Chapter

Toxicity can increase co-existence

Abstract

The composition of multispecies bacterial communities in various natural habitats, such as the microbiome in the human intestine, is determined by social interactions. Competition for resources, like nutrients and space, most often dominates. Nevertheless, bacteria can develop cooperative traits or aid others through accidental effects. For example, public resistance mechanisms based on intra- or extracellular enzymatic deactivation of antibiotics can offer cross-protection to sensitive neighboring cells, without co-evolution. In the first part of this chapter, we demonstrated that such accidental positive interactions could counterbalance competition, when the superior competitor relies on the cross-protection of the weaker competitor, thereby creating a stable co-culture. By specifically combining the probiotic strain *E. coli* Nissle and the pathogen *Salmonella* Typhimurium, our results indicate that cross-protection and its influence on microbial ecology can have major consequences for combinatorial therapy of antibiotics and probiotics in the medical sector. When the pathogen exhibits public resistance to the antibiotic and, in doing so, provides protection to the probiotic, the probiotic, in turn, will not be able to outcompete the pathogen. Conversely, in the second part of this chapter, we studied whether the introduction of competition could destabilize otherwise stable co-cultures depending on mutual cross-protection. Hereto, we compared the stability of intraspecies mutual cross-protecting communities with interspecies communities. Our study indicated a strong condition dependency for mutual cross-protection. Although it could be noted that in the presence of two antibiotics intraspecies mutual cross-protection led to a more stable community than interspecies cross-protection, the influence of interspecies competition seems to be limited. The variance in stability between the different communities was rather caused by variations in susceptibility to the antibiotics in combination with differences in degradation capacity and speed.

1.1 Introduction

Although, in the past, most research has been done on interactions between bacteria of the same species (intraspecies interactions), bacteria are social organisms that are most often embedded in complex, multispecies communities[3–5]. For example, several microbial ecosystems that are important to humans, such as the skin and gut microbiome, are heterogenous and consist of mixed species communities[303–308]. Therefore, over the past decades, the focus shifted towards interactions across species boundaries (interspecies interactions), revealing that the interactions between different species can have an important influence on the community, including structure, productivity and stability, as well as on tolerance to treatments, like antimicrobials[4–8].

These interspecies interactions in polymicrobial communities include competition for limited resources, such as nutrients and space[2,19,20,309], as well as cooperation, where bacteria evolve traits that have a positive influence on the survival of others. The best-known example of cooperation can be found in the cross-feeding of metabolites. The specialization in production of part of the metabolites and trading them with surrounding cells can be more beneficial than to work individually[202,310–315]. In addition to cooperation and competition, accidental effects, such as the detoxification of harmful environments, can occur. For example, β-lactamases, which are known to be secreted or contained in the periplasm of the cell, degrade β-lactam antibiotics, thereby lowering the antibiotic concentration in the medium and, consequently, protecting surrounding cells[9,316,317]. In addition, intracellular enzymatic deactivation of an antibiotic can lower the total concentration of active antibiotic in the environment, thereby also offering cross-protection. For instance, Tet(X2), a flavin-dependent monooxygenase, and CAT, a chloramphenicol acetyl transferase, can protect adjacent cells to tetracycline and chloramphenicol, respectively[50,316,318]. These degradation mechanisms are known as public resistance mechanisms, in contrast to private mechanisms, which focus on modifying the target, changing membrane permeability and limiting uptake or increasing efflux of the drug[319]. Public resistance mechanisms can be classified as commensalism, as the expression of the resistance mechanism is not driven by the presence of the sensitive surrounding bacteria, thus they are not evolutionary adaptations. In addition,

the fitness of the producer is, initially, unaffected by the benefit of the sensitive strain that profits from the offered protection[2].

