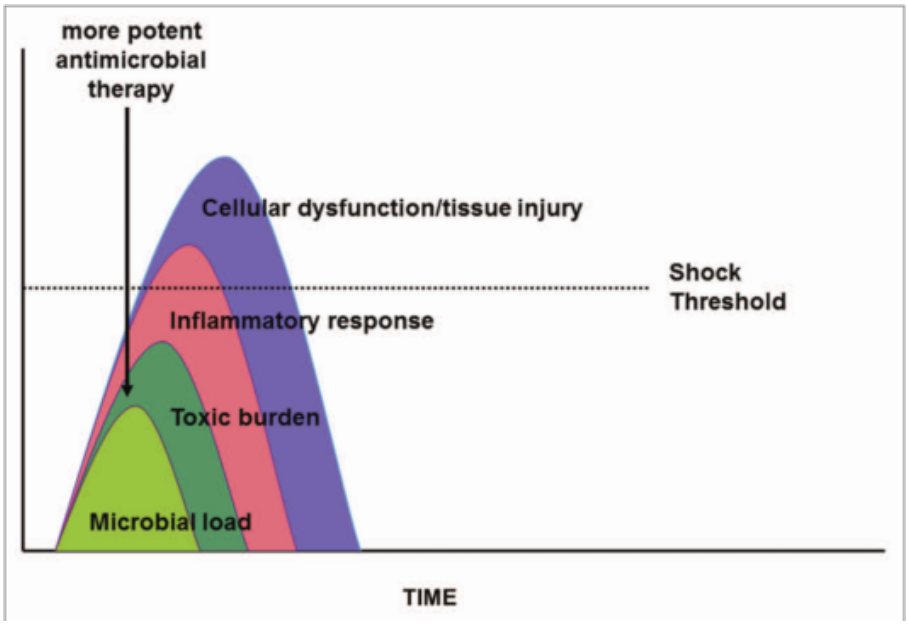
In order to select the appropriate antimicrobial dosing regime, we need to understand the pathophysiological changes that will impact the pharmacokinetic (PK) and pharmacodynamic (PD) properties of such drugs in the critically ill septic patient.[84]

The proinflammatory cascade of sepsis increases capillary permeability, which leads to an increased volume of distribution for hydrophilic drugs (beta-lactams, aminoglycosides, glycopeptides, linezolid, colistin), therefore reducing their plasmatic concentration. An increased initial cardiac output in early sepsis will also augment renal clearance, which can have secondary effects on antibiotic levels after initial dosing and drug distribution. Renal clearance can also become impaired throughout the septic process. These changes can lead to failure to achieve sustained therapeutic levels of the antimicrobials with standard dosing approaches.[85-88] Thus, a good strategy to achieve effective concentrations of antimicrobials from the first-treatment dose is to use loading doses.[89]

1.2.2 Antimicrobial potency

Not only dosing of antimicrobials is important to achieve appropriate bacterial kill, but potentially also the choice of antimicrobial strategy. Highly potent antimicrobials will provide rapid clearance of pathogens. This entails releasing less endogenous mediators with a more rapid resolution of hemodynamic instability and thus, improved survival should result (**Figure 6**).

Figure 6. Impact of more potent antimicrobial therapy in sepsis and septic shock[21]



The three ways of improving antimicrobial potency are: 1. choosing a cidal antimicrobial over a static one, 2. optimizing PK and PD of the antimicrobials and 3. improving synergistic effects by using appropriate antimicrobial combinations.

1.2.2.1 Cidal vs. static

Cidal antimicrobials kill the pathogen directly, while the static drugs stop the pathogen division and require an efficient immune response, where phagocytic cells will definitely clear the microorganism. Thus, by definition, cidal antimicrobial therapy should provide faster pathogen clearance than static antimicrobials.

However, there is no definitive clinical evidence to support this, since assessing a drug class effect in a clinical meaningful way is difficult, and most guidelines recommend the use of cidal drugs over static in life threatening infections.[90-92]

The best known study addressing this question is the 1951 paper by Lepper and colleagues, where treating life threatening bacterial meningitis with penicillin, a cidal drug, was superior than using chlortetracycline, bacteriostatic.[93]

Other old trials on bacterial endocarditis, osteomyelitis and neutropenic gram-negative bacteremia also showed better clinical cure rates when using cidal over static drugs.[94-97] A more recent RCT in invasive *Candida spp.* infections shows superiority of the cidal antifungal anidulafungin over fluconazole, static.[98]

Overall, the available evidence supports the superiority of cidal therapy over static in life-threatening infections. However, a recent metanalysis did not find enough evidence to support this statement in non-critical infections and a recent systematic review questions this statement altogether.[90,99] Additional studies are required to definitively address this question in septic shock, where the difference should be more profound.

1.2.2.2 Optimization of pharmacokinetics and pharmacodynamics

There is an increasing body of literature focused on the importance of dosing optimization strategies in sepsis over the last two decades, and the importance of PK/PD optimization to achieve appropriate antimicrobial concentrations at the site of infection.[89,100,101]

When choosing antimicrobials and their dose, we should be aware of their PK characteristics, the pathophysiological changes of sepsis in our patient (hemodynamic and fluid status, renal and hepatic function), and how these change the PK parameters (increased volume of distribution, augmented or impaired renal clearance). We should also know the location of the infectious source and the penetration of the antimicrobials in that tissue to appropriately dose and administer such drugs and achieve the PD targets required to successfully control the source of infection.

The PD targets change depending on the antimicrobial class.

1.2.2.2.1 Time dependent killing agents

For time dependent killing agents, like beta-lactams, carbapenems and lincosamides, the key PD target to optimize pathogen clearance is the percentage of time for which the drug concentration in plasma stays above the minimum inhibitory concentration (MIC) i.e. T>MIC. The few studies that examine the role of T>MIC in serious human infections, suggest that high T>MIC (>60-100%) is associated with better bacterial eradication and clinical cure.[102,103]

Continuous infusion of this class of antibiotics generates 100% T>MIC for sensitive and intermediately resistant pathogens, compared to intermittent administration. This administration strategy has shown improved clinical cure, shorter hospital stay and decreased mortality in observational and randomized trials in critically ill patients, most of whom would have been in septic shock.[104-108]

Of the latest five meta-analyses published on continuous infusion of beta-lactams versus intermittent dosing, the three more recent show a reduction in mortality of critically ill patients with continuous administration of beta-lactams, while the other two show a trend towards mortality benefit.[109-113] These results confirm the underlying hypothesis that PD target optimization reduces mortality in critically ill patients treated with beta-lactams.

1.2.2.2.2 Concentration dependent killing agents, with and without time dependency

Concentration dependent killing agents use different PD targets depending if there is a time dependent element (fluoroquinolones, glycopeptides, tigecycline) or they are mostly time independent (aminoglycosides).

For aminoglycosides, the key PD target is the ratio of the peak concentration (C_{max}) to the MIC of the pathogen, or C_{max}/MIC. For fluoroquinolones and glycopeptides the optimal PD target is the ratio of the area under the concentration curve (AUC) during 24h to the MIC of the pathogen, or AUC_{0-24}/MIC.[101]

Experimental animal models and human studies suggest certain ratios of these PD targets to optimize pathogen clearance and clinical cure.[114-120] Unfortunately, there are no human data linking fluoroquinolone or aminoglycosides PD target optimization to mortality and no studies of septic shock have yet been reported. There is evidence of emergence of resistance when using fluoroquinolone concentrations below the PD targets, this has been linked to poor outcomes and thus, an association between poor outcomes and PD targets not being met has been made.[121]

Vancomycin is another antibiotic whose efficacy is most closely related to combined concentration and time dependent PD indices. Retrospective studies of methicillin-resistant *S. aureus* (MRSA) bacteremia and pneumonia showed better clinical cure above an AUC_{0-24}/MIC of 400mg*h/L.[122] *In vitro*, failure to achieve this PD target was associated with appearance of vancomycin-resistant *S. aureus.* A retrospective study of MRSA septic shock identified an AUC_{0-24}/MIC target below which there was a higher mortality.[123]

Overall, antimicrobial PD target optimization has been linked to clinical and microbiologic response in multiple studies. To date we use these targets as therapeutic drug monitoring markers to make sure we achieve appropriate treatment goals. Unfortunately, there is limited evidence linking concentration dependent PD target optimization and survival or other relevant clinical outcomes. To the extent that this evidence exists, it tends to show a survival benefit in critically ill patients, especially in those with septic shock.

1.3 Combination therapy

Multidrug antimicrobial therapy is commonly used in the early treatment of sepsis and septic shock, following the recommendations of the Surviving Sepsis Campaign guidelines[2,39,40]. Delayed appropriate antibiotic therapy is known to be strongly associated with increased mortality in patients with septic shock[30], and broad spectrum multidrug therapy will increase the probability for appropriate therapy as compared to monotherapy.[2,30] Combination therapy, on the other hand, exhibits a synergistic effect of the antimicrobials used, causing an accelerated clearance of the pathogen; and leading to improved outcomes in severe infections and sepsis.[21,124-126]

1.3.1 Definition of combination therapy

The term combination therapy, as opposed to multidrug therapy, can be specifically defined as the concomitant use of two or more microbially appropriate agents with different mechanisms of action to cover the same pathogen.[2]

There are three major potential advantages to using combination anti-infective therapy for serious life-threatening infections:[127] 1) broader spectrum of coverage (addition of the spectrum of both antimicrobials), allowing a higher probability of appropriate initial therapy;[2,125] 2) reduced risk for emerging of resistance during therapy;[124,128] and 3) the potential synergistic effect (faster bacterial kill by the combined action of both antibiotics).[124,129-131] There are also potential disadvantages related to multidrug therapy, like increased risk for toxicity,

higher costs, possible antagonism between specific drug combinations and selection of resistant strains.[8]

1.3.2 Synergy

Synergism of an antibiotic combination is defined as a greater antibacterial activity of the combination regimen compared with either agent alone or together.[124,129] Thus, it leads to more rapid pathogen clearance, which may translate into improved patient outcome and may be the most directly clinically relevant advantage of combination therapy.[21,124-126,130]

Antimicrobial synergy, with increased bacterial clearance, has been best established for beta-lactam/aminoglycoside combinations for Gram-negative bacilli (GNB).[132] It has also been described in beta-lactam/fluoroquinolone combinations,[133,134] and there are some data that suggest additive effects or even synergism for beta-lactam/macrolides combinations[135]. These combinations have been effective *in vitro* not only with susceptible GNB, but also with multidrug-resistant GNB[136-140] and Gram-positive bacteria[141].

The existing evidence to date is insufficient to support the choice of combinations and explain the synergistic effects observed. *In vitro* models are not very satisfactory, and their synergistic data should be cautiously interpreted, since their clinical significance has not been determined.[142] However, they can be used to screen for effective combinations that can later be validated in animal or clinical studies.[143]

Published *in vivo* studies in animal models of infection have also shown a beneficial effect of combination therapy over monotherapy with respect to accelerated bacterial clearance, in sensitive and resistant strains of microorganisms.[131,144-146] There are no published data for antibiotic sequence and animal models of sepsis.

1.3.3 Clinical evidence of combination therapy

While empiric combination therapy for sepsis is widely used and recommended by clinical guidelines[2]; the published clinical evidence supporting combination therapy in sepsis, and related clinical studies in neutropenic infections, Gram-negative bacteremia,[147-149] and meta-analyses;[52,150-153] is diverse and contradictory.

The existing clinical evidence is not sufficient to support the use of definitive combination therapy in immunocompetent patients with sepsis without shock, since it could be associated with increased risk for toxicity, higher costs, and selection of resistant strains. Therefore, it is recommended to de-escalate antibiotic treatment to the most appropriate single agent as soon as the antibiotic susceptibility profile is known[5].

However, in situations where there is a significant risk of bacterial multi-drug resistance or significant risk of death like in immunosuppressed patients or septic shock, the use of combination therapy may be warranted to ensure that likely pathogens are sensitive to at least one antibiotic.[21,130,154-156]

There are highly suggestive retrospective meta-analyses that favour combination therapy in septic shock patients.[52,150] The potential clinical benefit of antibiotic combination therapy has been questioned by three previous meta-analyses and a Cochrane systematic review (same group of authors), which failed to show improvement in clinical outcomes with combination therapy.[151,152,157,158] These meta-analyses did not undertake subgroup analyses of the septic shock population, nor did they use the definition of combination therapy, and they included in their analysis multidrug therapy trials.

Given the contradictory clinical evidence of combination therapy in sepsis without shock, the major rational for using it remains septic shock and to broaden the spectrum of coverage.[2] Many questions remain to be answered. While waiting for appropriately designed randomized controlled trials, combination of empiric antibiotic therapy for several days with two drugs of different mechanisms of action, to which the bacteria are susceptible, is appropriate for patients in septic shock. Monotherapy is reasonable for patients who are not critically ill or at high risk of death.

1.4 Sequence of antibiotics in combination therapy

One of the hypothetical reasons why combination therapy may not always improve outcomes is that maybe the sequence of antibiotics maters. It is possible that the synergistic effect of beta-lactams and aminoglycosides or fluoroquinolones may be dependent on the order of antibiotics to affect accelerated pathogen clearance.

The mechanism of action of beta-lactams is to inhibit the bacterial cell wall biosynthesis by inhibiting a family of enzymes called penicillin binding proteins.[159] The biosynthesis of the bacterial cell wall happens mostly during bacterial division. The bactericidal effect of beta-lactams is indirect, their interference in the cell wall synthesis activates the autolytic cell system.[160]

Aminoglycosides' mechanism of action consists of interference with protein formation by binding to the 16S unit of the ribosome.[161] One pharmacodynamic characteristic of aminoglycosides is the post antibiotic effect, or the persistent bacterial growth suppression after the drug has been cleared.[162] Fluoroquinolones on the other hand, directly inhibit DNA synthesis and suppress bacterial growth.[163] In both cases, in order to get into the cytoplasm of the bacteria, these antibiotics have to actively cross the lipopolysaccharide membrane. Which explains why synergistic effects have been observed with cell-wall active agents like beta-lactams, since the aminoglycosides and fluoroquinolones may penetrate through the bacterial cell wall better when used after beta-lactams.

Thus, the right sequence of antibiotics may provide faster bacterial clearance. By giving beta-lactams first we can facilitate the entrance of aminoglycosides and fluoroquinolones in the bacteria. On the other hand, the opposite order may preclude beta-lactams from working appropriately and slow bacterial clearance, since the post-antibiotic effect of aminoglycosides and fluoroquinolones will suppress bacterial replication, which is key for beta-lactams mechanism of action.

To date the evidence on antibiotic combination sequence and its synergistic effects is sparce, old, and mostly *in vitro.*

1.4.1 *In vitro* evidence

The available evidence reduces to seven studies between 1982-2004.[164-170] These studies suggest that the sequence of antibiotic administration may have a significant impact on bacterial kill rates in *in vitro* pharmacodynamic models. Most of the *in vitro* evidence was performed on *P. aeruginosa,* with one paper on *E. coli*[165] and one on *S. aureus.*[169]

Of the studies that included beta-lactams, all but the one on *S. aureus*,[169] they favor the administration of beta-lactams first or simultaneously with the other agent (aminoglycoside or fluoroquinolone), but with inconsistent results, **Table 3**.

Table 3. Comparison of existing in vitro sequence publications.

Author	Journal	Year	Bacteria	Antibiotics	Effect
Zelenitsky et al[170]	Diagnostic Microbiology and Infectious Disease	2004	*P. aeruginosa*	1. Ceftazidime - ciprofloxacin 2. Ceftazidime - tobramycin	**Staggered** (beta-lactam first) or **simultaneous** better bacterial clearance
Goto et al[169]	International Journal of Antimicrobial Agents	1999	*S. aureus*	Netilmicin and minocycline	**Staggered** (netilmicin first) or **simultaneous** dosing optimal
den Hollander et al[168]	Antimicrobial Agents and Chemotherapy	1997	*P. aeruginosa*	Ceftazidime and tobramycin	**Simultaneous** dosing better
Barclay et al[167]	Antimicrobial Agents and Chemotherapy	1995	*P. aeruginosa*	Ceftazidime and gentamicin	**Staggered** dosing optimal (regardless of the order)
Dudley et al[166]	Journal of Infectious Diseases	1991	*P. aeruginosa*	Ciprofloxacin and azlocillin	**Simultaneous** dosing optimal
Konig et al[165]	Chemotherapy	1986	*E. coli*	Ampicillin and gentamicin	**Staggered** dosing optimal (beta-lactam first)
Haller et al[164]	Infection	1982	*P. aeruginosa*	Azlocillin and sisomycin	**No difference**

P. aeruginosa – Pseudomonas aeruginosa; S. aureus – Staphylococcus aureus; E. coli – Escherichia coli.

1.4.2 *In vivo* evidence

The only published animal evidence to date is the previously mentioned study by Goto et al, where they combined *in vitro and in vivo* evidence of antimicrobial sequence in *S. aureus.*[169] They demonstrated a higher survival rate (83%) with netilmicin followed by minocycline than with the opposite order of antibiotics (survival rate 42%) or simultaneous dosing (survival 30%) in a neutropenic mice model of MRSA pneumonia.

As per clinical evidence, the clinical report from Guggenbichler et al. is the only existing published evidence to the best of our knowledge.[171] They observed significant improvements in *P. aeruginosa* clearance from the sputum of 36 cystic fibrosis patients depending on the sequence of antibiotics, especially when beta-lactams were dosed four hours before aminoglycosides, as opposed to administered together.

1.5 Gaps in knowledge, purpose and hypotheses

With this thesis I want to further contribute to the knowledge around combination therapy, especially as it may pertain to septic shock.

1.5.1 Chapter 2: Antimicrobial combination therapy in sepsis: a systematic review and meta-analysis

Purpose: To evaluate the efficacy and safety of antimicrobial combination therapy in patients with sepsis following the appropriate definition of combination therapy, we will perform a systematic review of randomized clinical trials. And we will meta-analyze the subgroup of septic shock patients.

Hypothesis: When using the appropriate definition of combination therapy, we will find a difference in relevant clinical and safety outcomes that favours combination therapy in critically ill patients with sepsis and/or septic shock.

1.5.2 Chapter 3: Animal model of antibiotic sequence

Purpose: To study the effects of antibiotic sequence in bacterial clearance in a rat model of septic shock using different bacterial strains and antibiotic doses.

Hypothesis: Giving the cell-wall active agent first will improve bacterial clearance, physiologic responses, and will change the inflammatory mediator generation in our rat model of septic shock.

2 CHAPTER 2: Antimicrobial combination therapy in sepsis: a systematic review and meta-analysis

2.1 Introduction

As previously introduced in **Section 1.3.3**, given the contradictory existing evidence on combination therapy in critically ill patients to date; we decided to perform a systematic review to identify, critically-appraise and meta-analyze data on the efficacy and safety of antimicrobial combination therapy compared to monotherapy in sepsis and septic shock patients in randomized clinical trials using the appropriate definition of combination therapy.

2.2 Methods

2.2.1 Search strategy

We, the systematic review team (**Appendix 1**), conducted this systematic review in accordance to the Methodological Expectations of Cochrane Intervention Reviews (MECIR) guidelines using a pre-planned protocol.[172] It is reported as per the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) guidelines for systematic review and meta-analysis.[173] We searched the following electronic databases from inception to March 2017: PubMed (National Library of Medicine), EMBASE (Ovid), and CENTRAL (the Cochrane Library – Wiley). The Cochrane Highly Sensitive Search Strategy (HSSS) was used as a model for searching.[174] The search strategy was designed for PubMed with input from a library sciences specialist at the University of Manitoba and translated for

other databases. The following search terms were used: “sepsis”, “septic shock”, “combination therapy”, “antimicrobial”, “combination therapy”. The search strategy for PubMed is presented in **Appendix 2**. It was performed in two parts, part 1 for the indexed Medline database, where all the antimicrobial drug terms were included in the subject headings, and part 2 for the non-indexed entries, which does not include the drug terms. Included articles were used to perform a forward search in Scopus to identify additional citations. We searched the World Health Organization’s International Clinical Trials Registry Platform, in order to identify ongoing or planned clinical trials. In addition to electronic database searching, we searched abstracts and conference proceedings for the following societies from 2012 – 2017: American Thoracic Society, Society of Critical Care Medicine and European Society of Intensive Care Medicine. Finally, we hand searched the bibliographies of relevant reviews and included clinical trials for additional citations. Reference Management was performed in EndNote™ (ver. 16, Thomson Reuters, Carlsbad, CA, USA).

2.2.2 Study Selection and Outcomes

We included studies that met the following inclusion criteria: prospective randomized trials; adults (at least 80% of enrolled patients being 18 years of age or older); diagnosed with sepsis (with organ dysfunction) or septic shock following the definition of the 1991 Surviving Sepsis Campaign guidelines[175] (two Systemic Inflammatory Response Syndrome (SIRS) criteria and organ dysfunction, secondary to infection); with any kind of infection (bacterial, fungal or viral); treated with combination therapy in comparison to monotherapy (where combination

therapy comprises two or more antimicrobials with different mechanism of action, the comparator must include one of the antibiotics given in combination and the doses of the primary antibiotic must be the same in combination and monotherapy). Studies were excluded if the pathogens responsible for the septic episode where standard of care is combination therapy (i.e. *Mycobacterium tuberculosis*, *Mycobacterium avium* complex (MAC), human immunodeficiency virus (HIV)); or were observational study designs, quasi-randomized, crossover, or cluster-randomized trials.

After completing the literature search, citations were imported into a Microsoft Excel 2015 spreadsheet (Microsoft Corporation, Redmond, WA) for screening purposes. Two reviewers (GVG and CG) independently reviewed the title and abstract of each citation to determine if a study generally met the inclusion criteria. The full text of all citations listed as include or unsure by either reviewer at this stage of screening were retrieved for full-text review. The two reviewers then independently assessed the full text of each citation to determine if the study satisfied the inclusion and exclusion criteria. Studies meeting the inclusion criteria were reviewed independently and in duplicate for data extraction using a standardized data extraction form. Disagreements at each stage were resolved by discussion between the two reviewers. The following data were collected from each included study: study demographics (author, year of publication, country of study, funding source, publication status, duration of study, number of centers involved, inclusion and exclusion criteria, and risk of bias using the Cochrane Collaboration Risk of Bias tool);[176,177] patient demographics (age, sex, ICU

admission diagnosis, Acute Physiology and Chronic Health Evaluation (APACHE) II score[178] or other illness severity score, therapies received (vasopressors, mechanical ventilation, renal replacement therapy); details specific to the intervention (antimicrobials used in combination and in monotherapy); and details pertaining to the primary, secondary, and safety outcomes.

Our primary outcome was all-cause mortality reported at the longest follow-up (regardless of the primary outcome of the study). Pre-determined secondary outcomes were hospital and intensive care unit lengths of stay, ventilator, vasopressor and renal replacement therapy-free days and quality of life using a validated measurement scale. Our safety outcomes were any reported drug reactions and incidence of *Clostridioides difficile* colitis (previously known as *Clostridium difficile)*.

2.2.3 Risk of Bias Assessment

We used the Cochrane Collaboration Risk of Bias tool to assess the internal validity of included RCTs.[176,177] The overall risk of bias was considered low only if all six (sequence generation, allocation concealment, blinding, incomplete outcome data, selective outcome reporting, and "other" sources of bias) components were rated as having a low risk of bias.

2.2.4 Data Analysis

We analyzed data from the included studies using the Cochrane Review Manager (RevMan version 5.3.5, The Cochrane Collaboration 2014, Copenhagen, Denmark). For dichotomous data, we expressed summary measures of effect as

risk ratios (RR) with 95% confidence intervals (CI) using the Mantel-Haenszel method. We used a random-effects model for all analyses. A risk ratio of less than 1 suggests a lower rate of death among patients treated with monotherapy than those in the combination group. We assessed statistical heterogeneity of the data using the I^2 statistic. We investigated potential sources of heterogeneity using sensitivity analyses based on methodologic characteristics, quality and patient characteristics. Publication bias was assessed by viewing the overlap of the study confidence intervals and using funnel plot techniques.[179]

2.2.5 Subgroup and Sensitivity Analyses

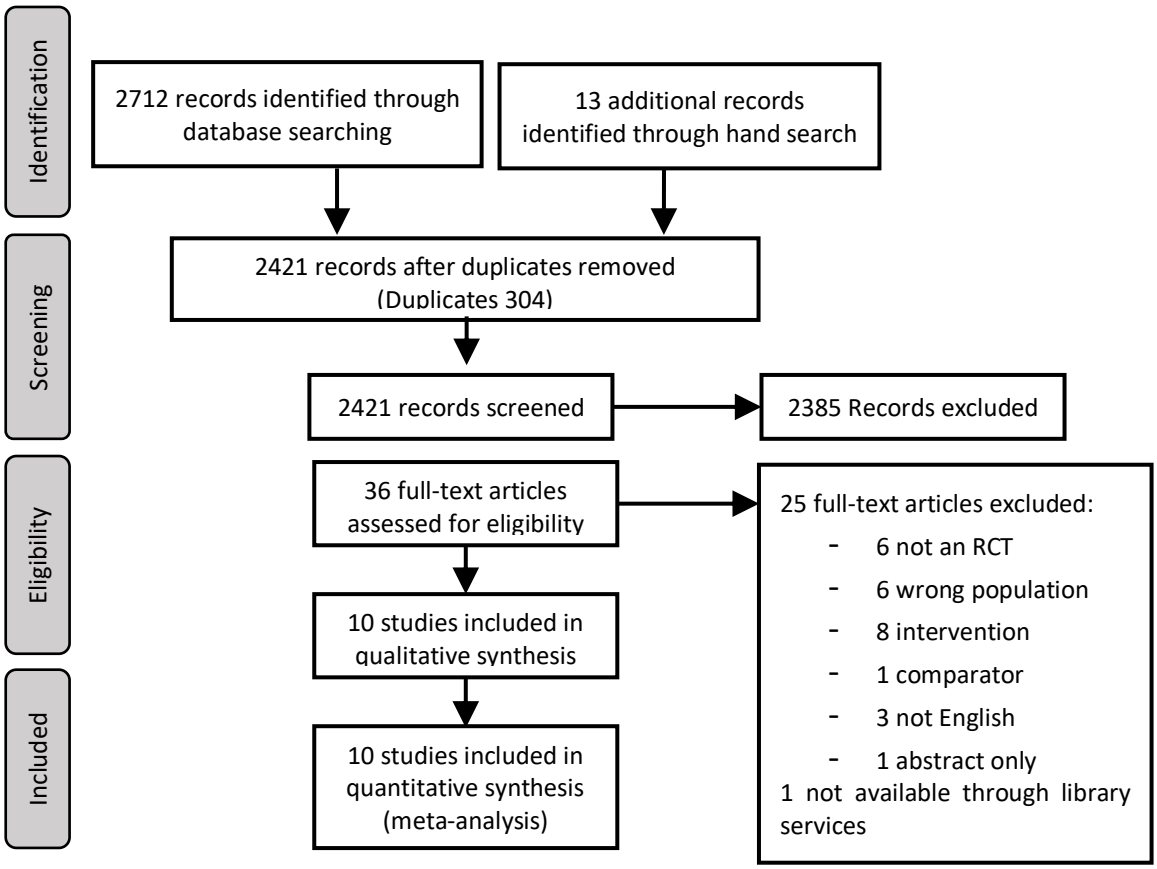
Subgroup and sensitivity analyses were performed to determine the effects of combination therapy in specific patient populations and to address other potentially confounding factors. Analyses were dependent on the number of studies and availability of appropriate outcomes. The following subgroup and sensitivity analyses were considered: methodological (North American vs. other; Industry funded vs. non-industry funded; low risk of bias vs. unclear / high risk of bias; Published vs. Grey literature / abstracts / conf. proceedings) and clinical (sepsis vs. septic shock; type of infection (bacterial vs. fungal vs. viral); carbapenems or extended spectrum beta-lactams ± aminoglycosides vs. carbapenems or extended spectrum beta-lactams ± fluoroquinolones vs. other beta-lactams ± aminoglycosides; high (25 or more) vs. low APACHE II score); immunocompromised vs. immunocompetent.

2.3 Results

Of the 2725 records identified through electronic and hand searches, we included 10 randomized trials (2318 participants) (**Figure 7**, **Table 4** and **Table 5**).[153,180-188] All were published in peer-reviewed, English-language journals. One trial was conducted in Asia,[188] two in North America,[185,187] and the other seven in Europe. Five were multicenter trials.[153,183-185,187] All studies were adjudicated to be of high risk of bias, due to lack of blinding; and the only one which performed blinding had unclear allocation and blinding[185] of outcome assessment (appendix **Figure 19**). All studies were non-industry funded trials.

All the trials met inclusion criteria; the details of the intervention varied between trials (**Table 4**). Klastersky et al[180] and Sage et al,[182] compared both antibiotics in monotherapy and then combined. Damas et al,[186] compared cefepime with two different secondary antibiotics, amikacin and levofloxacin. Only Rex et al compared antifungal monotherapy versus combination therapy for confirmed candidemia.[185] All other trials compared antibiotics in confirmed or suspected severe infections with signs of sepsis or septic shock.

Figure 7. PRISMA flow diagram



The PRISMA flow diagram shows the number of identified records; the ones that were screened after eliminating the duplicates; the ones excluded after not meeting eligibility in level I screening, the initial title and abstract screen; then the ones that were assessed for eligibility after level II screening, reading the full text; and the final included studies. We also disclose the reasons for exclusion of the level II assessed records.

Table 4. Characteristics of the included studies

Study	Year	Continent (Country)	Total N (monotherapy/ combination)	Single / Multicenter	Population	Primary AB	Secondary AB
Klastersky[180]	1973	Europe (Belgium)	70 (22/23/23)	Single center	Disseminated cancer and life-threatening infection	Carbenicillin Gentamicin	
Sculier[181]	1982	Europe (Belgium)	20 (10/10)	Single center	Neurosurgical patients with Gram negative VAP	Mezlocillin	Sisomicin
Sage[182]	1987	Europe (United Kingdom)	93 (34/32/27)	Single center	Hospitalized adults (>14) with suspected life-threatening sepsis by Enteriobacteriaceae or Staphylococci	Cefotaxime Netilmicin	
Cometta[183]	1994	Europe (Switzerland)	280 (142/138)	Multicenter	Adults (>16) with nosocomial pneumonia, nosocomial sepsis or severe diffuse peritonitis	Imipenem	Netilmicin
Dupont[184]	2000	Europe (France)	204 (99/105)	Multicenter	Adults (>18) with complicated intraabdominal infeccions (severe sepsis and or nosocomial)	Piperacillin Tazobactam	Amikacin
Rex[185]	2003	North America (United States)	219 (107/112)	Multicenter	>13 with confirmed candidemia and sepsis criteria	Fluconazole	Amphotericin B
Damas[186]	2006	Europe (Belgium)	59 (20/19/20)	Single center	Adults (>18) mechanically ventilated with confirmed VAP	Cefepime	Amikacin Levofloxacin
Heyland[187]	2008	North America (Canada)	739 (370/369)	Multicenter	Mechanically ventilated adults (>18) with suspected VAP	Meropenem	Ciprofloxacin
Jung[188]	2010	Asia (Korea)	83 (42/41)	Single center	Adults (>18) with suspected nosocomial pneumonia and sepsis (CPIS >6) and in MICU	Vancomycin	Rifampicin
Brunkhorst[153]	2012	Europe (Germany)	551 (273/278)	Multicenter	Severe sepsis and septic shock adults	Meropenem	Moxifloxacin

CPIS – clinical pulmonary infection score, MICU – medical intensive care unit, VAP – ventilator associated pneumonia.

This table summarizes the most important characteristics of each included study.

Table 5. Baseline characteristics of the population included in each of the studies.

Study	Intervention	Age mean year (SD)	Male (%)	APACHE II / other score (mean, SD)
Klastersky[180]		NR	NR	NR
Sculier[181]		NR	NR	NR
Sage[182]	Cefotaxime Netilmicin Combination	51 (18-75) 57 (14-85) 53 (15-82)	56.6% 39.2% 50%	NR
Cometta[183]	Monotherapy Combination	57.7 (17.7) 54 (18.3)	72.5% 71.7%	9.8, 4.5 9.6, 5.2
Dupont[184]	 Monotherapy Combination	 60 (17) 63 (18)	 61% 52%	SAPSII 30, 11 31, 11
Rex[185]	Monotherapy Combination	57 (2) 55 (2)	54.2% 47.3%	16.8, 0.6 15,0.7
Damas[186]	Cefepime (Cfp) Cfp + Amika Cfp + Levo	53.1 (22.1) 64.7 (19.1) 59.2 (14.8)	65% 52.6% 75%	17.1, 4.6 14.6, 6.8 16.5, 6.4
Heyland[187]	Monotherapy Combination	58.9 (17.7) 59.1 (17.9)	68% 71%	20, 6.2 19.9, 6.4
Jung[188]	Monotherapy Combination	71 (28-86) 66 (33-98)	83% 85%	24 (15-38) 24 (16-38)
Brunkhorst[153]	Monotherapy Combination	63.7 (14.4) 65.5 (14.5)	64.5% 64%	21.9, 7.1 21.3, 7.4

APACHE – acute physiologic assessment and chronic health evaluation, SAPS – simplified acute physiology score, SD – standard deviation, NR – not reported, Cfp – cefepime, Amika – amikacin, Levo – levofloxacin

In this table we describe the age, sex and severity of illness of the population in each of the included studies. The severity of illness was reported in seven of the ten studies, most of them used the APACHE II score, while Dupont et al. used the SAPS score. Both scores estimate the probability of mortality for ICU patients.

2.3.1 Primary Outcome

Combination therapy was not found to be associated with a significant reduction in all-cause mortality (RR 1.04, 95% CI 0.89 to 1.20; I^2 0%; 9 trials; 2206 participants, **Figure 8**).

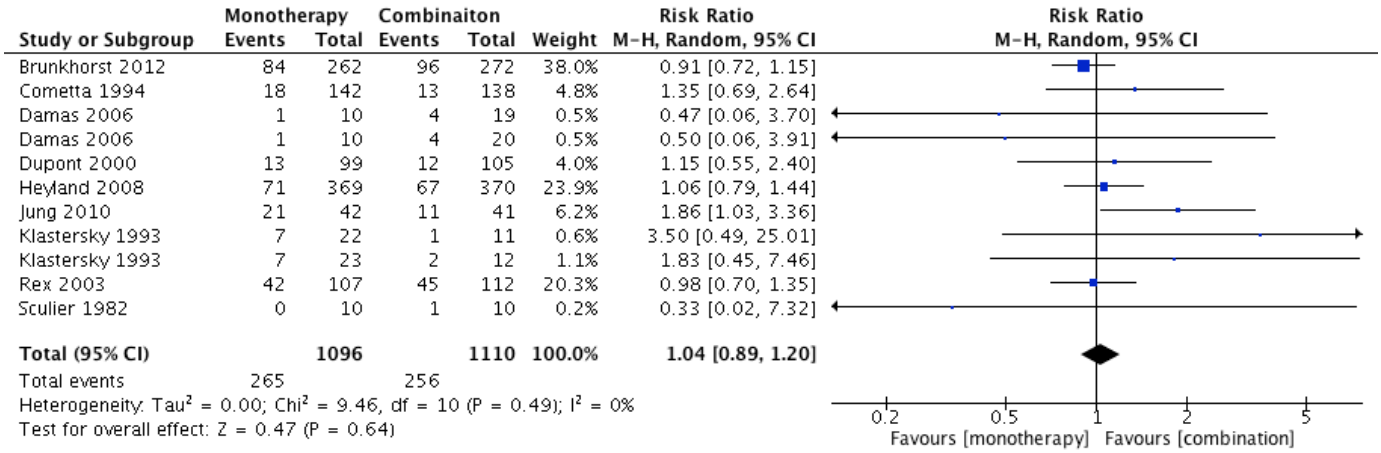
Funnel plot analysis, shown in the appendix, demonstrated a certain degree of asymmetry, missing some smaller positive studies for combination therapy and larger negative studies (appendix **Figure 20**). This is probably due to chance but could also represent some degree of publication bias.

None of the subgroup analyses performed showed any statistically significant data (appendix **Figure 21**).

2.3.2 Secondary Outcomes

Secondary outcomes were heterogeneously reported, no significant differences were found between combination therapy and monotherapy in ICU length of stay, hospital length of stay or ventilator free days (**Table 6**). We found no data reported regarding quality of life. Vasopressor and renal replacement therapy free days were only reported by Brunkhorst et al, who showed no differences between groups.[153]

Figure 8. Forrest plot of the primary outcome: Mortality



Using the Cochrane Review Manager (RevMan version 5.3.5, The Cochrane Collaboration 2014, Copenhagen, Denmark) we analyzed the compiled data of the nine included trials and their 2206 patients and obtained the following Forrest plot.

The x axis represents the Risk Ratio (RR), the size of the squares reflects the number of patients of each trial and the length of the lines represent the 95% confidence interval of the RR for each study. The black diamond represents the aggregated RR for our primary outcome.

We found no mortality benefit with combination therapy over monotherapy in the published studies (RR 1.04, 95% CI 0.89 to 1.20 and a statistical heterogeneity of I^2 = 0%, 9 trials, 2206 patients).

Most of the weight of the data comes from Brunkhorst et al.,[153] Heyland et al.[189] and Rex et al.[185] (38, 23.9 and 20.3% respectively), since they were the largest trials.

Table 6. Secondary and safety outcome measures

	# Studies	Participants		Effect estimate (95% CI)	I^2
		Monotherapy	Combination		
Secondary Outcomes					
ICU LOS	4	729	713	-0.19 (-1.77,1.40)	0%
Hospital LOS	2	320	321	2.90 (-0.69,6.49)	0%
Ventilator-free days	2	312	298	0.03 (-0.70,0.77)	0%
Safety Outcomes					
Adverse events	7	827	782	0.75 (0.60,0.93) Z= 2.59 **(P=0.01)**	15%
C. diff incidence	2	380	379	1.43 (0.83,2.47)	0%

ICU – Intensive Care Unit, LOS – length of stay, C. diff – Clostridioides difficile (previously Clostridium difficile), CI – confidence interval, I^2 – heterogeneity.

Using the Cochrane Review Manager (RevMan version 5.3.5, The Cochrane Collaboration 2014, Copenhagen, Denmark) we analyzed the compiled data for the secondary and safety outcomes.

We found no differences in any of our secondary outcomes, ICU length of stay, hospital length of stay or ventilator-free days when we compared monotherapy and combination therapy.

However, we found higher risk of adverse events in the combination therapy group (RR 0.75, CI 0.60 to 0.93, I^2 15%, 7 trials, 1609 patients, Z= 2.59, p=0.01). They considered renal injury as an adverse event. And there was some heterogeneity (I^2 of 15%) in the data, likely secondary to the inconsistent reporting of adverse events between trials. The trials that reported renal injury as an adverse event, used different definition criteria for renal injury.

There were no significant differences between monotherapy and combination therapy in C. difficile toxin presence. This was only reported in two trials.

2.3.3 Safety Outcomes

Combination therapy was associated with increased rate of adverse events (RR 0.75, CI 95% 0.6 to 0.93, I^2 15%; 7 trials; 1609 participants, Z=2.69, p=0.01) (**Table 6**). Most of the trials reported renal injury as an adverse event, owing to the fact that secondary antimicrobials were often nephrotoxic (aminoglycosides and amphotericin). There was a variable degree of reporting adverse events between studies, that may have introduced some heterogeneity in the data.

Incidence of *Clostridioides difficile* (previous *Clostridium difficile)* colitis was not reported, but positive *C. difficile* toxin was in two of the included trials and showed no difference between combination and monotherapy groups (RR 1.43, CI 95% 0.83-2.47, I^2=0%; 2 trials; 759 participants), see **Table 6**. Most (96.9%) of the weight of these data come from Brunkhorst et al trial.[153]

2.4 Conclusion

Combination therapy may not have a survival benefit over monotherapy in patients with sepsis. Our current analysis does not support combination therapy for the treatment of sepsis. As we were unable to discriminate based on severity of illness, a meta-regression adjusting by risk of death is needed to indicate whether severely ill patients may benefit from combination therapy. Large randomized controlled trials looking at patient-related outcomes are required to further explore the benefits and harms of this intervention.

3 CHAPTER 3: Animal model of antibiotic sequence

3.1 Introduction

In 2018, as part of the 68th World Health Assembly's global action plan to tackle antimicrobial resistance,[13] optimizing the use of antimicrobial agents was one of the proposed strategies. The use of combination therapy and in particular determining the best antimicrobial administration sequence may improve bacterial clearance and thus, clinical outcomes. In addressing this clinically relevant question, we will contribute to fulfill one of the "objectives" marked by the WHO for sepsis treatment.

The sparse existing evidence to date regarding antibiotic sequence, as previously described in **section 1.4**, showed that in *in vitro* pharmacodynamic models the use of beta-lactams first or simultaneously with aminoglycosides or fluoroquinolones can yield improved bacterial kill, although results among different models are inconsistent.

The rationale behind the potential effects of antibiotic sequence on bacterial clearance, and potentially on clinical outcomes, may be explained by the following two mechanisms. First, both fluoroquinolones and aminoglycosides need to cross the bacterial cell wall to be effective; cell-wall active agents may require to be already in place to facilitate their way inside the cell. Second, the post antibiotic effect with impaired bacterial growth after the administration of aminoglycosides and fluoroquinolones may interfere with the beta-lactam efficacy, since beta-lactams require the bacteria to be actively replicating to be effective.

3.2 Hypothesis

We hypothesize that the best antibiotic administration sequence for maximum bacterial clearance should be to administer the beta-lactam first followed by the aminoglycoside or fluoroquinolone. We propose that this sequence of administration of antibiotics will improve bacterial clearance, physiologic responses and inflammatory mediator generation in our rat model of monomicrobial peritonitis septic shock.

3.3 Objectives

3.3.1 Objective 1

Examine if pathogen clearance in the blood, organs and peritoneal fluid is improved depending on the antibiotic sequence in a rat model of peritonitis septic shock. These experiments were performed with two different bacterial strains (*S. pneumoniae* and *E. coli*) and with different doses of antibiotics.

3.3.2 Objective 2

Determine whether blood pathogen clearance is improved to a longer period of time or there is a greater extent of bacterial clearance depending on the order of the antibiotics in a rat model of peritonitis septic shock in two bacterial strains (*S. pneumoniae* and *E. coli*) and with different doses of antibiotics.

3.3.3 Objective 3

Assess if the sequence of antibiotics has an impact on the generation of inflammatory cytokines in a rat model of peritonitis induced septic shock.

3.4 Methods

3.4.1 Bacterial strains

Relevant pathogens that are responsible for most of the clinical burden of disease and are of international concern were studied.[20] Four different bacterial strains, all of which are virulent clinical isolates, two Gram positives and two Gram negative (one *Enterobacteriaceae* and one non-*Enterobacteriaceae*) were chosen.

- *E. coli:* ATCC 700973 serotype O18:K1:H7 (Bort), obtained from the American Type Culture Collection (ATCC). This strain was selected based on its virulence properties and its ability to cause septic shock in mice in previous studies.[27]
- *S. pneumoniae:* clinical strain 108246, obtained from the Clinical Microbiology lab at Health Sciences Centre (Winnipeg, Manitoba), courtesy of Dr. Zhanel.
- *S. aureus:* clinical strain 103421, obtained from the Clinical Microbiology lab at Health Sciences Centre (Winnipeg, Manitoba), courtesy of Dr. Zhanel.