While, from an evolutionary point of view, it is unfavorable to help strains that compete for the same resources, microbial communities are dynamic. The community composition and structure vary continuously due to large disturbances, like antibiotic exposure or shear force, changes in environmental conditions, invasion by novel strains or extinction[140,203,320]. As a result, the social environment of the strains within such multispecies communities alters and interactions with surrounding cells take place, without their behavior yet being adapted to this novel environment[4,321]. Consequently, it is possible that strains provide cross-protection to strains that compete for the same resources[9,316,317]. Moreover, it is possible that the positive interactions between the strains are stronger than the negative effect, allowing the positive interaction to evolve[13].

Moreover, there are some mechanisms that ensure the maintenance of public resistance mechanisms. One possible maintenance mechanism is the partial privatization of the public resistance mechanism[322–324]. At high antibiotic concentrations, for example, the initial degradation of the antibiotic will only be felt locally, before sufficient antibiotic has been broken down and the protection is shared with surrounding cells. Due to this partial privatization, it may also take some time before the resistant strain experiences disadvantages from the protection of the competing strain. Public resistance mechanisms can be maintained if the advantage of production and privatization is greater than the disadvantage of the competition, so that there is no selection against sharing the protection[322,323].

In this chapter, we investigate how cross-protection, either in one direction or in both directions, impacts the social interaction between competitors and the stability of the interspecies community. First, we aim to explore whether the competitive interactions, such as those involving a probiotic and a pathogen, can be compromised by a dependency for protection in a toxic environment, i.e. the presence of an antibiotic. We utilize a relevant model community consisting of two species that are known to interact *in situ* within the gastrointestinal tract. More specifically, we focus on the interactions between the pathogen *Salmonella* Typhimurium and the probiotic *Escherichia coli* Nissle 1917. *S*. Typhimurium displays the highest disease burden amongst

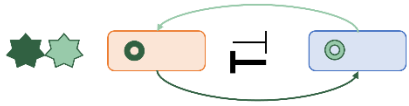
enteropathogens[58] and is becoming increasingly difficult to treat due to widespread antibiotic resistance. Indeed, in 2018 the EFSA reported that 40% of human *S*. Typhimurium isolates show multi-drug resistance[68]. Possible alternative treatment strategies include the use of probiotics[33,106,325] or combinatorial therapy of probiotics and antibiotics[326,327]. Hereto, the potential of the established probiotic *E. coli* Nissle to treat *S*. Typhimurium is often explored in humans as well as in animals[204,328–330]. *E. coli* Nissle 1917 is the most extensively studied probiotic within the Enterobacteriaceae family and is recognized for its array of positive effects on the host[328]. Moreover, it has been shown that *E. coli* Nissle 1917 can safeguard the host against *S*. Typhimurium infections through diverse mechanisms, including interference with the adhesion and invasion of the pathogen[204,330]. Besides, the probiotic can compete with *S*. Typhimurium for scarce nutrients, such as iron[329], and through the production of colicins and microcins[205]. Second, we aim to elucidate the importance of interspecies competition on the stability of communities relying on mutual cross-protection in the presence of two antibiotics. To achieve this objective, we compare the stability of intra- and interspecies communities over time. An overview of the most important experimental work to accomplish both objectives is represented in Figure 4.1.

A) One-sided interspecies cross-protection

1) EcN relies on ST

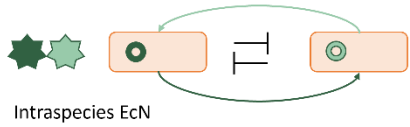
2) ST relies on EcN

B) Two-sided interspecies cross-protection

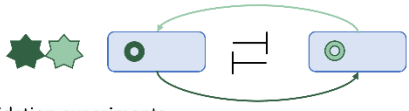


C) Two-sided intraspecies cross-protection

1) Intraspecies ST

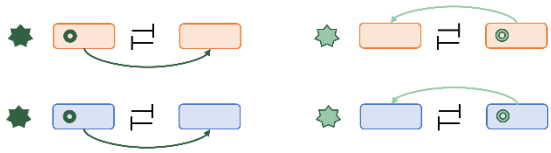


2) Intraspecies EcN

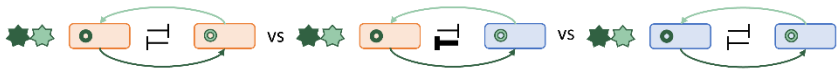


D) Validation experiments

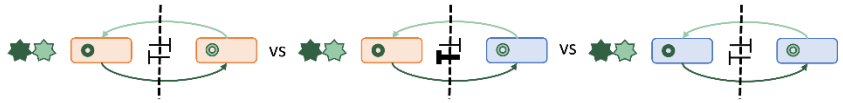
1) One-sided intraspecies cross-protection