- *Pseudomonas aeruginosa*: clinical strain 44302. Obtained from the Clinical Microbiology lab at Health Sciences Centre (Winnipeg, Manitoba), courtesy of Dr. Zhanel.

3.4.1.1 Determination of minimum inhibitory concentrations (MIC)

Sensitivities of the chosen pathogens to the selected antibiotics by determining the MIC were confirmed by the broth microdilution method following the Clinical and Laboratory Standard Institute guidelines.[190] All microorganisms were sensitive to the selected antibiotics.

3.4.1.2 Capsule preparation

3.4.1.2.1 Gelatin capsules

Size #4 gelatin capsules (Qualicaps Inc., USA) were sterilized by gamma irradiation using one million rads from a cobalt source at the Manitoba Agriculture, Food and Rural Initiative Veterinary services Branch of the Animal Health Center in Winnipeg. The sterilized capsules were stored in the refrigerator until they were used.

3.4.1.2.2 Inoculum

The bacterial strain of interest was cultured overnight in either Mueller-Hinton agar (MHA) petri plates or blood agar (BA) plates in the case of *S. pneumoniae*, at 37^{o}C and with an enriched CO_2 environment. All the bacteria from

at least 3 plates were collected with a sterile cotton swab and diluted in 2ml of normal saline (NS) for a dense mother inoculum.

Spectrophotometry (Spectronics-20 Genesys, Thermo Fisher Scientific, USA) optical density (OD) was used to achieve a daughter inoculum with a bacterial concentration of $1x10^8$ colony forming units (CFU)/mL. An OD of 0.08-0.085 at 625nm will yield this bacterial concentration. An aliquot of X µL of the mother inoculum was added to 3ml of NS and vortexed, the OD was then measured and the daughter inoculum was diluted or more inoculum was added until the desired OD was achieved. The daughter inoculum was then prepared using 4% fibrinogen (Sigma, F8630) instead of NS, and reserved.

The bacterial counts of the 4% fibrinogen daughter inoculum were confirmed by serial dilution and culture in duplicates in MHA or BA plates, as appropriate, incubating for 24 hours at 37°C.

3.4.1.2.3 Capsule generation and storage

In a sterile field inside the biosafety cabinet, the sterile gelatin capsules were opened and loosely filled up with previously autoclaved alpha-cellulose (Sigma, C8002), approximately 25-35mg. Then 100 µL of the 4% fibrinogen daughter inoculum was added to the capsules, for a total bacterial load of 10^7 CFU of the desired pathogen per capsule. Finally, 10 IU of Thrombin (Sigma, T6634) in 10 µL of distilled water were added.

After the capsules were filled, they were recapped in sterile conditions and frozen in individual sterile 1.5 mL Eppendorf tubes, first by placing the Eppendorf tubes in dry ice and then transferring the tubes to a -80^{o}C freezer until they were used.

3.4.1.2.4 Quantification of the inoculum in the capsules

To assure that the bacterial load of the capsules remained stable during their storage, the bacterial counts of the capsules were quantified one week, one month and every 3 months thereafter.

A single capsule was thawed in one mL of NS, serially diluted and then plated in duplicates on MHA plates. The plates were incubated at 37^{o}C for 24 hours, using an enriched CO_2 environment if required (*S. pneumoniae*). The bacterial load typically decreased to 5 x 10^6 to 10^6 CFU per capsule. This reduction on the bacterial load per capsule was consistent to this model and is due to the freeze-thaw cycle.[27,191]

3.4.1.2.5 Sham capsules

These capsules were made without the bacterial inoculum. The process was the same as previously explained in **section 3.4.1.2.3**, except that the 4% fibrinogen was sterile, did not have any bacterial inoculum added.

3.4.1.2.6 Higher inoculum capsules

If the standardization of the model showed that higher concentration capsules were needed to induce bacteremic septic shock; the same protocol that was previously described in **section 3.4.1.2.3** was followed and OD was used to guide the amount of inoculum per capsule. Ultimately the bacterial concentration of the capsules was determined by direct culture after thawing.

3.4.2 Antibiotics

Antibiotic combinations that had previously shown a synergistic effect in vitro against the selected pathogens were chosen. They included a beta-lactam and either an aminoglycoside or a fluoroquinolone. The pathogens were sensitive to both antimicrobials. The doses chosen were based on human sepsis dosing from the Sandford guideline,[192] extrapolated to the higher renal and hepatic clearance of drugs in rats, given the higher metabolism. We increased the dose by a factor of x2 and x4, see **Table 7**.[193,194] Since the duration of the experiment was a maximum of 9h none of the antibiotics used required redosing. All the antibiotics were procured commercially and stored at 4^{o}C.

Table 7. Antibiotic doses per organism.

Organism	Antibiotic	High dose	Sepsis dose
E. coli	• Cefotaxime • Gentamicin	120mg/kg 20mg/kg	60mg/kg 10mg/kg
S. pneumoniae	• Cefotaxime • Levofloxacin		60mg/kg 20mg/kg
S. aureus	• Cefazolin • Gentamicin		30mg/kg 10mg/kg
P. aeruginosa	• Ceftazidime • Tobramycin		500mg/kg 10mg/kg

3.4.3 Animals

Sprague-Dawley rats, of both sexes, weighing 300 ± 10 grams were used for all the experiments. All rats were purchased from the veterinary facility at the Bannantyne campus of University of Manitoba. The animals were bred in house and were housed in transparent cages in rooms with a 12h day/night cycle, and temperature controlled at 23 ± 2^{o}C. The animals were supplied with food and water *ad libitum*.

3.4.4 Animal ethics

The animal experiments and the experimental protocol were approved by the animal ethics committee at University of Manitoba, following the standards of the Canadian Council of Animal Care.

We complied with the recommendations of the “Minimum Quality Threshold in Preclinical Sepsis Studies” international consensus,[195] using the Modified Murine Sepsis Score described by Mai et al to determine the wellbeing of the septic animals.[196]

3.4.5 Rat model of peritoneal septic shock – Introduction and outline

The purpose was to establish a bacteremic model of monomicrobial peritonitis septic shock in Sprague-Dawley rats. This model was based on previous experiments in mice and rats done previously in Dr. Kumar’s lab.[27,191]

In order to generate the peritoneal model of septic shock two gelatin capsules were implanted in the peritoneum of the rats under inhaled generalized anesthesia. These capsules contained alpha cellulose, the bacterial inoculum and a fibrinogen-thrombin clot described in **section 3.4.1.2.** The gelatin capsule dissolves over several hours releasing its interior into the peritoneum of the animal, generating an inflammatory reaction and the development of the peritoneal infection.

After the capsule implantation the rat is woken up post anesthesia and recovered over a warm blanket. It is left overnight in its cage for the peritoneal infection to evolve. A peritoneal inflammatory reaction starts and the bacterial peritonitis continues to progress, evolving to septic shock within a known period of time established for each pathogen; usually between 12 to 14 hours.

The following morning, when the rat is in incipient septic shock, the rat is assessed using the Modified Murine Sepsis Score (**Table 12**)[196] and, if the score was below 2.5, the second surgery under general inhaled anesthesia was undertaken; cannulating the carotid artery and the jugular vein and performing a tracheostomy. After this surgery was completed, the rat was connected to mechanical ventilation and an invasive blood pressure monitoring until the development of septic shock.

Once the rat is in shock, our intervention was administered and then serial blood samples were taken until the experiment was completed and euthanasia was performed with an overdose of the general anesthetic. Then an autopsy was performed, where the peritoneal fluid, spleen, kidney, liver and lungs of the rat were retrieved for bacterial culture.

An outline of the experimental procedure is in **Table 8**. The following sections will describe all procedures used.

Table 8. Outline of an experiment over time

Day 1 **22:00**	• Infection: surgical implantation of the capsules
Over night	• Evolution of infection
Day 2 **8:00**	• Surgery: Intubation and cannulations (carotid artery and jugular vein). • Fluid resuscitation Baseline sample
10:00	• Septic shock • Administration of antibiotics iv and distribution 0h sample
	• Blood samples at different time points: 45', 1.5h, 3h, 6h, 9h
19:00	• Euthanasia and autopsy: Collection of organs and peritoneal fluid for culture

3.4.6 Infection

3.4.6.1 Induction, preparation, monitoring and anesthetic maintenance.

To start, the animal was weighed and taken to the procedure room, where it was anesthetized using an anesthesia box with 5% isofluorane. Once asleep the rat's hair around the neck and the abdominal left lower quadrant was shaved using an electric hair clipper. After being placed on the warming blanket, the anesthesia mask was placed over the rat's mouth and nose, and continuous inhaled isofluorane at 1-3% was applied throughout the procedure to get the optimal plane of sedation. During the surgery the depth of the anesthetic plane was confirmed by regularly checking for a pedal reflex.

The rat was then non-invasively monitored. First, the rectal thermometer (Kent Scientific Co., RoVent Physio suite, USA) was placed; then the blood pressure was monitored intermittently (every 3 minutes) with a non-invasive tail-cuff (Kent Scientific Co., CODA tail cuff, USA). The SpO2 was constantly monitored using an oximeter paw/foot sensor (STARR Scientific Corp., MouseOx, USA). Eye ointment was applied to protect the rat's corneas from drying during the experiment. All vital signs were recorded every five minutes in the electronic lab record sheet.

In order to control postoperative pain, a dose of buprenorphine (0.03 mg/kg in 0.75 ml of NS) was administered subcutaneously.

3.4.6.2 Capsule implant

After disinfection of the abdominal surgical site using iodine and 70% ethanol, a 0.5 cm incision in the skin and the abdominal muscle was performed using sterile scissors. Once the peritoneum was open, the two gelatin capsules were introduced with forceps and 2 mL of NS were instilled in the peritoneum. The abdomen was then closed in two planes using a 3 - 0 silk suture in discontinuous stiches and in order to reduce the local discomfort of the surgical site, a dose of local anesthetic with bupivacaine (0.2mg/kg in 0.5 ml of NS) was intradermally injected.

3.4.6.3 Anesthetic recovery

Once the surgery was completed, the rat recovered with oxygen over the warm blanket until awake and active. Then it was placed back in the cage with easy access to water and food *ad libitum*.

3.4.7 Evolution to septic shock

Overnight, the gelatine capsule would start dissolving, releasing its content into the peritoneum. Alpha cellulose, major dietary component of the rat's diet, and the fibrinogen-thrombin clot acted as foreign bodies, triggering an inflammatory reaction, and enabling the bacteria to persist and replicate. Generating over several hours a marked purulent peritoneal inflammation and infection. The bacterial peritonitis caused translocation of bacteria to the bloodstream. Over time, the peritoneal sepsis evolved to septic shock, causing persistent hypotension.

Depending on the bacterial pathogen and the inoculum, the time to septic shock varied from 12 to 14 hours (see standardization results per microorganism in **section 3.5.1**).

Approximately two hours before the rat is in septic shock (10-12 hours post infection), the rat was assessed using the Modified Murine Sepsis Score (appendix **Table 12**).[196] If the rat was found to have an average score above 2.5 the rat was euthanized for animal wellbeing and ethical considerations. Otherwise, the experiment proceeded.

3.4.7.1 Standardization of septic shock in each microorganism

The purpose of the standardization experiments was to establish a bacteremic model of peritonitis septic shock in Sprague-Dawley rats using four different bacterial strains. The model was based on previous experiments in mice and rats performed by Dr. Kumar's lab.[27,191]

One to two animals were used per group to standardize the model. Initially, one sham capsule and one capsule with inoculum were implanted in the first animal, following the previously described protocol (section **3.4.6**). The rats were closely followed, and assessed for animal distress using the Modified Murine Sepsis Scale[196] every hour after the initial eight hours post infection. Hourly blood samples of approximately 200 µL were drawn from the tail vein of the rat, for quantitative blood cultures.

If the first animal did not show bacteremia or did not reach septic shock within 18 hours, the standardization was repeated with a second animal. This time two capsules, both with bacterial inoculum, were used and repeated the same procedure. If the second animal failed to reach septic shock or bacteremia, new capsules with a higher inoculum were made and repeated the process.

Whenever a new batch of capsules was made, this standardization process was undertaken in one animal, to make sure that the capsules met the standards of the previous batch.

In order to reduce the number of animals, some of these standardization animals were used as training animals, in which case the blood samples were drawn from the central line instead of the tail vein.

3.4.8 Non-recovery surgery

3.4.8.1 Induction and monitoring

The rats were re-anesthetized using the anesthesia box with 5% isofluorane. Once unconscious, the rats were monitored non-invasively as described in **section 3.4.6.1** and all vital signs and isofluorane dose were recorded every 15 minutes in our electronic experimental record sheet. Volatile general anesthesia was used throughout the case with inhaled isofluorane ranging from 1-3%; a sustained and appropriate anesthetic plane was confirmed by checking the rat's pedal reflex every 15 minutes.

3.4.8.2 Jugular vein and arterial cannulation

After cleaning the neck with iodine and 70% ethanol, a 1.5cm midline incision was performed from mid-neck until the sternal notch. Next, a blunt dissection of the tissues, carefully exposed the trachea, the left carotid artery and jugular vein.

The vein was cannulated and then the artery with 3 French polyurethane catheters. In order to perform the cannulations, the vessels were distally ligated with a 4-0 silk suture. A second ligature was placed 0.5 cm proximal to the heart, but it was not initially tightened. The carotid blood flow was stopped by carefully

placing the forceps and applying tension to the vessel. An incision on the wall of the vessels was made using micro-scissors and the 3 French polyurethane catheter was advanced. The second ligature was then tightened, and the blood flow was restored once the forceps was repositioned. The positioning of the venous cannulation was then tested by withdrawing blood and flushing with NS. The arterial line was connected to a blood pressure transducer, which was connected to the pre-calibrated blood pressure analyzer (Digimed, HPA 400, Micro-Med Inc., USA). The arterial cannulation was confirmed by checking the waveform on the blood pressure analyzer. Both the arterial and venous catheters were heparinized using a concentration of unfractionated heparin of 100 IU/ml.

The central venous catheter was used to administer antibiotics, fluid and norepinephrine, and to collect all the blood samples. The arterial line was used to monitor the blood pressure and hemodynamic parameters throughout the experiment. All the hemodynamic parameters were recorded every minute using the software provided by the manufacturers of the blood pressure analyzer (Digimed, DMSI-400/4, Version 3.4 for Windows, Micro-Med Inc., USA).

3.4.8.3 Tracheostomy

After the arterial and vein cannulations, the tracheostomy was undertaken. An incision between the 5^{th} and 6^{th} tracheal rings was made with scissors, and a 14-gauge polyethylene catheter was carefully placed 1-2 cm in the trachea of the rat. The catheter was secured using 4-0 silk sutures. Immediately after, the rat was connected to mechanical ventilation using a small animal ventilator (Kent Scientific

Co., RoVent Physio Suite, USA). See mechanical ventilation **section 3.4.9.3** for ventilator settings. The oxygen saturation was monitored throughout the experiment by using a SpO_2 foot sensor (STARR Scientific Corp., MouseOx, USA) along with the software of the manufacturer to archive each experiment with readings every minute.

3.4.9 Supportive care

3.4.9.1 General anesthesia

Isoflurane inhaled anesthesia was administered via face-mask at the beginning of the surgery and via endotracheal tube once tracheotomized. The minimum amount of anesthetic was used to keep an appropriate sedation of the animal; with isoflurane doses ranging from 1-3%. The anesthetic plane was assessed by checking the pedal reflex every 15 minutes.

3.4.9.2 Temperature control

Using a rectal probe and a warming blanket, the rat's body temperature was maintained around 37 ± 0.2^{o}C. The temperature probe and the warming blanket were regulated by the Physio Suite ventilator software (Kent Scientific Co., USA).

3.4.9.3 Mechanical ventilation

The rats were connected to mechanical ventilation, using a small animal ventilator (Kent Scientific Co., RoVent Physio Suite, USA). The mode of mechanical ventilation chosen was volume assist control, following a lung

protection ventilatory strategy to avoid ventilatory induced lung injury. The tidal volume was 6ml/kg, the respiratory rate 80 breaths per minute, and the positive end expiratory pressure 5 cmH_2O. The ventilator was connected to a source of oxygen at 1 lpm and the anesthesia machine with the isoflurane vaporizer.

3.4.9.4 Fluid resuscitation

During the experiment the rat was resuscitated with a total of 3 mL of NS intravenously (iv), one milliliter after each surgical procedure (central line, arterial cannulation and tracheostomy); which represents an initial resuscitation of 10mL/Kg. Every time a blood sample of 0.3 mL was drawn, it was replaced it with 1mL of NS iv. There were continuous iv fluids running at a rate of 1-1.2 mL/h. If the hemodynamic goals were not met, up to three activations of 0.5 mL of NS were available as fluid boluses.

The urine output was monitored and the urine was collected at every blood draw time point by gently squeezing the bladder. Total fluids in and out were recorded, and an accurate fluid balance was performed at the end of each experiment.

3.4.9.5 Hemodynamic support

Heart rate, systolic, diastolic and mean arterial pressures (MAP) were measured invasively (Digimed, HPA 400) and recorded in our electronic experimental record every 15 minutes. The Digimed software recorded all the hemodynamic parameters every minute.

The hemodynamic goals were a MAP of 70 mmHg or greater and a systolic blood pressure greater than 100 mmHg.

In order to achieve these goals, we had up to three activations of 0.5 mL of NS iv, as fluid boluses, and a norepinephrine infusion that was titrated as needed. The total amount of fluid and the norepinephrine required was recorded for each experiment.

3.4.10 Interventions

There were a total of 5 treatment arms, experimenting with the sequence of antibiotics, and 2 control groups. Depending on the pathogen, the second antibiotic could be an aminoglycoside or a fluoroquinolone (see antibiotic **section 3.4.2**).

① β-lactam monotherapy

② Second antibiotic monotherapy

③ Simultaneous administration

④ β-lactam followed (20 min) by 2nd antibiotic

⑤ 2nd antibiotic followed (20 min) by β-lactam

⑥ Untreated controls (UTC)

⑦ Sham controls

To maintain the total amount of saline given with the intervention at a fixed volume of 1 ml for all animals, the concentration of the antibiotics was adjusted, even if receiving two antibiotics. In the UTC and sham control groups we used NS instead of antibiotics.

3.4.11 Blood samples for quantitative culture and cytokine assay

Blood and urine samples were collected at seven time points (eight in the *E. coli* high antibiotic dose cohort): Baseline (after fluid resuscitation), 0h (when septic shock was stablished, and after our intervention, once antibiotics were distributed), 45 minutes, 1.5h, 3h, 4.5h, and 6h in all animals; a 9h sample was collected in the *E. coli* high dose of antibiotic, *E. coli* UTC cohorts and sham rats. All blood samples were 300 µl, and the animal was then resuscitated with 1 ml of NS iv.

Immediately after collection, the blood was centrifuged at 10,000 rpm for ten minutes (Centrifuge 5418, Eppendorf Scientific Inc, Germany); the serum (with antibiotic) was removed and frozen at -80^{o}C for later cytokine and lactate analysis. The same amount of NS was then added to resuspend the pellet.

Subsequently, serial dilution and determination of bacterial counts in duplicates was undertaken and the samples were plated on MHA or BA plates as appropriate, depending on the organism, and the plates were incubated overnight at 37^{o}C and with 5% CO_2.

The bacterial count reading was taken by two independent readers. The limit of detection of bacterial counts from blood was 100 CFU/mL of blood.

3.4.12 Euthanasia, autopsy and tissue samples

After the end of the non-recovery experiment, all the animals were euthanized using an overdose of isoflurane and exsanguination. The protocol was approved by the animal care committee and veterinary services at University of Manitoba.

Once the animals were euthanized, an autopsy was conducted to collect peritoneal fluid and tissue samples. Using a sterile technique, the peritoneum of the animal was opened and 1ml of peritoneal fluid was collected for culture. Then the spleen, kidneys, liver and lungs were retrieved and washed thrice in NS. A portion of all the organs was placed in 10% formalin. The other half was homogenized with 1ml of NS.

The homogenates and the peritoneal fluid were serially diluted and plated in duplicate on MHA or BA plates, depending on the microorganism, for quantitative cultures. The plates were incubated overnight at 37^{o}C with 5% CO_2. The bacterial count reading was taken by two independent readers. The limit of detection of bacterial counts from tissues was 100 CFU/ml of homogenate.

3.4.13 Cytokine assay

Millipore's rat cytokine magnetic bead panel 6-plex kit was used to detect plasma levels of the following cytokines: IL-1 beta, IL-2, IL-6, RKC (rodents' functional equivalent for human IL 8), IL-10, TNF alpha.

All samples and controls were processed in duplicates following the manufactures recommendations.

3.4.14 Sample size calculation

The sample size calculation was based on the bacterial clearance seen in previous rat data from Dr. Kumar's lab,[197] where the standard deviation of bacterial clearance was 0.8 $\log_{10}$ CFU/ml. A 1.6 $\log_{10}$ CFU/ml reduction of blood bacterial counts was expected to be detected. For a 5% α error and a power (1−β) of 90%, the constant C will be 10.51. Using **Equation 1,** the sample size required is 6 rats per arm.

Equation 1 $$n = 1 + 2C\left(\frac{s}{d}\right)^2$$

N is the sample size; C a constant dependent on the value of α and β errors selected; s is the standard deviation of the population; and d the difference the investigator wishes to detect.

Since septic shock is a severe pathology with a high mortality rate, we added one more animal in the UTC cohort for a total of seven UTC animals per microorganism. The total number of animals per bacterial strain was 37. The sham control group was six rats.

3.4.15 Statistical analysis

The primary outcome was bacterial clearance from the blood over time, expressed as a logarithm in base ten of the colony forming units per milliliter (log_{10} CFU/ml) of blood. The logarithmic transformation is a routine variance-stabilizing and normalizing transformation used to reduce both unequal variance and skewness seen in count data. We also subtracted the baseline counts to all blood counts measurements to control for bacterial load at baseline. To account for the within-animal correlations due to repeated measures, we used a linear mixed-effect model with animal-specific random intercept term. The hypotheses regarding coincident trajectories over time were tested using the likelihood ratio test. The end of experiment blood, organ and peritoneal counts were compared using conventional ANOVA. We controlled for bacterial load at baseline and time to antibiotics.

For the cytokine analysis we replaced values reported as below limit of quantification with one-half of the reported lower level of detection reflected in the outcome data of each plate. And replaced values reported as above the upper limit of quantification with that value. We used the logarithm in base 10 transformation to stabilize the variance and reduce skewness. We averaged the duplicate measurements of the logarithm in base 10 concentration values. We used a similar linear mixed-effect model for repeated measures and compared cytokine trajectories between antibiotic treatments using likelihood ratio tests and compared cytokine levels at the beginning and the end of the experiments using conventional

ANOVA. We controlled for rat-to-rat variation by adjusting for baseline cytokine level in the end-of-experiment analysis.

Basic summary statistics reported include the mean and standard deviation for normally distributed variables and median and interquartile range for non-normally distributed variables. Given the multiple hypothesis, we decide to adjust the conventional alpha = 0.05 level, after applying the Bonferroni-correction, to 0.025 to be considered significant. The secondary and other analyses were performed at the alpha = 0.05 level, without adjustment for multiple inference.

Statistical analyses were performed using R (v 4.1.1), and the R packages nlme (version 3.1) for the modeling and ggplot2 (v. 3.3.5) for the figures.

3.5 Results

3.5.1 Standardization of the rat model

3.5.1.1 Escherichia coli

Based on Dr. Kumar's lab previous experience with this model,[191] a microbial load of 1-2 x10^6 CFU caused septic shock in a 300 ±10g Sprague-Dawley rat in 12 to 13 hours. The resulting bacteremia was consistently between 10^6-10^8 CFU/ml of blood at the time of shock.

3.5.1.2 Streptococcus pneumoniae

For *S. pneumoniae*, a bacteremic model of septic shock was achieved with a microbial load of 2 x10^6 CFU; time to septic shock was 12 to 15 hours and the bacteremia achieved at that time was 10^5-10^7 CFU/ml of blood.

To fulfill all experiments with *S. pneumoniae*, two different batches of capsules were used, there was a discrepancy in the time to septic shock between groups, the first batch required 12-13 hours and the second batch 14-15 hours.

3.5.1.3 Staphylococcus aureus

After using 12 animals, and five different capsule batches with increasing bacterial load, up to an inoculum of 10^{11} CFU per capsule, there was no success in generating a bacteremic model of septic shock with *S. aureus*. The rats would get septic, although not in shock, and the rats were not bacteremic. The capsules were repeated, making sure that *S. aureus* was in the logarithmic growth phase when used to make the inoculum. Despite the efforts there was no success in

generating a bacteremic septic shock model using a pan-sensitive *S. aureus.* This model was not pursued.

3.5.1.4 Pseudomonas aeruginosa

A bacteremic septic shock model was obtained after 12-13 hours of infection with a microbial load of 1-2 x 10^9 CFU. The peritoneal fluid counts maintained around 10^6 CFU/ml, and the organ bacterial counts were between 10^4-10^7 CFU/ml of tissue homogenate. However, the bacteremia was only 10^3-10^4 CFU/ml of blood in UTC and did not increase over time.

The eight animals used were in profound septic shock and unstable, but not bacteremic enough for us to be able to see the 1.6 log difference between sequences of antibiotics after treatment, when our detection threshold is 100 CFU/ml. This model was not pursued as recommendation of my committee.

3.5.1.5 Sham controls

Eight sham controls were performed, where two sterile capsules were implanted in the peritoneum of the rats. Two of these animals were euthanized at the beginning of the experiment due to equipment malfunctioning. One rat had a hemorrhagic shock, with hemoperitoneum likely secondary to bleeding from the capsule implant surgical site. The other five rats had no bacterial counts in any of the blood samples, neither in the peritoneum or any organ and will be included in the analysis.

3.5.2 Escherichia coli results

Of the 67 rats included in the *E. coli* arm, 52% were females, and had a mean weight of 302.35 g (SD 11.96 g). Time to septic shock was on average 12.01 hours (SD 0.59 h), 85% of the animals were febrile, see **Table 9.**

The *E. coli* peritonitis septic shock model proved to be very stable, with a mean bacteremia load at baseline, of 6.41 log_{10} CFU/ml (SD 0.48 log_{10} CFU/ml, n=67), despite using different batches of *E. coli* capsules, see **Table 9.**

Table 9. E. coli experiments animal baseline characteristics by antibiotic cohort

	N	Sex F N (%)	Weight g (SD)	Fever %	Initial HR bpm (SD)	Time to septic shock h (SD)	Time to antibiotics h (SD)	BL blood bacterial load log_{10} CFU/ml (SD)	Early death N (%)
Total E. coli rats	67	35 (52 %)	302.35 (11.96)	85 %	373.7 (38.7)	12.01 (0.59)	12.18 (0.60)	6.41 (0.48)	19 (28 %)
• **UTC**	7	4 (57 %)	308.57 (11.41)	85 %	354.14 (58.96)	11.93 (0.60)	12.33 (0.70)	6.37 (0.61)	6 (85 %)
• **Normal antibiotic dosing**	30	14 (46 %)	303.57 (11.82)	96 %	376.97 (30.29)	12.16 (0.48)	12.28 (0.49)	6.48 (0.46)	5 (17 %)
• **High antibiotic dosing**	30	17 (56 %)	299.7 (11.86)	70 %	375.17 (40.78)	11.87 (0.67)	12.04 (0.68)	6.33 (0.48)	8 (27 %)

F, female; g, grams; SD, standard deviation; HR, heart rate; bpm, beats per minute; h, hours; BL, baseline; log_{10}, logarithm in base 10; CFU, colony forming units; ml, milliliter; UTC, untreated controls.

Two different doses of antimicrobials were used; high and normal septic shock antibiotic dosing (see **sections 3.5.2.1** and **3.5.2.2**). 30 animals were included in each antibiotic cohort; six animals per treatment arm. The antibiotics dosage was based on human septic shock guideline recommendations and were

adapted to the rat metabolism in comparison with humans, see **section 3.4.2**. The seven UTC animals served as comparator for both cohorts of antibiotic dosing. As expected, the proportion of early death in the UTC *E. coli* infected animals was higher than in the treatment cohorts.

3.5.2.1 Normal sepsis antibiotic dose

The same protocol that was previously described in **section 3.4.5** was followed, with the following major differences:

3.5.2.1.1 Material and Methods – differences from main protocol

Capsules: two different batches of *E. coli* capsules, made in different days, were used to complete all experiments. The two batches behaved similarly and caused septic shock around 12 hours post infection. There were no differences in time to shock or baseline blood bacterial counts between the different batches.

Antibiotics: 60mg/kg of cefotaxime and 10mg/kg of gentamicin.

Duration of the experiment: six hours after administration of the antibiotics.

3.5.2.1.2 Results for normal sepsis antibiotic dosing cohort

3.5.2.1.2.1 Baseline characteristics

Of the 30 rats included in the *E. coli* normal septic shock dosing arm, 46% were females, and had a mean weight of 303.56 g (SD 11.82 g), see **Table 9.** Time to septic shock was 12.16 hours (SD 0.47 h), 96% of the animals were febrile. See the baseline characteristics described in **Table 10.**

Table 10 E. coli experiments baseline characteristics by treatment arm

	N	Sex N (%) F	Weight g (SD)	Fever %	Initial HR bpm (SD)	T -> shock h (SD)	T -> Abx h (SD)	BL blood bacterial load log_{10} CFU/ml (SD)	Early death
Total E. coli rats	67	35 (52 %)	302.35 (11.96)	85 %	373.7 (38.7)	12.01 (0.59)	12.18 (0.60)	6.41 (0.48)	19 (28 %)
• UTC	7	4 (57 %)	308.57 (11.41)	85 %	354.14 (58.96)	11.93 (0.60)	12.33 (0.70)	6.37 (0.61)	6 (85 %)
• Normal ABx dosing									
• Gentamicin	6	3 (50 %)	302.67 (17.77)	100%	402.83 (45.49)	12.39 (0.40)	12.53 (0.42)	6.43 (0.70)	0 (0 %)
• Cefotaxime	6	3 (50 %)	310.33 (5.20)	100%	365.83 (23.68)	12.33 (0.22)	12.45 (0.26)	6.48 (0.49)	2 (33 %)
• Simultaneous	6	3 (50 %)	302 (9.9)	100%	376.67 (33.25)	12.22 (0.48)	12.34 (0.47)	6.63 (0.34)	0 (0 %)
• Cef -> Gent	6	2 (33 %)	299 (14.38)	83%	373.33 (15.23)	11.75 (0.66)	11.88 (0.68)	6.46 (0.50)	1 (16 %)
• Gent -> Cef	6	3 (50 %)	303.83 (8.91)	100%	366.17 (15.74)	12.12 (0.37)	12.22 (0.36)	6.51 (0.44)	2 (33 %)
• High ABx dosing									
• Gentamicin	6	4 (83 %)	297.17 (13.79)	83%	370.67 (38.37)	11.89 (0.82)	12.08 (0.78)	6.54 (0.60)	3 (50 %)
• Cefotaxime	6	4 (83 %)	300 (14.79)	50%	352.33 (21.55)	11.93 (0.94)	12.22 (1.01)	6.18 (0.33)	2 (33 %)
• Simultaneous	6	2 (33 %)	293.5 (9.85)	83%	384 (65.01)	11.68 (0.24)	11.78 (0.25)	6.23 (0.39)	1 (16 %)
• Cef -> Gent	6	3 (50 %)	303.83 (8.28)	50%	375.17 (37.65)	11.93 (0.50)	12.04 (0.49)	6.46 (0.45)	1 (16 %)
• Gent -> Cef	6	2 (33 %)	304 (12.03)	83%	393.67 (30.18)	11.94 (0.81)	12.08 (0.80)	6.21 (0.54)	1 (16 %)

N – number of animals; F – female; g, grams; SD – standard deviation; HR – heart rate; bpm – beats per minute; T, time; h, hours, ABx, antibiotics; BL, baseline; log_{10}, logarithm in base 10; CFU, colony forming units; ml, milliliter; UTC, untreated controls; Cef, cefotaxime; Gent, gentamicin.

3.5.2.1.2.2 Blood bacterial counts

The mean baseline bacteremia for the 37 animals included in the normal antibiotic dosing cohort was 6.48 log_{10} CFU/ml of blood (SD 0.46 log_{10} CFU/ml), see **Table 9** and **Table 10.**

The different interventions showed different blood bacterial counts over time, shown in **Figure 9.** All treatment arms significantly reduced the blood bacterial load over time ($p<0.001$) when compared to UTC; showing different trajectories (**Figure 9**).

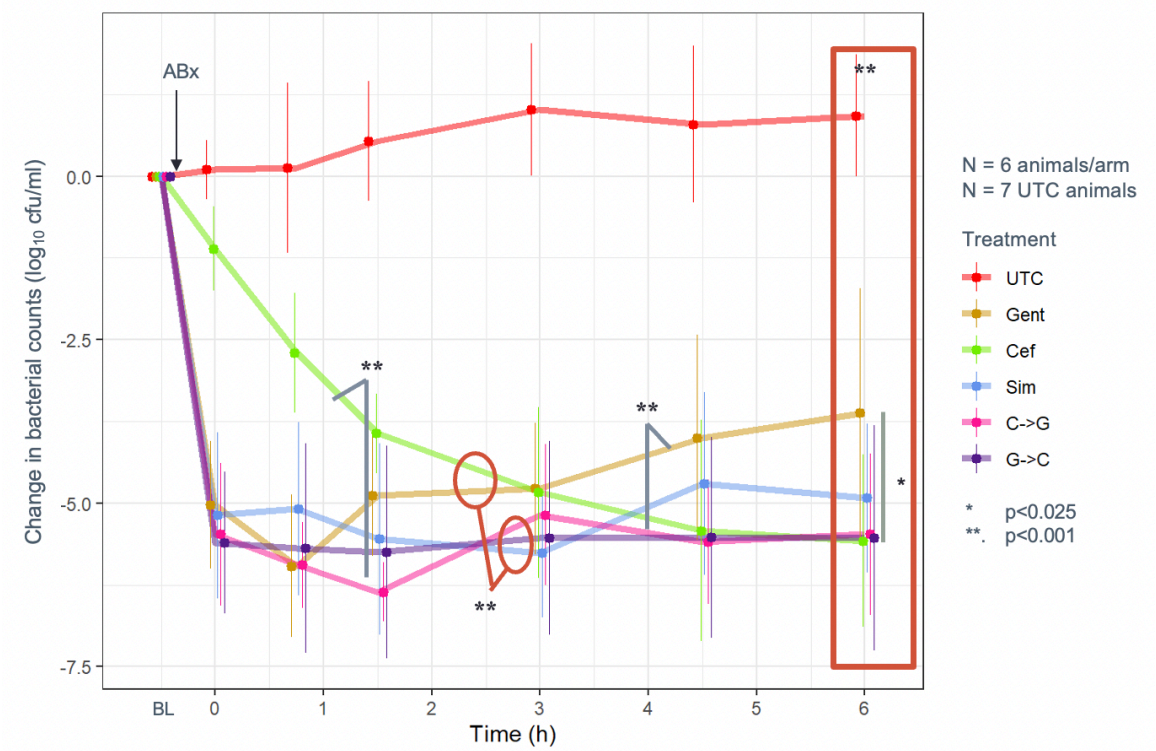
The UTC rats, as expected, showed an increased bacterial blood concentration over time. Of the initial seven UTC animals only three rats survived to hour six; their bacterial blood concentration at the end of the experiment had increased on average 0.93 log_{10} CFU/ml (SD 0.37 log_{10} CFU/ml). All antibiotic treatments had a significant bacterial load reduction when compared to UTC ($p<0.001$).

When comparing the different treatment arms among them, we didn't find statistical significant differences between the bacterial clearance trajectory of any of the three sequences of combination therapy ($p>0.025$ in all three comparisons). While the trajectories in blood bacterial clearance was statistically significant when comparing combination therapy and monotherapy ($p<0.001$), showing a synergistic effect of combination therapy, with faster initial blood bacterial clearance that maintained over time (**Figure 9**).

Cefotaxime monotherapy's bacterial clearance trajectory was significantly different from any other treatment arm ($p<0.001$ in all comparisons); showing a slower initial bacterial clearance with a steady decline (**Figure 9**). Gentamicin monotherapy's bacterial clearance over time was significantly different to all treatment arms ($p<0.001$), except for simultaneous combination therapy ($p=0.064$), they showed similar rapid initial bacterial clearance with regrowth over time (**Figure 9**).

When we compared the bacterial counts at the end of the experiment using ANOVA at six hours, we found no differences between treatment arms, except for gentamicin monotherapy when compared with C->G ($p=0.025$); this analysis almost reached significance when comparing gentamicin monotherapy with G->C ($p=0.028$).

Figure 9. Change in *E. coli* blood bacterial counts over time using normal septic shock antibiotic dosing.



*ABx – antibiotic administration, N – number of animals, UTC – Untreated controls, Gent – gentamicin, Cef – cefotaxime, Sim – simultaneous, C->G – cefotaxime then gentamicin, G->C – gentamicin then cefotaxime, log_{10} – logarithm in base ten, cfu – colony forming units, mL – millilitre, BL – baseline, h – hour, ** – statistically significant p<0.001, * – statistically significant p<0.025.*

This figure represents quantitative change blood bacterial clearance over time with the different antimicrobial administration strategies. There are six animals per arm, except for UTC, where there are seven animals. We represent the average bacterial load change from baseline and the 95% CI (whiskers) of the blood bacterial count reduction at each timepoint.

Cefotaxime monotherapy shows a slow clearance of the bacterial load in the blood over time, while gentamicin shows a post antibiotic effect, with regrowth after 1.5 hours. Of the three combination sequences, they all behave initially as gentamicin monotherapy, with a fast initial decline in bacterial counts, that maintains over time, meeting the clearance of cefotaxime monotherapy by 4.5 hours. The simultaneous dosing arm experiences regrowth by 4.5 hours.

We performed trajectory analysis to compare the different treatment strategies, using C->G as reference. There were no differences between combination sequences ($p>0.025$), although each monotherapy was different to each combination strategy ($p<0.001$) except simultaneous dosing and gentamicin that showed no differences ($p=0.64$). Combination therapy showed a significantly different trajectory from monotherapy ($p<0.001$) (indicated by red circles in the figure).

The ANOVA at experiment end (red rectangle) showed statistically significant differences for UTC ($p<0.001$) and gentamicin monotherapy ($p<0.025$). There were no significant differences between combination sequences.

3.5.2.1.2.3 *P*eritoneal fluid bacterial counts

All treatment arms showed similar bacterial load in the peritoneum (mean 5.9 log_{10} CFU/ml, SD 0.69 log_{10} CFU/ml, n=30), this was statistically significant different ($p<0.001$) when compared to UTC (mean 9.40 log_{10} CFU/ml, SD 0.55 log_{10} CFU/ml, n=7), see **Figure 10 A**.

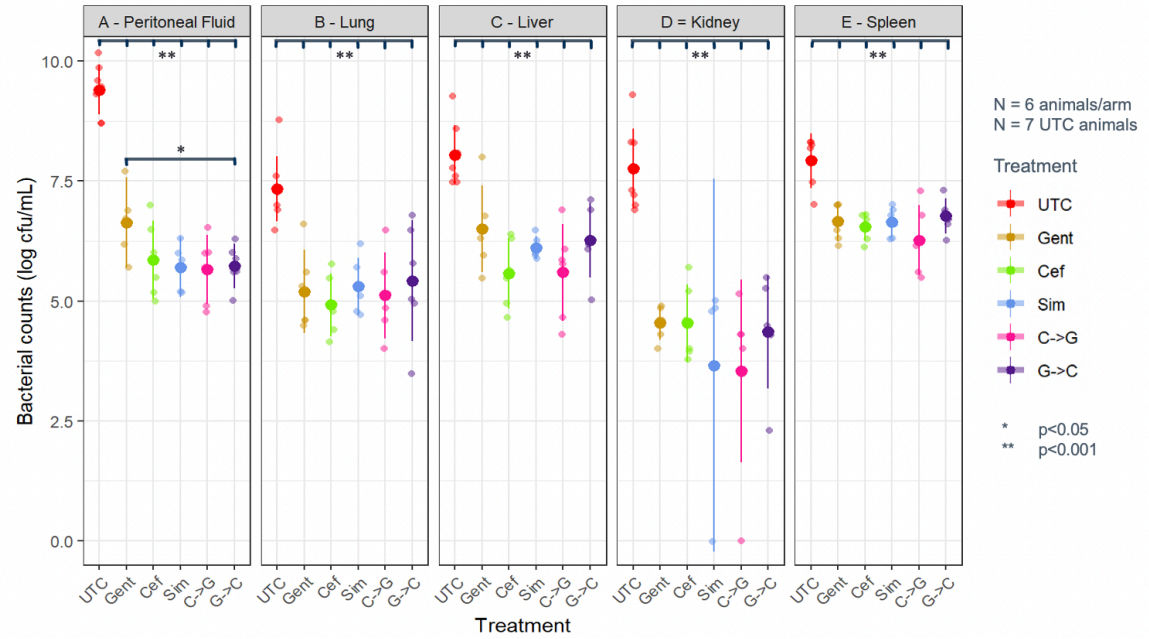
There were no statistically significant differences between combination sequences regarding bacterial clearance in the peritoneal fluid (**Figure 10 A**).

Gentamicin monotherapy had slightly less inoculum clearance at the target infection site, reaching statistical significance ($p=0.02$) when compared to C->G combination strategy; and almost reaching statistical significance when compared to the other two combination strategies ($p=0.031$ G vs. G->C; and $p=0.033$ G vs. Sim), see **Figure 10 A**.

3.5.2.1.2.4 Organ bacterial counts

Similarly, the organ bacterial counts were significantly different between treated and untreated animals ($p<0.001$), but there were no statistically significant differences between treatment arms in any of the organs, **Figure 10 B-E.**

Figure 10. E. coli peritoneal bacterial counts and organ counts using normal septic shock antibiotic dosing.



*N – number of animals, UTC – untreated controls, Gent – gentamicin, Cef – cefotaxime, Sim – simultaneous antibiotic administration, C->G – cefotaxime then gentamicin administration, G->C – gentamicin then cefotaxime administration, log – logarithm in base ten, cfu – colony forming units, mL – milliliter; ** - Statistical significance p<0.001, * - statistical significance p<0.025*

In this graph we represent the peritoneal fluid bacterial and the organ bacterial counts at the end of the experiment in E. coli septic shock rats treated with normal antibiotic septic shock dosing. Each animal is represented by a single dot. We also represent the mean and 95% CI of the bacterial load in the peritoneal fluid and the organs for each treatment arm.

All UTC bacterial counts were significantly higher than treated animals (p<0.001). There were no differences between treatment arms in organ bacterial counts to the p<0.025 level of significance. The only comparison that reached significance was the counts in peritoneal fluid when comparing C->G with gentamicin monotherapy (p=0.020).

3.5.2.1.3 Conclusion

In our *E. coli* peritonitis septic shock rat model treated with normal septic shock antibiotic dosing, we found no significant differences in bacterial kill in the peritoneal fluid or the organ counts between the three antibiotic combination sequences. Altering the antibiotic sequence had no significant impact in bacterial clearance from the blood over time, neither at the end of experiment (6 hours).

Combination therapy resulted in significantly different blood bacterial kill over time than monotherapy ($p<0.001$). Showing synergistic effects of both antibiotics in combination, with a rapid initial bacterial kill and a maintained bacterial clearance from the blood over time. Staggered dosing strategies had more stable bacterial clearance at the end of the experiment, while simultaneous dosing showed a slight regrowth after 4.5 hours, this difference was not statistically significant.