2) Ratio experiments (1:999 – 999:1)



3) Supernatants experiments



Legend

Strains

Salmonella Typhimurium (ST)

E. coli Nissle (EcN)

Antibiotics

Cefotaxime

Chloramphenicol

Resistance mechanisms

pGDPI CTX-M-15

pGDPI CAT

Social interactions

Competitive inhibition

Cross-protection

Interaction strength

Figure 4.1: Schematic overview of the experimental work in this chapter. First, we want to investigate whether dependency on cross-protection in a toxic environment can neutralize competition between a probiotic and a pathogen. **A)** After verification of the competitive overhand of *E. coli* Nissle (EcN) compared to *Salmonella* Typhimurium (ST), the one-sided interspecies cross-protection is studied. We compare the stability of the duo-cultures in which the strong EcN relies on the protection offered by ST and vice versa. **A1)** We hypothesise that, if EcN relies on ST for survival, the pathogenic strain will not be completely outcompeted, but rather oscillatory dynamics will occur. If EcN becomes too abundant and starts to outcompete ST, the cross-protection concurrently decreases, ultimately leading to a reduction in EcN. The subsequent competitive relieve allows ST to regrow and a novel cycle can start. **A2)** Conversely, when ST relies on EcN, we predict that the competitive advantage of the probiotic increases, leading to collapse of the ST population. **B)** Second, we aim to gain a better understanding of the importance of interspecies competition on the stability of mutual cross-protective communities. Therefore, a short-term co-culture experiment in the presence of both antibiotics is performed. As the survival of one strain depends on the degradation of the antibiotic by the other strain, again oscillatory dynamics are expected. **C)** As a reference, the stability of both the ST and EcN intraspecies communities are studied. In these intraspecies communities, competition is believed to be limited to competition for resources. As the used strains within one species are isogenic, solely differing in fluorescent label and the public resistance mechanism, no differences in growth rate are expected. **D)** After assessing the stability of both inter- and intraspecies communities, additional validation experiments are conducted to elucidate the underlying mechanisms responsible for the observed differences in stability between the communities. **D1)** To gain insights in the strength of cross-protection offered by each strain in the absence of interspecies competition, one sided intraspecies cross-protection experiments were performed. **D2)** To assess the extent of cross-protection across various strain ratios and densities, ratio experiments were conducted in both inter- and intraspecies communities in the presence of both antibiotics. Ratios ranging from 1:999 to 999:1 were tested. **D3)** Finally, to gain a better understanding of the sensitivity to and degradation rate and capacity of the antibiotics of the different strains, supernatants experiments were conducted.

1.2 Materials & methods

1.2.1 Bacterial strains and plasmids

To visually distinguish *E. coli* Nissle and *Salmonella* Typhimurium, or to differentiate between strains that contained the different resistance mechanisms, we worked with fluorescent labels. Hereto, we selected green and red fluorescent proteins, that were easily separable during CytoFLEX S measurements as well as under the Illumatool. For *E. coli* Nissle, the fluorescent proteins sfGFP and tdTomato were used, while *Salmonella* was labelled with either sfGFP or mCherry via scarless genome editing. The expression of all fluorescent markers was controlled by a constitutive p_{λ}-promoter. To keep consistent, the chloramphenicol resistant strains was always labelled with the green fluorescent protein, while the cefotaxime resistant strain was red. The *ramR* knock-out mutant of *Salmonella* Typhimurium SL1344 was generated using a protocol adapted from Datsenko and Wanner[268]. TOP10 cells were used during the construction of different plasmids. LB broth (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) and LB agar (LB broth, 15 g/L bacteriological agar) were used during the construction of the different strains and plasmids used throughout this chapter (Table 4.1, Table 4.2).

Table 4.1: Overview of the bacterial strains used throughout this chapter

Bacterial strain	Identifier	Reference
Salmonella Typhimurium SL1344	*Salmonella* Typhimurium WT	[229,230]
Salmonella Typhimurium SL1344 sfGFP	*Salmonella* Typhimurium sfGFP Or ST sfGFP	MiCA lab, unpublished
Salmonella Typhimurium SL1344 mCherry	*Salmonella* Typhimurium mCherry Or ST mCherry	MiCA lab, unpublished
Salmonella Typhimurium SL1344 Δ*ramR*	*Salmonella* Typhimurium Δ*ramR*	MiCA lab, unpublished
Escherichia coli Nissle 1917	*E. coli* Nissle WT	[331]
Escherichia coli Nissle 1917 sfGFP	*E. coli* Nissle sfGFP Or EcN sfGFP	MiCA lab, unpublished
Escherichia coli Nissle 1917 tdTomato	*E. coli* Nissle tdTomato Or EcN tdTomato	MiCA lab, unpublished
Escherichia coli TOP10	TOP10	Invitrogen

The pGDPI:CTX-M-15 plasmid was constructed using restriction enzymes. Hereto, the *CTX-M-15* gene was amplified from the pACYC184:CTX-M-15 by Q5 PCR, attaching a XbaI and XhoI restriction site at 5' end and 3' end, respectively, using primers S&P-02070 and S&P 02071. For the backbone, pGDPI:/ was used as a template that already contained the requested restriction sites. For the restriction and ligation, the protocol supplied by New England Biolabs was followed. The construction of the novel plasmid was verified via Sanger sequencing, using primers S&P-02041 and S&P02072 for the first halve, and primers S&P-02073 and S&P-02042 for the second halve. Thereafter, the plasmid was electroporated in the strains of interest, which was verified via Taq-PCR using primers S&P-02041 and S&P-02042.

For the construction of the pGDPI:CAT plasmid, the *CAT* gene was amplified from the similar pGDP3-backbone. Therefore, a compatible combination of restriction enzymes between both plasmids was found in NcoI and XhoI. Restriction and ligation were performed following the protocol New England Biolabs. The construction of the plasmid was validated by Sanger sequencing, while the electroporation in the strains of interest was verified by Taq-PCR, both based on the primers S&P-02041 and S&P-02042.

The *floR* gene and putative promoter region were introduced in the pGDPI backbone via the NEBuilder Gibson Assembly® Protocol (New England Biolabs) in TOP10 cells, using primers S&P-02395 and S&P-02396 for the amplification of the gene of interest and attachment of overlapping tails with the backbone during Q5-PCR, while the backbone was amplified using primers S&P-02397 and S&P-02398. All used primers are shown in Table S. 4.1.

Table 4.2: Overview of plasmids used throughout this chapter

Plasmid	Characteristics	Reference
pGDPI:/	Plasmid constructed by the Wright lab, containing an origin of replication derived from the pBR322 plasmid, a multiple cloning site (MCS), a β-lactamase promoter (P_{bla}) and a kanamycin (Km^R) selection marker.	[332]
pGDPI:CTX-M-15	pGDP1 plasmid backbone with the *CTX-M-15* gene cloned into the MCS, encoding a β-lactamase, conferring resistance to cefotaxime.	This work
pGDPI:CAT	pGDP1 plasmid backbone with the *CAT* gene cloned into the MCS, encoding a chloramphenicol acetyl transferase, conferring resistance to chloramphenicol	This work
pGDPI:FloR	pGDP1 plasmid backbone with the *floR* gene and putative promoter region into the MCS, encoding a phenicol-specific efflux pump, conferring resistance to chloramphenicol	This work
pGDP3:CAT	Plasmid constructed by the Wright lab, containing an origin of replication derived from the pBR322 plasmid, a β-lactamase promoter (P_{bla}) controlling the *CAT* gene and an ampicillin (Ap^R) selection marker.	[332]
pACYC184:CTX-M-15	Low copy plasmid with *the CTX-M-15* gene inserted using the BamHI and EcoRV restriction sites, containing a tetracycline resistance cassette.	MiCA, unpublished
p39R861-4	Plasmid, obtained from Ruth Hall, containing resistance against chloramphenicol and florfenicol (FloR), sulphonamides and tetracycline.	[333]