All therapies showed statistically significant reduction of bacterial load in blood, organs and peritoneal fluid in our *in vivo* model of *E. coli peritonitis* septic shock when compared to untreated controls ($p<0.001$).

3.5.2.2 High septic shock antibiotic dose

The same protocol that was previously described in **section 3.4.5** was followed, with the following major differences:

3.5.2.2.1 Material and Methods – differences from main protocol

Microorganism: *E. coli* Bort.

Capsules: one batch of capsules was used for all experiments.

Antibiotic dose: 120mg/kg of cefotaxime and 20mg/kg of gentamicin.

Duration of the experiments: nine hours after the distribution of antibiotics.

3.5.2.2.2 Results for high septic shock antibiotic dosing cohort

3.5.2.2.2.1 Baseline characteristics

Of the 30 rats included in the *E. coli* high dose of antibiotics cohort, 56% were females, and had a mean weight of 299.7 g (SD 11.86 g). Time to septic shock was 11.87 hours (SD 0.67 h), 70% of the animals were febrile. See **Table 9** and **Table 10**.

3.5.2.2.2.2 Blood bacterial counts

The mean baseline bacteremia for the 37 animals included in the high antibiotic dosing cohort was 6.33 $\log_{10}$ CFU/ml of blood (SD 0.48 $\log_{10}$ CFU/ml), see **Table 9** and **Table 10**.

All treatment arms showed a reduction of bacterial counts over time, while UTC, as expected, showed an increased bacterial blood load over time (**Figure 11).** Only two UTC rats survived to hour nine, their bacterial blood concentration

at nine hours had increased on average 1.31 $\log_{10}$ CFU/ml (SD 0.01 $\log_{10}$ CFU/ml). All treatment arms showed a significantly different blood bacterial count trajectory during the nine-hour experiment, as well as a significant blood bacterial load reduction at the end of the experiment when compared to UTC ($p<0.001$).

The different interventions showed different blood bacterial count trajectories over time, shown in **Figure 11**. All combination arms showed a rapid initial bacterial kill, similar to gentamicin monotherapy, with a maintained bacterial clearance over time, except for the simultaneous sequence, where we saw a slight late regrowth after hour six. When comparing the different treatment arms, the three combination strategies showed not to have statistically significant differences in trajectory over the nine-hour experiment ($p>0.025$ in all three comparisons).

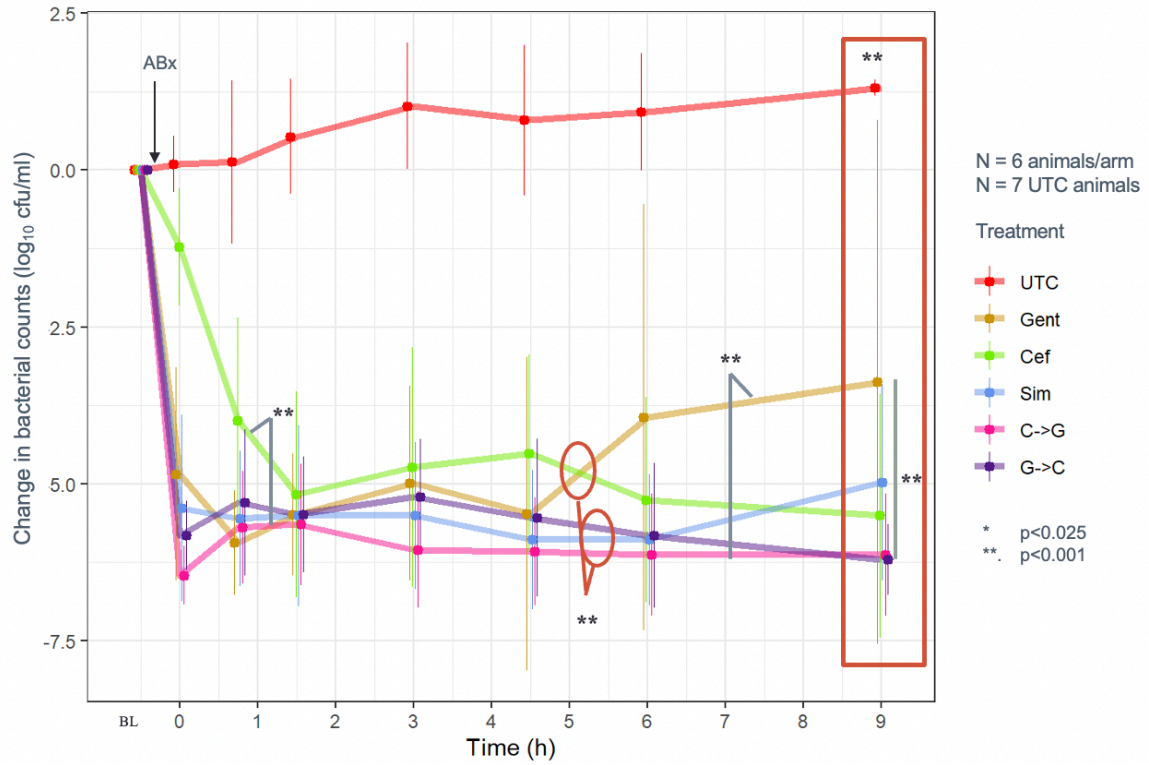
Monotherapy treatments' aggregate trajectory was significantly different when compared to combination therapy ($p<0.001$). Cefotaxime monotherapy's trajectory had a slower initial bacterial clearance with a sustained bacterial clearance after 1.5 hours, that showed significant trajectory differences when compared to any of the combination sequences ($p<0.001$ in all three comparisons). While gentamicin monotherapy's trajectory had a rapid initial bacterial decline with regrowth after 4.5 hours; and also showed statistical differences when compared to the staggered combination strategies ($p<0.001$ for both comparisons) as well as the simultaneous dosing arm ($p=0.017$), see **Figure 11.**

At experiment end (nine hours), we found statistically significant differences between gentamicin monotherapy and staggered antibiotic administration strategies ($p=0.007$ for G vs C->G and $p=0.005$ for G vs G->C). No other analysis

showed differences to the level of $p<0.025$. See **Figure 11** and the appendix for the statistical tables.

When reviewing the data for high and normal antibiotic dosing (**Figure 9** and **Figure 11**), we could see a difference in blood clearance trajectory between the monotherapy arms. Observing a faster initial bacterial clearance for cefotaxime monotherapy high dose and delayed regrowth in the gentamicin high dose case, when compared to their normal dose counterparts. As this was not identified as our a priori primary or secondary outcomes, it just represents a finding that may be worth analyzing in a future project or as a post hoc analysis to generate hypotheses.

Figure 11. Change in *E. coli* blood bacterial counts over time using high septic shock antibiotic dosing.



*ABx – antibiotic, N – number of animals, UTC – untreated controls, Gent – gentamicin, Cef – cefotaxime, Sim – simultaneous dosing, C->G – cefotaxime then gentamicin, G->C – gentamicin then cefotaxime, log – logarithm in base ten, cfu – colony forming units, mL – milliliter, h – hours, ** – statistically significant p<0.001, * – statistically significant p<0.025.*

This figure represents quantitative blood bacterial clearance over time with the different antimicrobial therapeutic strategies. There are six animals per treatment arm, and seven UTC. We represent the average bacterial load change from baseline and the 95% CI (whiskers) of the reduction in blood bacterial counts at each timepoint.

Cefotaxime monotherapy showed a slower initial clearance of the bacterial load in the blood. Gentamicin monotherapy showed a post antibiotic effect, with regrowth after 4.5 hours. Of the three combination sequences, they all behaved initially as gentamicin monotherapy, with a fast initial decline in bacterial counts, that maintains over time meeting cefotaxime monotherapy clearance by 1.5h. The simultaneous dosing arm experiences regrowth by 6 hours.

We performed trajectory analysis to compare the different treatment strategies, using C->G as reference for the model. We found no differences between the bacterial clearance trajectories of the three combination sequences ($p>0.025$). Both monotherapy treatment trajectories were significantly different from the three combination administration strategies ($p<0.001$) when compared individually. Bacterial clearance trajectories were statistically different when comparing monotherapy to combination therapy ($p<0.001$) (indicated by red circles in the figure).

At the end of experiment (red box) gentamicin monotherapy blood bacterial counts were statistically higher than either of the staggered combination strategies ($p<0.025$ in both cases).

3.5.2.2.2.3 Peritoneal fluid bacterial counts

All treatment arms showed similar bacterial load in the peritoneum (mean 5.37 log_{10} CFU/ml, SD 1.11 log_{10} CFU/ml, n=30), this was statistically significantly different ($p<0.001$) when compared to UTC (mean 9.40 log_{10} CFU/ml, SD 0.55 log_{10} CFU/ml, n=7), see **Figure 12 A**.

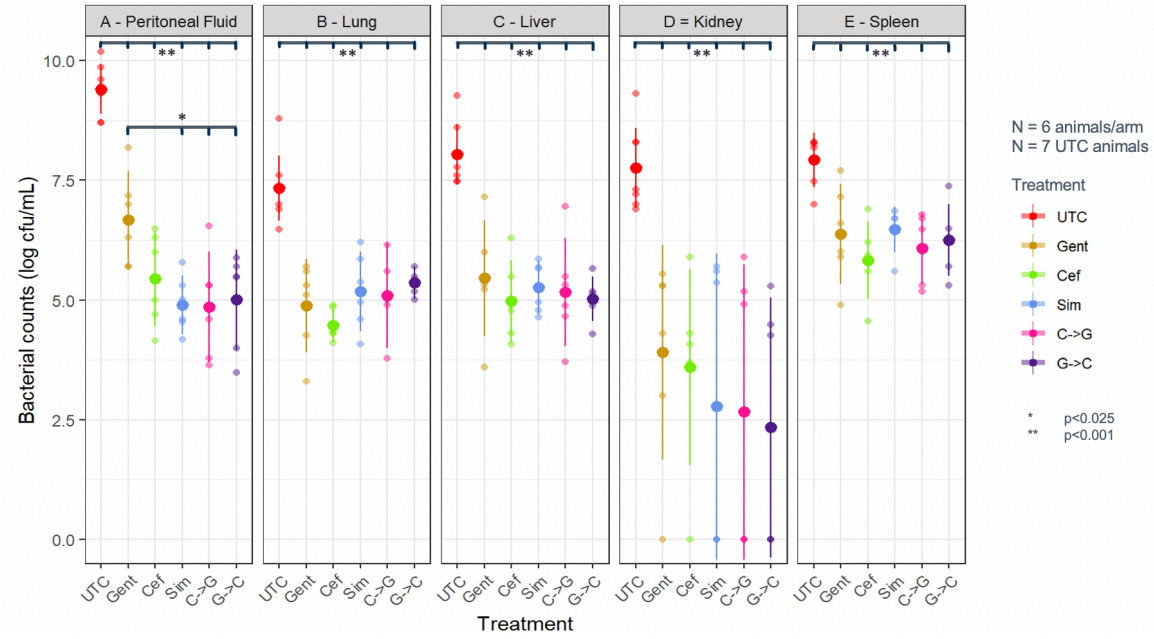
There were no statistically significant differences between combination sequences regarding bacterial clearance in the peritoneal fluid, $p>0.025$ (**Figure 12 A**).

Gentamicin monotherapy had lower inoculum clearance at the target infection site, reaching statistical significance when compared to either of the three combination strategies ($p<0.005$ for all three comparisons); likely driving the differences between combination therapy and monotherapy ($p<0.003$), see **Figure 12 A**.

3.5.2.2.2.4 Organ bacterial counts

Similarly, the organ bacterial counts were significantly different between treated and untreated animals ($p<0.001$), but there were no statistically significant differences between treatment arms in any of the organs, **Figure 12 B-E.**

Figure 12. E. coli peritoneal and organ bacterial counts using high septic shock antibiotic dosing.



*N – number of animals, UTC – Untreated controls, Gent – gentamicin, Cef – cefotaxime, Sim – simultaneous antibiotic dosing, C->G – cefotaxime then gentamicin, G->C – gentamicin then cefotaxime, log – logarithm in base ten, cfu – colony forming units, mL – milliliter, ** – statistically significant p < 0.001, * – statistically significant p < 0.025.*

In this graph we represent the peritoneal fluid and the organ bacterial counts at the end of the experiment in E. coli septic shock rats treated with high antibiotic septic shock dosing. Each animal is represented by a single dot. We also represent the mean and the 95% CI of the bacterial load of the peritoneal fluid and the organs for each treatment arm.

All UTC bacterial counts were significantly higher than in treated animals (p<0.001). There were no differences between treatment arms in organ bacterial counts to the p<0.025 level of significance. The only significant findings between treatments were the peritoneal fluid counts of gentamicin monotherapy when compared to either of the three antibiotic combination strategies (p<0.005 for all three comparisons).

3.5.2.2.3 Conclusion

Combination therapy of the first dose of antibiotics in our *E. coli* rat model of peritonitis septic shock treated with high dose of antibiotics showed no significant impact in bacterial clearance from the blood, organs or peritoneal fluid between the three antibiotic combination sequences ($p>0.025$). Altering the antibiotic sequence neither had an impact in blood bacterial clearance at experiment end between the different combination strategies ($p>0.025$).

However, combination therapy showed synergistic effects with a rapid and maintained blood bacterial clearance over time. While none of the trajectories of the combination therapy arms differ significantly from each other ($p>0.025$), combination therapy showed a significant difference in bacterial clearance when compared to monotherapy ($p<0.001$).

When comparing higher and normal antibiotic dosing, blood bacterial clearance seemed to be improved in the monotherapy arms, however this is just a finding that should be reviewed in the future.

Overall, therapeutic strategies showed significant reduction of the bacterial load in blood, organs and peritoneal fluid of our *in vivo* model of *E. coli* septic shock when compared to UTC ($p<0.001$). However, altering the antibiotic sequence had no significant impact in bacterial clearance from the organs or the peritoneal fluid ($p>0.025$).

3.5.3 Streptococcus pneumoniae

The same protocol that was previously described in **section 3.4.5** was followed, with the following major differences:

3.5.3.1 Material and Methods – differences from main protocol

Microorganism: *Streptococcus pneumoniae* clinical bacterial strain 108246.

Capsules: two set of capsules were used.

Antibiotics: Cefotaxime 60 mg/kg and levofloxacin 20 mg/kg.

Duration of the experiment: six hours after antibiotic administration.

3.5.3.2 Results for S. pneumoniae peritonitis septic shock model

3.5.3.2.1 Baseline characteristics

Of the 37 animals infected with *S. pneumoniae,* 56% were female, their average weight was 302.35 g (SD 14.63 g), 91% were febrile. Time to septic shock had a bimodal distribution, associated with the two batches of capsules used. One batch generated septic shock by hour 12-14 and the other by hour 15-17, the median time to septic shock was 15.15 hours (IQR 12.8-16.88 hours). See **Table 11.**

Table 11. S. pneumoniae experiments baseline characteristics per treatment arm.

Treatment arm	N	Sex F N (%)	Weight g (SD)	Fever %	Initial HR bpm (SD)	T -> shock h (IQR)	T -> Abx h (IQR)	BL blood bacterial load log CFU/mL (SD)	Early death
• UTC	7	5 (71%)	301.43 (12.83)	86%	363 (31.94)	13 (12.4-14)	13.1 (12.5-14.1)	5.49 (0.50)	0 (0%)
• Levofloxacin	6	3 (50%)	314.5 (6.02)	100 %	377.67 (19.19)	12.3 (12.2-12.7)	12.37 (12.3-12.8)	5.44 (0.55)	0 (0%)
• Cefotaxime	6	4 (66%)	301 (13.86)	100 %	359.33 (28.82)	14.8 (12.9-15.5)	14.9 (13.1-15.6)	5.36 (0.33)	1 (16%)
• Simultaneous	6	4 (66%)	293.5 (14.60)	100 %	362.5 (17.78)	16 (15.4-16.7)	16 (15.4-16.7)	5.35 (0.37)	1 (16%)
• Cef -> Levo	6	2 (33%)	308.67 (16.68)	83 %	360.5 (48.86)	15.9 (14.4-17.2)	15.9 (14.5-17.3)	5.36 (0.32)	1 (16%)
• Levo -> Cef	6	3 (50%)	295.17 (15.47)	100 %	365 (27.89)	17 (16.9-17.3)	17 (17-17.4)	5.58 (0.37)	0 (0%)

N – number of animals; F – female; g – grams; SD – standard deviation; HR – heart rate; bpm – beats per minute; T – time; T -> shock – time to septic shock; h – hours; IQR – interquartile range; T -> ABx – time to antibiotics; BL, baseline; log – logarithm in base 10; CFU – colony forming units; mL – milliliter; UTC, untreated controls; Cef -> Levo, cefotaxime then levofloxacin; Levo -> Cef – levofloxacin then cefotaxime.

3.5.3.2.2 Blood bacterial counts

The mean baseline bacteremia for the 37 animals was 5.43 $\log_{10}$ CFU/ml of blood (SD 0.37 $\log_{10}$ CFU/ml). See **Table 11** for baseline bacterial blood counts per treatment arm.

The different interventions showed different blood bacterial counts over time, shown in **Figure 13**. All treatment arms significantly reduced the blood bacterial load over time ($p<0.001$) when compared to UTC, showing different blood clearance trajectories (**Figure 13**).

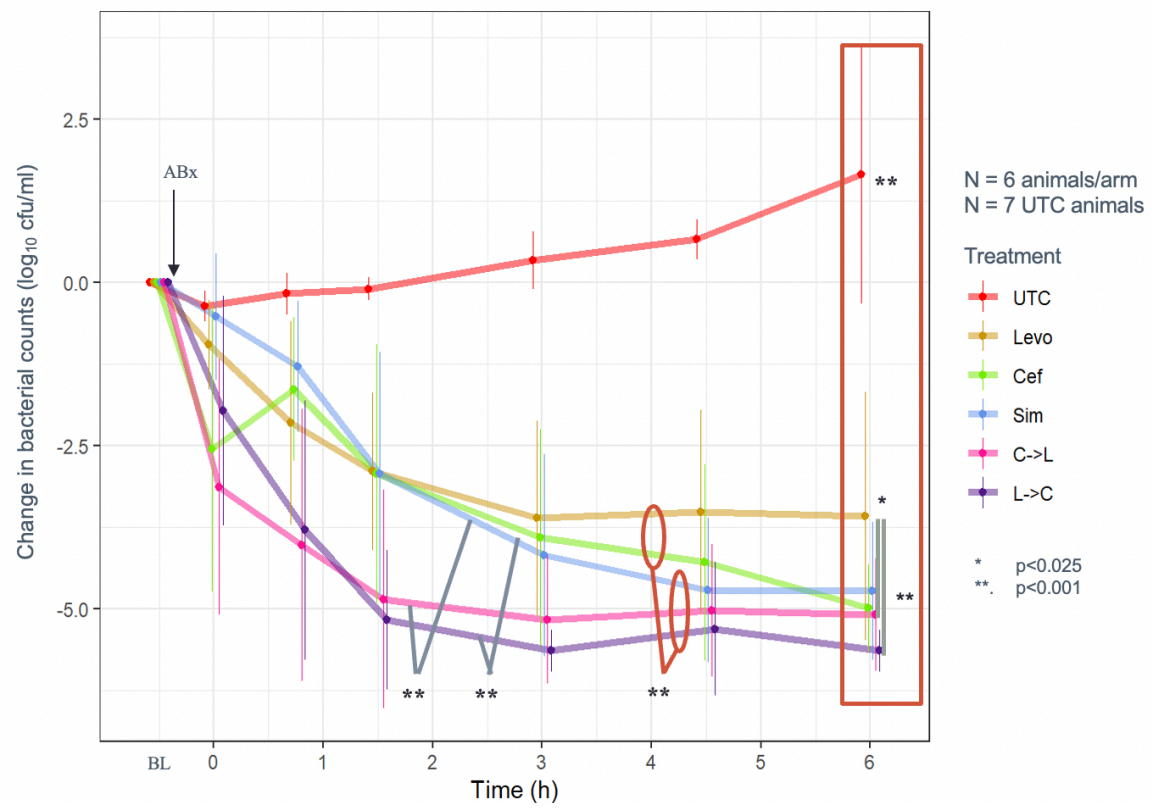
The UTC rats, as expected, showed increasing bacterial blood load over time. All UTC rats survived to hour six; their bacterial blood concentration at six hours had increased on average 0.85 $\log_{10}$ CFU/ml (SD 0.48 $\log_{10}$ CFU/ml). All antibiotic treatments had a significant bacterial load reduction when compared to UTC ($p<0.001$).

When comparing the different combination treatment sequences among them, we found that simultaneous antibiotic dosing showed a statistically different trajectory of blood bacterial clearance than either of the staggered administration strategies ($p<0.001$ for both comparisons). Staggered antibiotic administration showed a faster and more sustained blood bacterial clearance over time, without significant difference among both strategies ($p=0.32$) (**Figure 13**). Showing a synergistic effect of staggered administration of combination therapy, independently of the order, with faster initial blood bacterial clearance that maintained over time (**Figure 13**).

The trajectories in blood bacterial clearance were statistically significant when comparing combination therapy, as a group, to monotherapy ($p<0.001$). However, cefotaxime monotherapy, levofloxacin monotherapy and simultaneous dosing of antibiotics behaved similarly over time, showing a progressive and slower blood bacterial kill and no significant differences in bacteriemia clearance trajectory among them ($p>0.025$). All three showed significant differences in blood bacterial clearance trajectories when compared to the staggered combination strategies ($p<0.001$ in all comparisons).

When we compared the bacterial blood counts at experiment end using ANOVA we only found statistically significant differences in blood bacterial clearance between levofloxacin monotherapy and the two staggered combination strategies ($p=0.012$ C->L vs L; and $p=0.001$ L->C vs L).

Figure 13. S. pneumoniae bacterial blood counts over time.