1.2.2 Co-culture experiments

Growth conditions

For the co-culture experiments and monoculture growth controls, single colonies were picked, inoculated in 5 mL CFA (broth, 10 g/L casamino acids, 1.5 g/L yeast extract, 50 mg/L $MgSO_4$, 5 mg/L $MnCl_2$, adjusted to pH 7.4) and grown overnight at 37°C, shaking at 200 rpm. Thereafter, all overnight cultures were adjusted to an OD_{595} of 2.5 and further diluted. Each strain was added to the co-culture or monoculture experiment at a final density of 1.5 10^6 CFU/mL. Co-culture experiments were performed by mixing the strains of interest in 200 µL CFA in 96-well plates. To test cross-protection, chloramphenicol was added from the start of the experiment at a final concentration of 16 µg/mL, while cefotaxime was supplemented after 2 hours at a concentration of 0.25 µg/mL, unless mentioned otherwise. After preparation, the 96-well plates were sealed with a gas-permeable, sterile membrane (Greiner Bio-one) and a lid and incubated at 37°C for 24 hours, shaking at 200 rpm.

For the 24-hour cross-protection validation experiment, the cell densities were measured using a spot-plating method after 24 h of incubation. For the short-term co-culture experiments, the cultures were diluted daily (1/100) in fresh CFA medium, supplemented with chloramphenicol, while cefotaxime was added 2 hours post dilution, where necessary. Every cycle, the cell densities of both populations in the co-culture were measured using flowcytometry.

Cell density quantification

Spot-plating: To limit the detection of dead, fluorescent cells and determine the cross-protection more accurately, the first 24-hour cross-protection validation experiment in the presence of both antibiotics was plated out using a spot-plating method. Hereto, 10 µL of a tenfold serial dilution of each sample was spotted in triplicate (on LB agar, LB agar supplemented with cefotaxime and LB agar with chloramphenicol). Agar plates with spots were incubated at 37°C overnight, after which the different fluorescent cells were counted under the Illumatool Tunable Lighting System (Lightools Research, Encinitas, California, United States).

Flowcytometry: The inoculum density as well as the daily cell counts of the co-culture experiments were measured after dilution in filtered PBS (1.24 g/L K_2HPO, 0.39 g/L KH_2PO_4, 8.8 g/L NaCl) by the CytoFLEX S (Beckman Coulter) using the following settings: FSC 1000, SSC 1000, FITC 2000 and ECD 2000. Only dilutions with 100-2000 events/s were taken into account. Each sample was measured for exactly one minute at 20 µL/min. During data processing, the first ten seconds were excluded to limit the influence of carry-over from previous samples and allow stabilization of the measurement. Besides, the gates that distinguish the different fluorescent populations were adjusted daily, but were kept constant for all measurements that day. As a negative control, sterile filtered PBS was measured following the same settings. For all samples, the average number of measured events in the negative control (n = 5) in the same gate was subtracted prior to further processing, which includes the calculation of the original cell density in the undiluted sample by taking into account the dilution. The LOD was calculated as three times the standard deviation of the sterile PBS samples within the same gate. Thereafter, this LOD was per sample corrected for the applied dilution, giving rise to different LODs for different samples on the same day. Cell count values below the LOD were substituted by LOD/2.