*ABx – antibiotic administration, N – number of animals, UTC – Untreated controls, Levo – levofloxacin, Cef – cefotaxime, Sim – simultaneous antibiotic dosing, C->L – cefotaxime then levofloxacin, L->C – levofloxacin then cefotaxime, log – logarithm in base ten, cfu – colony forming units, mL – milliliter, ** – statistically significant p<0.001, * – statistically significant p<0.025.*

This figure represents quantitative blood bacterial clearance over time in our S. pneumoniae peritoneal septic shock rat model treated with different antimicrobial sequences in combination as well as monotherapy and UTC. There are six animals per treatment arm, and seven UTC. Here we represent the average bacterial load change from baseline and the standard deviation for the 95% CI (whiskers) of the blood bacterial counts at each timepoint.

Staggered dosing regimens showed a synergistic effect, with faster initial blood bacterial clearance that maintained over time after 1.5 hours. These trajectory differences showed to be statistically significant when compared to simultaneous administration of antibiotics or either of the monotherapy strategies (p<0.001 in all three comparisons). Bacterial clearance trajectories were statistically different

when comparing monotherapy to combination therapy ($p<0.001$) (indicated by red circles in the figure).

Cefotaxime and levofloxacin monotherapy, as well as simultaneous dosing showed a slower initial bacterial kill, and showed no differences between their blood bacterial clearance trajectories ($p>0.025$).

At experiment end (red box), levofloxacin monotherapy had the lowest bacterial clearance, showing significant differences with either of the staggered combination strategies ($p=0.012$ C->L vs. L and $p=0.001$ L->C vs. L).

3.5.3.2.3 Peritoneal fluid bacterial counts

All treatment arms showed similar bacterial load in the peritoneum (mean 5.78 log_{10} CFU/ml, SD 1.42 log_{10} CFU/ml, n=30), this finding reached statistical significance ($p<0.001$) when compared to UTC (mean 9.21 log_{10} CFU/ml, SD 0.44 log_{10} CFU/ml, n=7), see **Figure 14 A**.

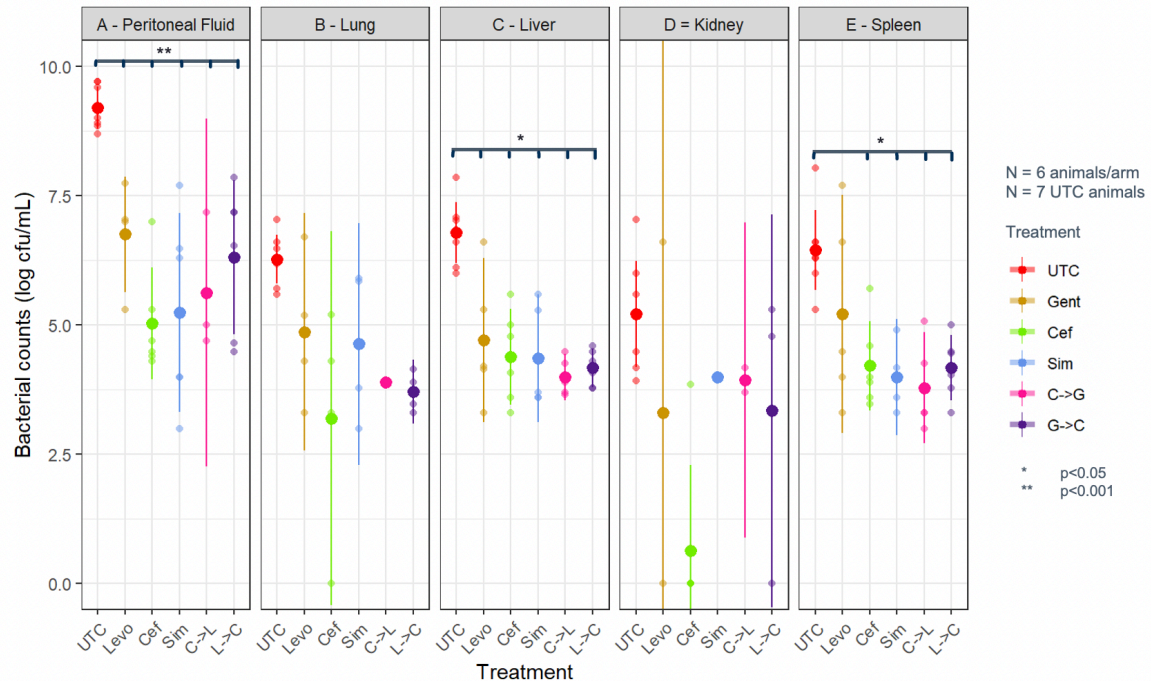
There were no statistically significant differences between any treatment arms or when comparing combination therapy to monotherapy ($p>0.025$), see **Figure 14 A.**

3.5.3.2.4 Organ bacterial counts

We present the organ bacterial counts in **Figure 14 B-E**. UTC showed significant differences in organ bacterial counts in the spleen and liver when compared to treatment arms ($p<0.001$) with the exception of the levofloxacin monotherapy arm, where there were no significant differences ($p>0.025$), see **Figure 14 C and E.**

In the lung we only found significant differences in bacterial counts between UTC and L->C, $p<0.001$ (**Figure 14 B**). We were not able to extract any conclusions from the kidney bacterial counts, since we had a significant number of sample losses due to equipment malfunctioning and technician processing expertise in this cohort of experiments, see **Figure 14 D**.

Figure 14. S. pneumoniae peritoneal fluid and organ bacterial counts.



*N – number of animals, UTC – Untreated controls, Levo – levofloxacin, Cef – cefotaxime, Sim – simultaneous antibiotic administration, C->L – cefotaxime then levofloxacin, L->C – levofloxacin then cefotaxime, log – logarithm in base ten, cfu – colony forming units, mL – milliliter, ** – statistically significant p < 0.001, * – statistically significant p < 0.05.*

This graph represents the peritoneal fluid bacterial counts at the end of the experiment in S. pneumoniae septic shock rats. Each animal is represented by a single dot. We also represent the mean and the 95% CI of the bacterial load of the peritoneal fluid and the organs for each treatment arm.

The peritoneal fluid bacterial load of UTC was significantly higher than all other treatment arms (p<0.001). UTC organ bacterial counts were higher than the treatment arms, but only significant in the liver and spleen when compared to combination therapies and cefotaxime monotherapy (p<0.05).

There were no other significant differences between treatment arms in peritoneal fluid or organ bacterial counts.

3.5.3.3 Conclusion

The sequence of combination therapy of the first dose of antibiotics in our *S. pneumoniae* septic shock rat model showed to be relevant in bacterial clearance. Staggered administration strategies showed better initial blood bacterial clearance that maintained over time. This finding proved to be statistically different when compared to the blood bacterial clearance trajectory of the simultaneous administration of antibiotics ($p<0.001$ for both comparisons). There were no differences in bacteremia clearance between staggered administration strategies ($p>0.025$). The blood bacterial clearance at the end of the experiment was not significantly different among the three combination strategies ($p>0.025$).

The aggregate trajectory of combination therapy bacteremia clearance compared with monotherapy was significantly different ($p<0.001$), showing faster and prolonged bacterial clearance over time.

Each antibiotic in monotherapy and simultaneous dosing had a slower and decreased initial bacterial clearance. These three treatments showed no differences in blood bacterial clearance trajectory among them ($p>0.025$ in all comparisons).

Altering the antibiotic sequence had no significant impact in bacterial clearance from the organs or the peritoneal fluid in our *in vivo* model of *S. pneumoniae* septic shock ($p>0.025$).

3.5.4 Inflammatory mediator profile

3.5.4.1 Cytokine profile of E. coli rats treated with septic shock high dosing antibiotic strategy.

At baseline, approximately 12 hours after the surgical implantation of the capsules, the only cytokines that showed statistical differences between infected animals and sham controls were RKC and IL-10 ($p<0.001$ for both), **Figure 15**.

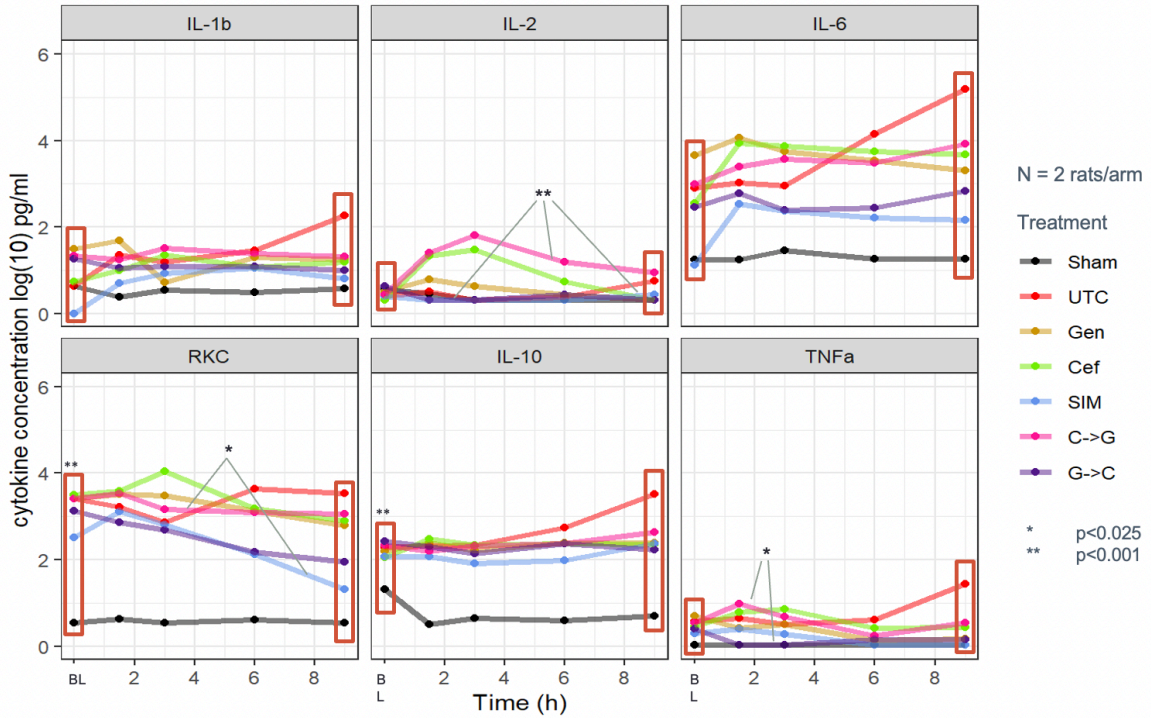
Sham controls showed an almost flat trajectory over time for all cytokines (**Figure 15**). When comparing the trajectories of the cytokines for sham and UCT rats, we found the following significant differences in cytokine trajectory: IL-1 beta, IL-6, IL-10 and TNF alpha ($p<0.001$ for all comparisons).

As expected, UTC showed an upward trajectory of all proinflammatory cytokines over time, that showed higher levels of all cytokines except of IL2 at experiment end (**Figure 15**). However, the differences in cytokines at the end of the experiment only showed to be statistically significant between UTC and sham rats for IL-6 ($p=0.028$), IL-10 ($p=0.006$) and TNF alpha ($p=0.011$), after controlling for baseline levels.

Between combination arms, the sequence of antibiotic administration only showed significant differences in cytokine trajectory for: IL-2 ($p<0.001$ for both C->G compared to SIM and G->C), RKC ($p=0.002$ for C->G vs. SIM), and TNF alpha ($p=0.002$ between staggered strategies). **Figure 15.**

At the end of the nine-hour experiment, approximately 21 hours post infection, and when controlling for baseline cytokine levels, there were no significant differences between the different combination sequences.

Figure 15. Cytokine profile of E. coli rats treated with high antibiotic septic shock dosing:



*IL-1b – interleukin 1 beta, IL-2 – interleukin 2, IL-6 – interleukin 6, RKC – rat cytokine KC (similar to human interleukin 8), IL-10 – interleukin 10, TNFa – tumor necrosis factor alpha, N – number of animals per treatment arm, UTC – untreated controls, Gen – gentamicin, Cef – cefotaxime, SIM – simultaneous administration of antibiotics, C->G – cefotaxime then gentamicin, G->C – gentamicin then cefotaxime, h – hour, log(10) – logarithm in base ten, pg – picograms, ml – milliliter, ** – statistically significant p<0.001, * – statistically significant p<0.025.*

In this graph we represent the average cytokine levels of E. coli rats treated with the high antibiotic dosing strategy. Cytokine expression levels were measured in duplicates using Milliplex magnetic beads. We measured the cytokine levels at five different timepoints throughout the experiment in two rats per treatment arm. We normalized the data by applying logarithm in base ten, averaging the duplicate samples and then the rats per treatment, and controlling for baseline levels for the experiment end analysis, to reduce the variability between animals.

This graph only represents statistically significant differences for our primary outcome, differences between sequence of antibiotics, other findings are described but not included in the graph for simplicity. Baseline analysis was performed between infected animals and Sham controls and is represented in the

left red square on each graph. Comparisons at experiment end are represented in the right red square on each graph.

When comparing the baseline cytokine levels of the infected rats vs. sham controls (represented by the left red square in every graph), we found significant differences in RKC and IL-10 ($p<0.001$).

The trajectory analysis of sham controls vs UCT was only different for IL-1b, IL-6, IL-10 and TNFa ($p<0.001$ in all comparisons). At the end of the experiment (red square to the right of each graph), UTC cytokine levels were only significantly different from sham controls for TNFa and IL-10 ($p=0.011$ and $p=0.006$ respectively).

The sequence of antibiotics given in combination showed differences in the trajectory of the logarithmic levels of cytokines of IL-2 ($p<0.001$ when comparing cefotaxime first with the other two strategies), RKC ($p=0.002$ for C->G vs SIM) and TNF alpha ($p=0.002$ between staggered strategies).

3.5.4.2 Cytokine profile of E. coli rats treated with septic shock normal antibiotic dosing strategy.

The cytokine levels at baseline were statistically different when comparing infected animals versus sham controls for IL-6 (p=0.008), RKC (p<0.001), IL-10 (p<0.001) and TNF alpha (p=0.048). See **Figure 16.**

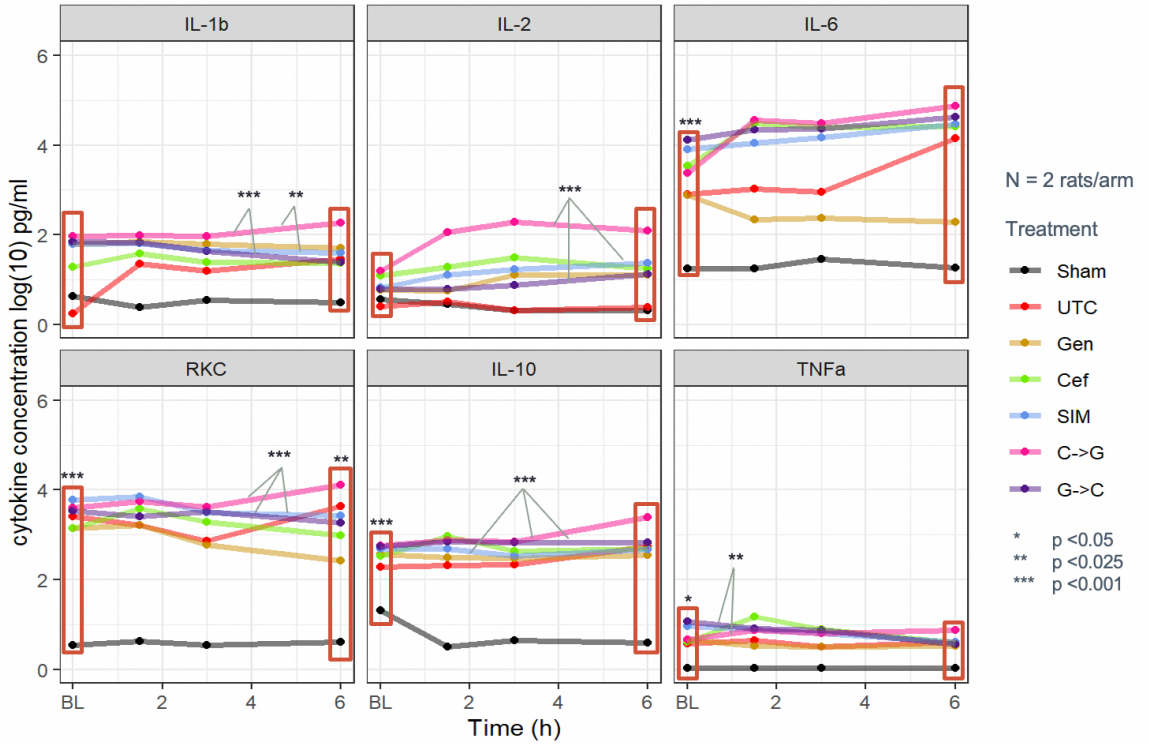
The trajectories of cytokine levels in sham controls appears to be mostly flat for all cytokines. These trajectories are statistically significantly different from UTC for the following cytokines: IL-6 (p=0.001) and IL-10 (p<0.001), see **Figure 16.**

Interestingly UTC cytokine trajectories, in contrast with the high dosing of antibiotics, does not show a clear increase over time except for IL-6; and UTC don't represent the highest levels of cytokines when compared to treated rats (**Figure 16**). There were no differences between UTC and other strategies at experiment end after controlling for baseline cytokine levels.

Antibiotic combination showed some significant differences in trajectory depending on the sequence. Giving the beta-lactam before the aminoglycoside proved to be statistically different for all cytokines except IL-6, see **Figure 16**. Cefotaxime then gentamicin had the highest levels of cytokines.

At experiment end, only RKC showed statistically significant differences between combination strategies (p=0.008 when comparing staggered dosing, and p=0.005 between C->G and SIM). All other exploratory analysis for RKC and C->G compared to monotherapies proved significant as well (p=0.001 for Gentamicin and p=0.023 for cefotaxime). See **Figure 16**.

Figure 16. Cytokine profile of E. coli rats treated with normal antibiotic septic shock dosing:



*IL-1b – interleukin 1 beta, IL-2 – interleukin 2, IL-6 – interleukin 6, RKC – rat cytokine KC (similar to human interleukin 8), IL-10 – interleukin 10, TNFa – tumor necrosis factor alpha, N – number of animals per treatment arm, UTC – untreated controls, Gen – gentamicin, Cef – cefotaxime, SIM – simultaneous administration of antibiotics, C->G – cefotaxime then gentamicin, G->C – gentamicin then cefotaxime, h – hour, log(10) – logarithm in base ten, pg – picograms, ml – milliliter, ** – statistically significant p<0.001, * – statistically significant p<0.05.*

In this graph we represent the average cytokine levels of E. coli rats treated with the high antibiotic dosing strategy. Cytokine expression levels were measured in duplicates using Milliplex magnetic beads. We measured the cytokine levels at five different timepoints throughout the experiment in two rats per treatment arm. We normalized the data by applying logarithm in base ten, averaging the duplicate samples and then the rats per treatment, and controlling for baseline levels for the experiment end analysis, to reduce the variability between animals.

This graph only represents statistically significant differences for our primary outcome, differences between sequence of antibiotics, other findings are described but not included in the graph for simplicity. Baseline analysis was performed between infected animals and Sham controls and is represented in the

left red square on each graph. Comparisons at experiment end are represented in the right red square on each graph.

Baseline comparison of cytokine levels between infected animals and sham controls proved to be statistically significant for IL-6 ($p=0.008$), RKC ($p<0.001$), IL-10 ($p<0.001$) and TNF alpha (0.042) (red left square of each graph). Trajectory analysis of the cytokines between UTC and sham rats was only significant for IL-6 ($p=0.001$) and IL-10 ($p<0.001$).

The cytokine trajectories of cefotaxime then gentamicin, proved to be significantly different from the other combination sequences for most cytokines: IL-1b ($p<0.001$ for G->C, $p=0.008$ for SIM), IL-2 ($p=0.001$ for G->C, $p=0.13$ for SIM), RKC ($p=0.002$ for G->C, $p=0.001$ for SIM), IL-10 ($p<0.001$ for both G->C and SIM) and TNF alpha ($p =0.02$ when compared to G->C).

At the end of the experiment (right red square on each graph) C->G showed higher levels of RKC than all other treatment strategies ($p<0.025$ for all comparisons)

3.5.4.3 Cytokine profile of S. pneumoniae rats

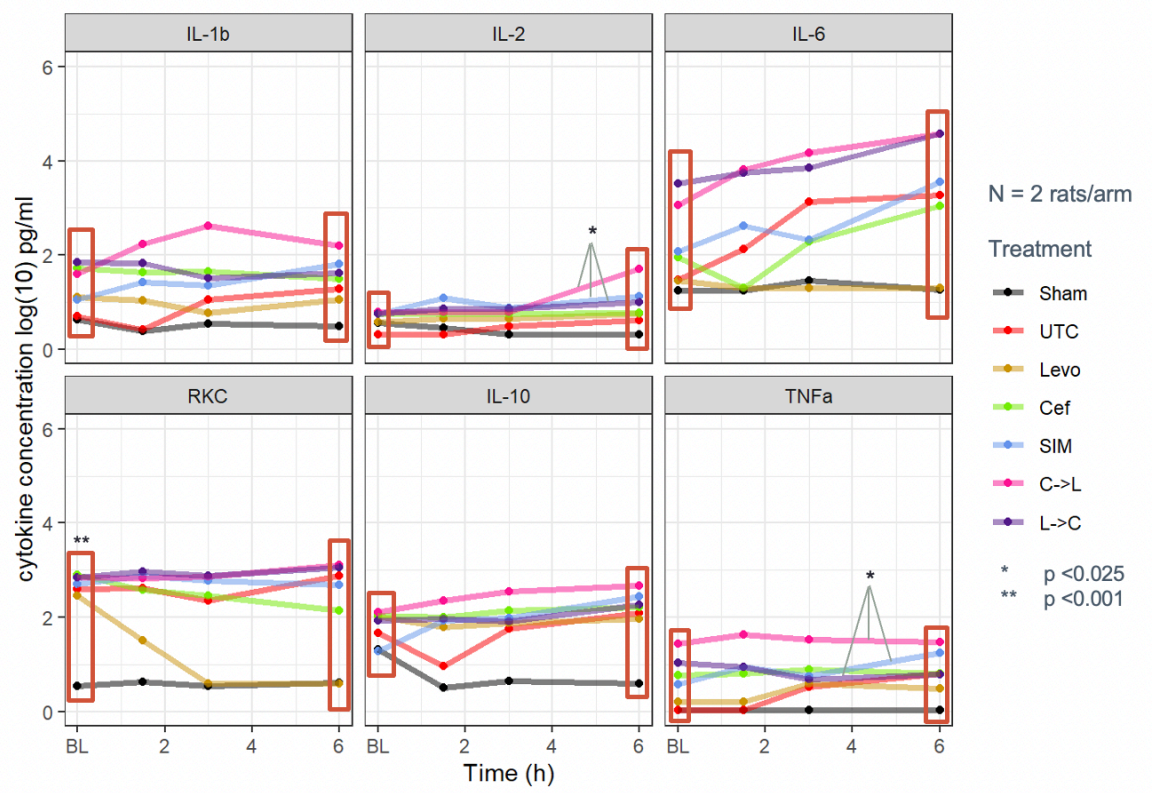
Baseline cytokine levels were similar between infected animals and sham controls. The only statistical difference found was RKC ($p<0.001$), see **Figure 17**.

The cytokine trajectories for sham controls were almost flat, while for UTC animals they showed some increase in cytokine levels overtime (**Figure 17).** Interestingly, UTC cytokine levels were not the highest as we would have expected. The trajectory analysis between untreated and sham rats proved to be significant for all cytokines but IL-2 (IL-1b $p=0.019$, IL-6 $p=0.005$, RKC $p<0.001$, IL-10 $p=0.002$, TNFa $p=0.007$).

The sequence of antibiotics proved to have different cytokine trajectories for IL-2 and TNFa. In the case of IL-2 giving cefotaxime first had a different trajectory that the other two combination strategies ($p=0.007$ for L->C and $p=0.1$ for simultaneous dosing). While for TNF alpha, all three combination strategies had a different cytokine trajectory ($p<0.025$ for all three comparisons), see **Figure 17**.

At the end of the six-hour experiment, and controlling for baseline cytokine levels, we found statistical differences between combination therapies and monotherapies for RKC ($p<0.001$) and between sham and UTC for IL-10 ($p=0.023$).

Figure 17. Cytokine profile of S. pneumoniae septic shock rat model



*IL-1b – interleukin 1 beta, IL-2 – interleukin 2, IL-6 – interleukin 6, RKC – rat cytokine KC (similar to human interleukin 8), IL-10 – interleukin 10, TNFa – tumor necrosis factor alpha, N – number of animals per treatment arm, UTC – untreated controls, Gen – gentamicin, Cef – cefotaxime, SIM – simultaneous administration of antibiotics, C->G – cefotaxime then gentamicin, G->C – gentamicin then cefotaxime, h – hour, log(10) – logarithm in base ten, pg – picograms, ml – milliliter, ** – statistically significant p<0.001, * – statistically significant p<0.025.*

In this graph we represent the average cytokine levels of E. coli rats treated with the high antibiotic dosing strategy. Cytokine expression levels were measured in duplicates using Milliplex magnetic beads. We measured the cytokine levels at five different timepoints throughout the experiment in two rats per treatment arm. We normalized the data by applying logarithm in base ten, averaging the duplicate samples and then the rats per treatment, and controlling for baseline levels for the experiment end analysis, to reduce the variability between animals.

This graph only represents statistically significant differences for our primary outcome, differences between sequence of antibiotics, other findings are described but not included in the graph for simplicity. Baseline analysis was performed between infected animals and Sham controls and is represented in the

left red square on each graph. Comparisons at experiment end are represented in the right red square on each graph.

Baseline RKC levels (red left squares) were statistically different between infected animals and UTC ($p<0.001$). UTC and sham rats cytokine trajectories were significantly different for most cytokines IL-1b $p=0.019$, IL-6 $p=0.005$, RKC $p<0.001$, IL-10 $p=0.002$, TNFa $p=0.007$.

Combination therapy strategies showed different cytokine trajectories for IL-2 ($p=0.007$ between staggered strategies and $p=0.01$ between simultaneous administration and beta-lactam first) and TNF alpha (all 3 sequences proved different trajectories $p<0.025$).

At experiment end (red right squares) IL-10 levels were higher in UTC than sham rats ($p<0.001$) and RKC. There were no differences between antibiotic sequences.

3.5.4.4 Cytokine analysis summary

At baseline the only consistent cytokine difference between infected and uninfected animals was RKC, the murine analog of human IL-8.

Untreated animals showed a consistent difference in cytokine trajectory when compared to the sham controls for IL-6 and IL-10. Although for the other four cytokines this was not the case. Neither did UTC show the highest levels of proinflammatory cytokines in the six hour post intervention models, despite having been infected for 18 hours on average by the end of the experiment.

The sequence of antibiotics didn't seem to have a major impact on the cytokine trajectories. Giving the beta-lactam first using a staggered strategy showed significant differences in IL-2 and TNF alpha trajectories for all three cohorts when compared to the other combination strategies. Although these trajectories wouldn't be the ones expected given the existing literature;[27,198] since they showed higher cytokine levels than the other two combination strategies (see **Figure 15, Figure 16** and **Figure 17**).

At experiment end, after controlling for baseline cytokine levels, the six-hour post intervention cohorts showed differences in RKC levels for combination vs. monotherapy, showing higher cytokine levels in the combination cohorts. While the nine-hour *E. coli* model showed higher levels of IL-6, IL10 and TNF alpha in UTC when compared to sham rats.

3.6 Animal model results conclusion

Combination therapy as the first dose of antibiotics in a peritonitis animal model of septic shock showed synergistic effects with rapid and maintained bacterial clearance from the blood in *E. coli* and *S. pneumoniae.*

Altering the antimicrobial sequence had no significant impact in bacterial clearance from the blood, the peritoneal fluid or the organs in our *E. coli* model of septic shock, regardless of the dose of antibiotics given.

However, we did observe faster and more sustained blood bacterial clearance trajectory using staggered antibiotic combination strategies in our *S. pneumoniae model when compared to simultaneous dosing ($p<0.001$ for both comparisons)*. Yet, at experiment end, the blood bacterial clearance between all three combination strategies wasn't statistically different. Neither did combination sequence impact the bacterial clearance from the organs or the peritoneal fluid in our *S. pneumoniae* septic shock model.

Higher antibiotic dosing showed faster bacterial kill in cefotaxime monotherapy and a delayed appearance of regrowth in gentamicin monotherapy and simultaneous administration of antibiotics in our *E. coli* model.

The cytokine profile showed significant differences between combination strategies in all three cohorts for IL-2 and TNF alpha. Giving the beta-lactam first and then the second agent (gentamicin in the *E. coli* models and levofloxacin in *S. pneumoniae)* showed a different trajectory that the opposite order or simultaneous dosing, with higher cytokine levels through the experiment. There were no

differences on cytokine levels between combination strategies at experiment end. However, no conclusion should be drawn from our findings, given the small sample size and the heterogeneous results.

The impact of antibiotic sequence on bacterial clearance and clinical outcomes remains to be clearly answered. Future research should take a deeper look into antibiotic combination sequence as a potential tool to better fight against sepsis and septic shock.

4 CHAPTER 4: Discussion and final summary

4.1 Discussion of the systematic review

In our systematic review, we assessed outcomes of critically ill patients with severe infection randomly assigned either a single antimicrobial agent (monotherapy) or this same agent in combination with a secondary antimicrobial with non-redundant mechanism (combination therapy). We found no significant difference in mortality (assessed over a range of varying time points in different studies) between those assigned monotherapy and combination therapy. There was also no significant difference in hospital or ICU lengths of stay or ventilator free days. Days free of renal replacement therapy or vasopressors were only addressed in one of the reviewed trials, which reported no significant difference in these measurements. None of the reviewed trials assessed quality of life.

Although none of the reviewed studies reported incidence of *C. difficile* colitis, two of the studies did report *C. difficile* toxin incidence, but there was no difference between study groups. Adverse reactions were, overall, more common in patients assigned to combination therapy. This difference in adverse reactions is likely due to toxicity of the use of more than one drug and could be associated with the aminoglycosides and amphotericin used as secondary antimicrobials in the combination group.

The potential clinical benefit of antibiotic combination therapy has been questioned by three previous metanalysis and a Cochrane systematic review on this subject; most performed by the same authors.[151,152,157,158] These systematic reviews failed to show improvement in clinical outcomes with combination therapy

in immunocompetent patients with sepsis and /or Gram-negative bacteremia when compared to monotherapy. However, these metanalyses included studies that compared combinations of different antimicrobials, without following the current Surviving Sepsis definition of combination therapy. Further, many of the included studies failed to use the same antibiotic in monotherapy and in combination, leading to questions regarding the relevance of these SR as they pertain to combination therapy definition.

On the other hand, combination therapy has been supported by a meta-analysis and a propensity matched study of septic shock;[52,150] where a survival benefit was suggested in patients with a mortality risk greater than 25% treated with combination therapy. This suggests that the potential survival benefit of combination antimicrobial therapy may be restricted to high-risk groups (e.g., septic shock). However, the aforementioned meta-analysis (incorporating a meta-regression analysis) also suggested the possibility of increased mortality risk with combination therapy in low mortality-risk patients (i.e. patients without septic shock and a mortality risk less than 15%). Additionally, the meta-analysis included observational studies as well as randomized clinical trials (although the meta-regression analysis results hold in subset analysis restricted to randomized controlled trials).

To the best of our knowledge, this is the first systematic review of randomized controlled trials which uses the current definition of combination therapy (as two or more antimicrobials of different mechanistic classes meant to cover a single pathogen).

The great heterogeneity of populations included in the analyzed studies may obscure subtle differences between monotherapy and combination therapy groups. The overall mortality of the study populations was quite low compared to what would be expected for septic shock, and thus we can infer that many of the patients would not fall into this category. Given this mortality, most patients in the studies include in this analysis had sepsis or severe sepsis without shock whose mortality is typically less than 15% in developed countries. Unfortunately, the reviewed studies did not provide adequately separated data to discriminate outcomes of those with more severe illness (APACHE score >25) from those with less severe illness. This is an important subgroup analysis that should be made, since previous studies have suggested that a survival benefit of combination therapy compared to monotherapy may be restricted to those at greatest risk of death.[52] This difference in the subject populations of the various meta-analyses-meta-regression studies may account for the differing results of these analyses.

The fact that the data in our Forrest plot trend in either direction, makes us think that there might be other explanations for this variability. Lower severity studies may have been included (as you can see in **Table 5** the severity scores varied significantly between studies), or different definitions of severe infections and signs of sepsis and septic shock may have been used. Another option that may explain this variability is the potency of the antibiotics used in monotherapy; if they were too potent, we may not benefit when adding a secondary agent.

The biggest limitation of our systematic review is the high risk of bias of the included studies, as assessed using the Cochrane Collaboration Risk of Bias tool. Another caveat of our analysis is that reporting of our secondary outcomes was inconsistent between studies, leading to incomplete analysis. As well as our modified search, that may have left out important studies. Thus, a further SR with a complete search should be considered to try to answer the question of whether combination therapy has an impact in mortality in septic shock patients.

4.2 Discussion animal model of septic shock and sequence of antibiotic combination

Combination of antimicrobial treatment could be an easy and cost-effective strategy to optimize the use of antimicrobial agents and may be key to improve bacterial kill, and thus, clinical outcomes, in septic shock. The sequence in which antimicrobials are initially administered may have a significant impact on their antimicrobial activity. Addressing this question will contribute to fulfill one of the "objectives" marked by the WHO for sepsis treatment: antimicrobial optimization.[13]

As previously described in **section 1.4**, there is a paucity of literature regarding antibiotic sequence, the majority of the evidence is from the 1980s and 1990s and is mostly based on pharmacodynamic *in vitro* models. The available evidence shows inconsistent results regarding antibiotic sequence (**Table 3**).

Overall, the existing *in vitro* evidence suggests that the sequence of antibiotic administration may have a significant impact on bacterial kill; although this may depend on the pathogen, its resistance profile, the chosen antibiotics and the time to the administration of antibiotics from the initiation of septic shock and the interval between antibiotics.

In the studies where they used beta-lactams, using beta-lactam first or simultaneously with aminoglycosides or fluoroquinolones was associated with greater bacterial kill.[165,166,168-170] Of note, most of this evidence is based on *Pseudomonas aeruginosa in vitro* pharmacodynamic models, only one of the six published manuscripts used *E. coli*[165] *(*see **Table 3**).

Of the *in vivo* evidence to date, there is only one animal model of MRSA pneumonia in neutropenic mice[169] and one clinical report from 1988 in 36 cystic fibrosis patients with pseudomonal respiratory tract infecitons.[171] Both studies observed better outcomes depending on the sequence of antibiotic administration. The former mice study showed higher survival when the aminoglycoside netilmicin was followed two hours later by the tetracycline minocycline.[169] On the clinical side, Guggenbichler et al. showed substantial improvement in pseudomonal eradication when the beta-lactams were dosed first and four hours before the aminoglycosides, as opposed to the opposite sequence or simultaneously.[171] This evidence is in keeping with our observation in an unpublished retrospective clinical septic shock cohort, and supports and inspired our hypothesis that antimicrobial sequence could be key to improve sepsis outcomes.

The findings of this thesis show no differences in blood bacterial clearance depending on antibiotic sequence in any of the *E. coli* experimental cohorts (high and normal septic shock antibiotic dosing strategies). With evidence of synergy of the combination therapy over monotherapy ($p<0.001$ in both *E. coli* cohorts), showing a faster initial bacterial clearance that sustained overtime. Staggered dosing strategies had more stable bacterial clearance at the end of the experiment, while simultaneous dosing showed a slight regrowth, this difference was not statistically significant, see **Figure 9** and **Figure 11**. Higher doses of antibiotics prolonged the post-antibiotic effect and delayed the appearance of bacterial regrowth in the blood. However, we found no differences between any combination strategy in blood bacterial clearance at experiment end (**Figure 9** and **Figure 11**).

While in the *S. pneumoniae* cohort the sequence of the first dose of antibiotics given in combination proved to be relevant in blood bacterial clearance trajectory. Staggered administration strategies showed a greater and faster initial blood bacterial kill, that maintained over time, in contrast with simultaneous administration of both antibiotics ($p<0.001$), see **Figure 13.** However, at experiment end, the blood bacterial clearance between al three combination strategies weren't statistically different.

There were no significant differences in peritoneal or organ bacterial clearance between any treatment strategies in all of the three experimental cohorts (**Figure 10**, **Figure 12** and **Figure 14**).

With regards to the cytokine analysis, overall, we didn't find the expected trajectories in cytokine levels for UTC or treatment arms. Interestingly, UTC didn't significantly differ from sham controls in most of the cytokine trajectories (see the above sections **3.5.4.1**, **3.5.4.2** and **3.5.4.3**). Treatment arms didn't show a decrease in proinflammatory cytokines as we would have expected from previous published research[27,198] (see IL-2 behaviour in **Figure 15** and **Figure 16**). Antibiotic sequence was not a clear or consistent cause for different cytokine trajectories over time or at experiment end. And if there were any significant trajectory differences, they showed higher cytokine levels in the combination strategies than in monotherapy (see IL-2 behaviour in **Figure 15** and **Figure 16**).

With these results we cannot extrapolate clear conclusions. One possible explanation for our findings is our sample size, that may have been underpowered to detect the differences in blood bacterial clearance that we ambitioned. Low

sample size has definitively been the cause for our cytokine lack of results or inability to extrapolate conclusions, since an n of 2, despite duplicate samples, is too small to generate clear trajectories given the high animal to animal variability and the heterogeneity of sepsis.

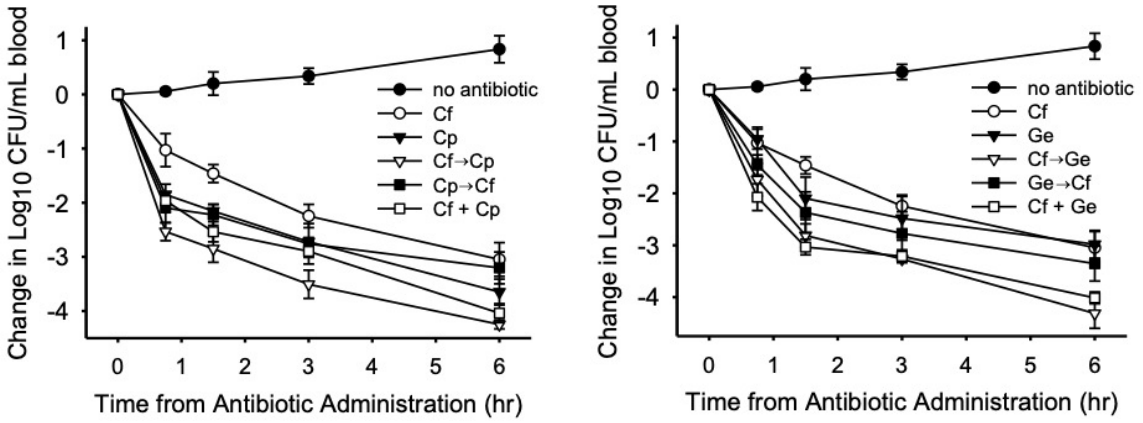
Another possible explanation for our results is that the time between antibiotics was not enough to observe differences between combination strategies. We chose 20 minutes between antibiotics as the minimum time that would take in the clinical setting to get the second antibiotic into the patient; and the time for *E. coli* to replicate in optimal conditions. In the previous published *in vivo* evidence from Goto et al.[169] and Guggenbichler et al.[171] they utilize a time window of two to four hours between antibiotics. Maybe longer time between antibiotics would have allowed us to see a difference in blood bacterial clearance depending on the sequence of antibiotic administration. Although Dr. Kumar et al. did see a difference in bacterial clearance with 20 minutes between antibiotic staggered combinations in their rat model of peritoneal septic shock.[197]

Other possible explanation for our mixed results with our current model is that the key factor to see differences in bacterial clearance depending on the sequence of antibiotics is the bacteria that causes septic shock or the type of infection. It could also be dependent on the resistance profile of the bacterial strains used. While our initial hypothesis may still stand, it may only apply to certain microorganisms or to resistant bacterial strains. The existing *in vivo* evidence was obtained from resistant bacteria (MRSA and *P. aeruginosa*), and in both cases there were significant outcome benefits depending on the antibiotic

sequence.[169,171] However, the preliminary evidence in which we based this thesis did show significant bacterial clearance improvement in sensitive *E. coli* depending on the order of antibiotics.[197] This hypothesis should be tested in future studies.

Preliminary work by Dr. Kumar et al, with a previous version of our rat model of *E. coli* peritoneal septic shock, showed improved bacterial kill when the beta-lactam was used before the aminoglycoside or the fluoroquinolone, **Figure 18**.[197]

Figure 18. Preliminary data on E. coli blood bacterial clearance over time in a previous rat model of E. coli peritoneal septic shock by Dr. Kumar et al.[197]



Cf: cefotaxime; Cp: ciprofloxacin; Ge: gentamicin; +: simultaneous dosing; ->: staggered dosing

However, our results in this thesis do not support their previous findings. While using the same strain of *E. coli*, there were no significant differences in blood bacterial kill between the staggered dosing cohorts in *E. coli* (with either dose of antimicrobials) (**Figure 9** and **Figure 11**). Although we did observe greater blood bacterial kill with staggered dosing than with simultaneous dosing by experiment end in both high and normal doses of antibiotics these differences were not statistically significant (see **Figure 9** and **Figure 11)**

Interestingly, when comparing the blood bacterial clearance over time between the preliminary model by Dr. Kumar et al. and the data in this thesis, we

can observe a faster and greater extent of blood bacterial clearance in all treatment arms despite using the same antibiotic and the same doses (see **Figure 9** and **Figure 18**). This observation is particularly obvious in treatments that contained gentamicin. While the reason behind this observation is unclear, it may have to do with the antibiotics we used, the gentamicin itself, or the dosing administration (iv bolus over ten minutes versus two minutes), that significantly improved bacterial clearance by over two-fold in all *E.coli* experiments treated with gentamicin. Cefotaxime monotherapy behaved similarly in both animal models, although we also had an increased bacterial clearance by hour six in our current model (**Figure 9** and **Figure 18**). UTC on the other hand, have similar bacterial counts over time in both models, with a slow increase that reaches approximately one logarithm by hour six.

While both models were very similar, there were significant differences between them. In the previous model by Dr. Kumar et al, they utilized smaller rats (250 +/- 10g), and a lesser bacterial inoculum (10^5 CFU), that required longer time to achieve septic shock (16 hours). They used intramuscular ketamine sedation as the anesthetic and they did not have a standardized intra venous fluid resuscitation strategy, nor vasopressor support with norepinephrine. The invasive hemodynamic monitoring and definition of shock has been refined in our model, as well as the use of mechanical ventilation given the profound shock and the use of volatile anesthetic. But none of these differences seem to affect the UTC rats, since they behave very similarly. This last finding points to the antibiotics as the responsible cause of the differences in bacterial clearance between the previous

by Dr. Kumar et al and our current model. The statistical analysis used in this thesis is also more refined, using trajectory analysis, a more rigorous significant threshold, and avoiding multiple comparison analysis.

4.2.1 Study strengths

To the best of our knowledge this is the first animal model of septic shock where the sequence of antimicrobials has been extensively tested. We have designed a high-quality, robust and reproducible rat model of peritonitis septic shock.

Our animal model follows most recommendations described by the Minimum Quality Threshold in Preclinical Sepsis Studies (MQTiPPS) consensus to represent a good preclinical sepsis model.[195,199-201]

We developed a consistent monomicrobial peritonitis model of infection using a fixed inoculum, that mimics well the evolution of clinical sepsis. The model exhibits a time dependent progression of infection evolving into sepsis and septic shock.