1.2.3 Ratio experiments

Ratio experiments were set up like co-culture experiments (section 4.2.2), except that the total cell density over both strains was kept constant at 3 10^6 CFU/mL and the ratio between the different strains was varied. Inoculum densities per strain varied between 3 10^3 and 3 10^6. The cell density after 24 h was determined via flow cytometry (section 4.2.2).

The theoretical, exponential growth of the chloramphenicol resistant strain after two hours was calculated by doubling the cell number for every half hour. In the alternative representation of the ratio experiment, the relative growth of the chloramphenicol resistant strain was calculated as the difference in ratio (chloramphenicol resistant cells/total cell count) at the end of the experiment compared to the inoculum ratio.

1.2.4 Minimum inhibitory concentration experiments

For the determination of the MIC, single colonies were picked, inoculated in 5 mL CFA and grown overnight at 37°C, shaking at 200 rpm. Thereafter, all overnight cultures were adjusted to an OD_{595} of 2.5 and further diluted to a

concentration of 1 10^6 CFU/mL. Then, a 96-well plate was filled with 50 µL CFA, except for the first column. The first column of the 96-well plate was filled with 100 µL antibiotic working solution (256 µg/mL) and a two-fold serial dilution was made by transferring 50 µL to the next column, ultimately reaching a concentration of 0.0156 µg/mL. Thereafter, 50 µL of the adjusted cell culture was added to each of the wells, diluting it to a concentration of 5 10^5 CFU/mL. In addition, a positive growth control without antibiotics and a negative, sterile control without cell culture were included. Finally, the plates were sealed with a gas-permeable, sterile membrane and a lid and incubated at 37°C for 24 hours, shaking at 200 rpm. After incubation, the growth of the strains was measured as the OD_{595}. The MIC value was determined per bacterial strain as the lowest antibiotic concentration were there was no measurable increase in OD_{595} compared to the negative sterile control, thus the lowest concentration that fully inhibited growth.

After the short-term co-culture experiments in the presence of one or both antibiotics, the MIC tests were repeated on the surviving strains. No change in MIC was observed for any of the tested colonies, leading to the conclusion that no evolution of resistance had occurred.

1.2.5 Supernatants experiments

To start the supernatants experiment, single colonies of the sensitive as well as the resistant strains were picked, inoculated in 5 mL CFA and grown overnight at 37°C, shaking at 200 rpm. Then, all cultures were adjusted to an OD of 2.5 and further diluted. The resistant strains were added to falcons containing 10 mL CFA, supplemented with either 16 µg/mL chloramphenicol for the strains carrying the pGDPI:CAT plasmid, or 0.25 µg/mL cefotaxime for the strains with the pGDPI:CTX-M-15 plasmid, at final concentrations of 3 10^3, 3 10^4, 3 10^5 and 3 10^6. The falcons with chloramphenicol were incubated at 37°C, shaking for 2 or 6 hours, while the falcons with cefotaxime were incubated for 1, 2 or 4 hours. Thereafter, the cultures were spun down and the supernatants was collected. After filter sterilization with a Millex-GP 0.22 µm Membrane Filter Unit (Merck Millipore, Burlington, Massachusetts, United States), the supernatants were used as growth medium for the sensitive strains. Hereto, the sensitive strains were added at a final concentration of 3 10^3, 3 10^4, 3 10^5 and 3 10^6 in 200 µL supernatants in a 96-well plate. After 20 hours, the growth was measured as the absorbance at

OD_{595}. The absorbance of the supernatants itself was subtracted during data processing as a correction. Besides, the supernatants of the same strains, but in the absence of any antibiotics, was used as a positive growth control.