We established a detailed study design that makes this model of sepsis easily replicable. All therapeutic interventions were performed after septic shock was established.

We used a balanced gender population of animals without comorbidities and embedded into our protocol important animal ethics considerations. By applying the Murine Sepsis Score,[196] we monitored the well-being of the animals

before starting the experiment and choose standardized criteria for euthanasia based on this scale.

The microorganisms that we chose for our animal model of sepsis are commonly found in human sepsis and are clinically relevant. They are responsible for generating most of the clinical burden that we see in the ICUs internationally, as shown by Vincent et al in their international prevalence study of ICU infections in 2009.[20]

We confirmed that combination therapy has a better blood bacterial clearance profile over time than monotherapy in all pathogen cohorts and treatment dosing strategies, see section 3.6.

4.2.2 Study limitations

We were unable to establish a bacteremic model with *Pseudomonas aeruginosa,* this is likely due to the virulency of the pathogen. The baseline bacteremia was 10^3-10^4 CFU/ml in UTC. The eight animals used were in profound septic shock and unstable, but not bacteremic enough for us to be able to see the 1.6 $\log_{10}$ CFU/ml difference between sequences of antibiotics after treatment, given the fact that our detection threshold is 100 CFU/ml.

While we could have pursued this model and look at organ pathogen clearance as our primary outcome, it would not have complied with the Canadian Council of Animal Care three “R” policy (Reduce, Replace and Refine), since we would have required to euthanize multiple animals at multiple timepoints. Thus, after discussing with my committee, we decided not to pursue it further.

Given that most of the published *in vitro* evidence on antibiotic sequence is with *P. aeruginosa,* it would be important to develop this model further and obtain a stable bacteremic model to reassess this important question.

Our *S. aureus* peritonitis model was also unsuccessful; in this case, we were not able to generate septic shock despite using a very high bacterial inoculum in the infection. We took all the steps required to assure that *S. aureus* was in the exponential growth phase when we made the capsules. But regardless of our efforts we were unable to generate septic shock. We hypothesize that our results could have been related to the high exposure of rodents to *S. aureus* environmentally, which would have allowed rats to develop some mechanisms of resistance to this pathogen to better adapt to their surroundings. We may be more successful if we use a resistant staphylococcal strain in the future, or a neutropenic thigh infection model.

Using more than one batch of capsules in the different experimental cohorts could have introduced some degree of variability in our infection model. We tried to be mindful of this in our statistical analysis and we normalized the data and set a stricter significance threshold.

However, one of the biggest limitations of our animal model was our primary outcome. Survival would have been a likely better choice, but the ethics board of University of Manitoba didn't consider it to be aligned with the standards of the Canadian Council of Animal Care. Twenty four hours is a short period of time to assess bacterial clearance in septic shock, as we can observe in our peritoneal fluid and organ bacterial counts at the end of our experiments (**Figure 10**, **Figure**

12 and **Figure 14**), the remaining bacterial load is significant. It will likely take days to clear the microbial burden from the peritoneum. However, given the complexity of our animal model, which required constant observation of the animals, extending our observation to 72-96 hours was not feasible. Had we gotten ethics approval, we would have likely modified the protocol and used a mice version of the animal model that did not require mechanical ventilation.

Other possible limitation of our model could be our choice of antimicrobials. They may be described by some as “old”, but they are still relevant antibiotics that are very commonly used in the clinical ICU setting every day. Beta-lactams all share the same mechanism of action, and our choice of beta-lactam wouldn’t change the principle behind our hypothesis. Fluoroquinolones and aminoglycosides remain among the most commonly used supplemental agents in most of the developed world. While they may not be the current initial empiric choice to treat septic shock patients in the ICU, given their spectrum of coverage; they could again become standard of care with regards to initial therapy once rapid pathogen detection systems are available and we can select targeted antimicrobials from the very first dose of antimicrobial treatment. Thus, our approach to use two antimicrobials that are effective to cover the pathogen may be more relevant once rapid pathogen detection systems are readily available at the bedside.

As previously mentioned, our time between antibiotics may be another limitation of our model. Given that the previous *in vivo* evidence showed differences between antibiotic sequences and they used longer time in between

antibiotics. However, we did show significant differences in the *S. pneumoniae* model using this timeline. And Dr. Kumar et al. had previously observed differences in their preliminary rat model of septic shock using this timeline. Nevertheless, it is true that we may have found different results had we used a different amount of time in between antibiotics. The *in vitro* evidence, on the other hand, showed differences using longer time between antibiotics.[123] Future research in combination therapy should likely look to answer this question.

Another consideration to make regarding our animal model, is the dose of antibiotics. Our dosing choice was extrapolated from clinical guidelines and adapted to the rat's faster metabolism.[193,194] Higher antibiotic doses improved the initial bacterial clearance with cefotaxime monotherapy and prolonged the post-antibiotic effect of gentamicin monotherapy and simultaneous dosing. However, higher doses of antibiotics did not show a difference in bacterial kill depending on the sequence of antibiotic dosing. Peritoneal fluid and organ bacterial counts were non-significantly reduced in the higher dosing cohort when compared to the normal septic shock dosing and did not show any differences between treatment arms, see **Figure 10** and **Figure 12.** This could be related to the duration of the experiment, and we may have observed some bacterial clearance differences had we observed the rats for a longer period of time.

Higher antibiotic dosing may also be important for resistant strains. We hypothesize that higher plasma MICs of two antibiotics may improve bacterial clearance in resistant pathogens. In such population, we may be able to see a significant difference depending on the sequence of high dose of antibiotics.

4.3 Summary of thesis results

The current guideline recommendations are to use combination therapy as a way to broaden the initial spectrum of coverage in sepsis and septic shock, and to treat some multi resistant bacteria; with early de-escalation to appropriate targeted antibiotics.[2]

Previous existing evidence didn't recommend combination therapy, as shown in previous SR, but they were not using the appropriate definition of combination therapy.

Overall, our SR showed that there's no current evidence to support the use of combination therapy in sepsis and septic shock. Although, this could have been attributed to differences in study design, variable degree of severity and maybe to sequence of antibiotic administration.

Our retrospective analyses of the sepsis database showed a survival benefit depending on the sequence of antibiotics, favoring giving the beta-lactam first.

However, we were not able to show a bacterial kill benefit in our rat model of peritonitis septic shock caused by *E. coli.* We did confirm the better initial bacterial kill of combination therapy when compared with monotherapy, but there were no significant differences between the sequences throughout the experiments, regardless of the antibiotic dose.

In the case of our *in vivo* model with *S. pneumoniae*, we did find statistical differences in blood bacterial kill, favoring staggered antibiotic administration. However, there were no differences in blood bacterial counts at experiment end between sequences, neither in the peritoneal fluid or the organs.

Our cytokine results showed significant differences depending on combination sequence for IL-2 and TNF alpha, with higher trajectory levels when using the beta-lactam first and then the second agent. This finding goes against what we had previously expected given the published literature.

Overall, our results show inconsistent differences between antibiotic sequence strategies. There are many considerations to make; we may have different results with resistant pathogens, six to nine hours may not be the enough time to assess a bacterial clearance difference depending on the antibiotic sequence, the chosen timeline between antibiotic administration may not be enough to show bacterial clearance differences, our sample size may have been underpowered to have a good estimate of the effects of antibiotic sequence.

The impact of antibiotic sequence on bacterial clearance and clinical outcomes may depend on the pathogen, its resistance profile, the chosen antibiotics and the time to and between antibiotics.

4.4 Future considerations

Redo the SR with a more complete search targeted to our combination therapy strategy and using the appropriate definition of combination therapy. This is actually underway, and will likely be published in the Fall of 2021.

Full retrospective analysis of the sepsis database, to confirm our previous observation, and a prospective clinical observational study looking into sequence and mortality benefit.

Repeat the animal model with resistant pathogens and a longer time between antibiotics, as well as a survival septic shock animal model.

If all this research were to show equipoise to use different sequence of antibiotics in the clinical setting to improve survival, we would ideally design a randomized clinical trial to answer this question. In order to optimize resources, we would likely do it in parallel or embedded with other important dosing questions to optimize resource usage.

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Appendix

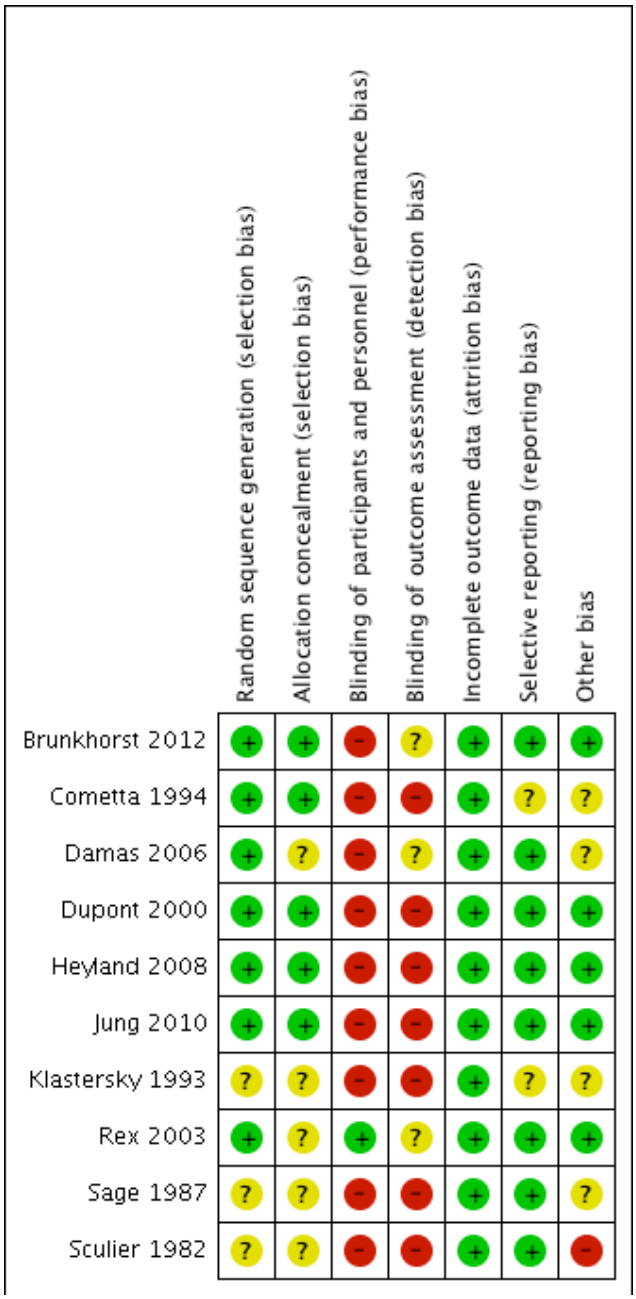
Appendix tables

Table 12. Murine Sepsis Score[196]

Murine Sepsis Score (MSS)				
Score	0	1	2	3
Appearance	Smooth coat	Slightly ruffled fur	Majority of fur on back is ruffled	Piloerection, puffy appearance
Level of consciousness	Active	Active, avoids standing upright	Active only when provoked	Non-responsive, even when provoked
Activity	Normal	Suppressed eating, drinking, or running	Stationary	Stationary, even when provoked
Response to stimulus	Normal	Slowed response to auditory or touch stimuli	No response to auditory, slowed response to touch	No response to touch stimuli
Eyes	Open	Not fully open, potentially secretions	Half closed, potential secretions	Mostly or completely closed
Respiration quality	Normal	Periods of labored breathing	Consistently labored breathing	Labored breathing with gasps

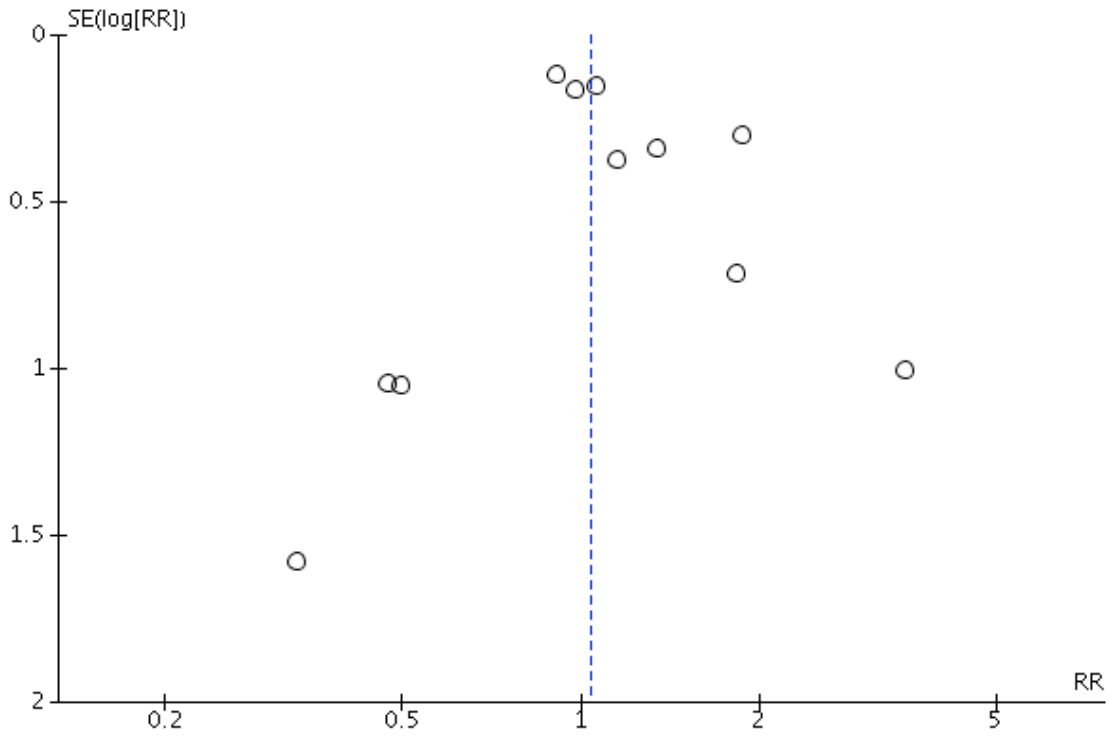
Appendix figures

Figure 19. Risk of bias assessment.



We utilized the Cochrane Collaboration Risk of Bias assessment tool to assess the internal validity of the included studies. Only one study included blinding of the participants and personnel, but unclear detection bias. All the studies were considered to have a moderate risk of bias.

Figure 20. Funnel plot. Publication bias: Mortality in monotherapy vs. combination therapy.

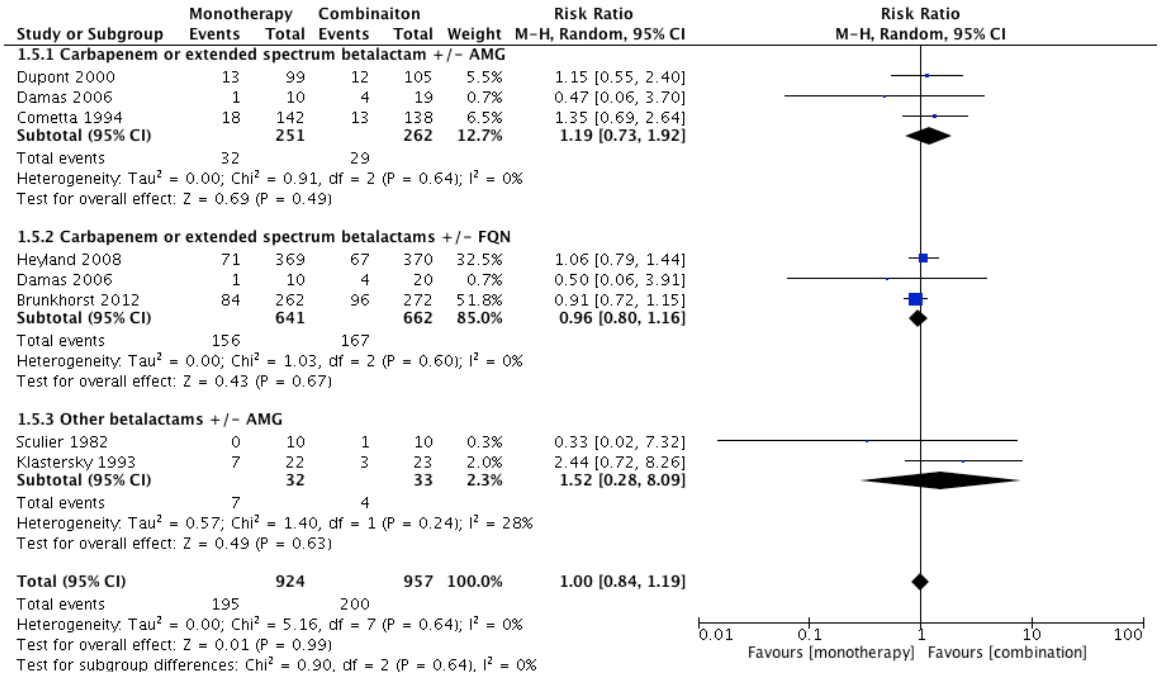


The funnel plot graph is designed to assess publication bias. The studies with high precision are plotted near the average, and studies with low precision will be spread evenly on both sides of the average.

This funnel plot represents all the published included trials in our systematic review as dots, their location depends on their risk ratio (RR) for mortality. Results <1 favor monotherapy, while results >1 favor combination therapy.

In our included studies there seems to be a lack of published lower precision studies supporting combination over monotherapy and a lack of high precision trials supporting monotherapy. Thus, there might be a publication bias; although this finding could also be due to chance.

Figure 21. Mortality by type of antibiotic combination.



AMG – aminoglycoside, FQN - fluoroquinolone

In this Forrest plot, we show one of the subgroup analysis that we performed to try and assess whether there was a mortality benefit depending on the family of beta-lactam used.

There were no differences in mortality in any of the subgroups:

- *Carbapenem or extended spectrum beta-lactam +/- aminoglycoside (RR 1.19, CI 0.73 to 1.92, three trials, and 513 patients, I^2 0%)*
- *Carbapenem or extended spectrum beta-lactam +/- fluoroquinolone (RR 0.96, CI 0.80 to 1.16, three trials, 1303 patients, I^2 0%)*
- *Other beta-lactams +/- aminoglycosides (RR 1.52, CI 0.28 to 8.09, two trials, 65 patients, I^2 28%). The wide confidence interval and the high statistical heterogeneity in this group are explained by the small number of events and patients.*

Appendix 1: Systematic review team

One critical care physician (GVG) coordinated all aspects of the review, including conduct of the literature search, screen relevant material, extract and analyze data, interpret the results of the meta-analytic procedures, investigate bias, and prepare the final report; the second reviewer was a PhD student in Medical Microbiology (CG), who conducted independent duplicate screening and data extraction. A critical care and infectious disease physician (AK) will provide content expertise and methodological advice. One librarian (HL) with expertise in systematic review search methodology provided advice regarding search strategies. A senior statistician and methodologist (RR) with extensive systematic review experience provided statistical expertise and oversight. One clinician/researcher with expertise conducting systematic reviews provided technical and methodological advice (AMAS) and a hematologist and critical care physician provided content expertise, as well as methodological and statistical guidance (RZ).

Appendix 2: Ovid Medline search strategy

1. exp sepsis/
2. shock, septic/
3. fasciitis, necrotizing/
4. Intestinal Perforation/
5. peritonitis/
6. exp pneumonia, bacterial/
7. ((blood or bloodstream) adj3 poison*).tw,kf.
8. py?emia*.tw,kf.
9. pyoh?emia*.tw,kf.
10. sepsis.tw,kf.
11. bacter?emia.tw,kf.
12. endotox?emia.tw,kf.
13. fung?emia.tw,kf.
14. candid?emia.tw,kf.
15. (shock adj3 (septic or endotoxic or toxic)).tw,kf.
16. (intestinal adj2 perforation*).tw,kf.
17. (necrotizing adj3 fasci?ti*).tw,kf.
18. peritonitis.tw,kf.
19. (pneumonia* adj2 (bacterial or chlamyodial* or mycoplasma or atypical or pneumococcal or rickettsial or staphylocc*)).tw,kf.
20. or/1-19
21. exp critical illness/
22. critical care/
23. exp emergency service, hospital/
24. (emergenc* intensive* or acute* or severe* or serious* or critical*).tw,kf.
25. or/21-24
26. 20 and 25
27. exp anti-bacterial agents/
28. exp antifungal agents/
29. 27 and 28
30. anti-infective agents/
31. exp sepsis/dt
32. shock, septic/dt
33. fasciitis, necrotizing/dt
34. intestinal perforation/dt
35. peritonitis/dt
36. exp pneumonia, bacterial/dt
37. or/30-36
38. exp drug therapy, combination/
39. drug synergism/
40. drug combinations/
41. ((combin* or dual) adj5 (drug* or therap* or chemotherap* or chemo-therap*)).tw,kf.
42. (polychemotherap* or poly-cheomtherap*).tw,kf.
43. (drug* adj3 (synergism* or augmentation* or potentiation*)).tw,kf.
44. or/38-43
45. 37 and 44
46. 29 or 45
47. 26 and 46
48. randomized controlled trial.pt.
49. controlled clinical trial.pt.
50. clinical trials as topic.sh.
51. (randomi#ed or randomly or RCT$1 or placebo*).tw.
52. ((singl* or doubl* or trebl* or tripl*) adj (mask* or blind* or dumm*)).tw.
53. trial.ti.
54. or/48-53
55. 47 and 54
56. animals/ not (animals/ and humans/)
57. editorial.pt.
58. (letter not (letter and randomized controlled trial)).pt.
59. 57 or 58
60. child/ not (exp adult/ and child/)
61. infant/ not (exp adult/ and infant/)
62. or/60-61
63. 55 not 56
64. 63 not 59
65. 64 not 62