1.3 Results

1.3.1 *E. coli* Nissle is a stronger competitor than *Salmonella* Typhimurium in the absence of antibiotics

Before studying the change in interspecies dynamics upon addition of a stressor in the growth medium, we first characterized the baseline interspecies interactions in the absence of antibiotics. When the probiotic *E. coli* Nissle and the pathogen *Salmonella* Typhimurium are put together in co-culture conditions in which both strains can grow and are diluted 1/100 daily into fresh growth medium, *E. coli* Nissle exhibits a progressive competitive advantage. It gradually takes the upper hand of *Salmonella* over the course of four days, ultimately leading to up to two-log differences (Figure 4.2). By extrapolating these results over time, we hypothesize that competitive exclusion will ultimately occur. The competitive advantage of *E. coli* Nissle can be two-fold, including both exploitative and interference competition. On the one hand, previous research in our laboratory in similar *in vitro* experiments showed that *E. coli* Nissle has a higher growth rate than *Salmonella* Typhimurium. On the other hand, *E. coli* Nissle is known for its production of microcins and colicins, proteinaceous toxins that can inhibit other bacterial strains, including *Salmonella* Typhimurium[199–201]. Monoculture experiments, containing two isogenic strains that only differ in fluorescent label, were performed in parallel for *S*. Typhimurium as well as *E. coli* Nissle as a growth control (Figure S. 4.3 & Figure S. 4.4, respectively).

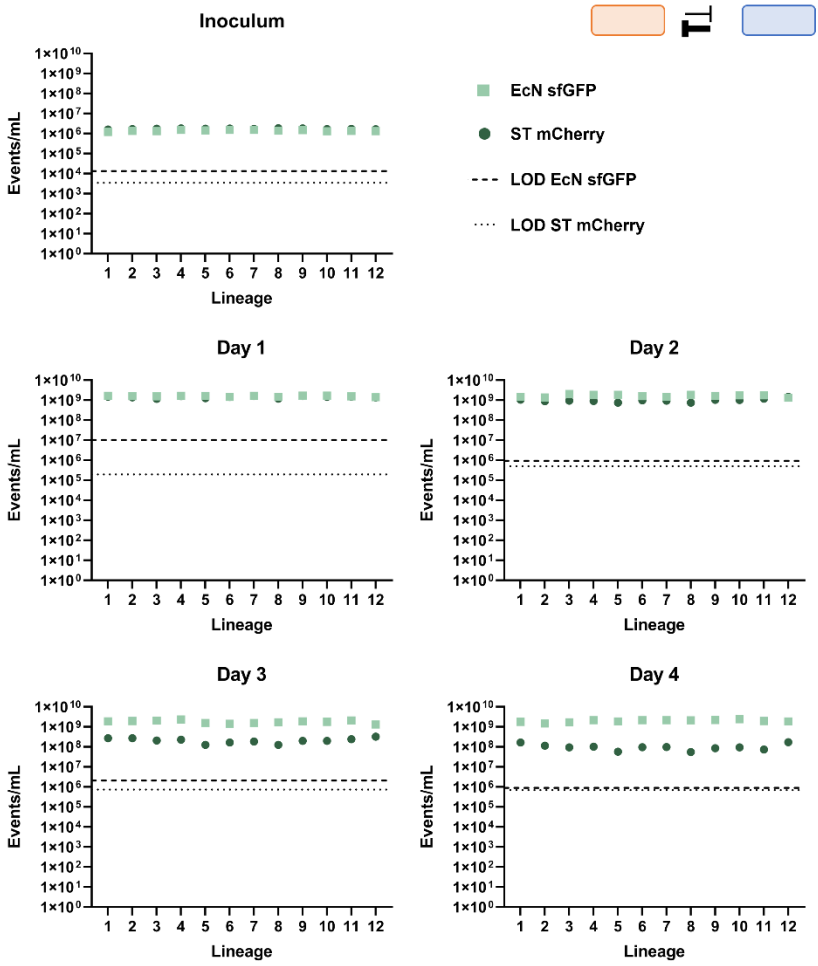


Figure 4.2: Competition experiment between *E. coli* Nissle and *Salmonella* Typhimurium in the absence of antibiotics. The cell counts of twelve biological repeats, originating from different overnight cultures, were followed over the course of four days. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Events/mL, representing cell counts, were measured each cycle using the CytoFLEX S.

1.3.2 Cross-protection against a toxic environment can increase co-existence

Thereafter, we studied whether cross-protection can neutralize the previously observed competition and, thus, can increase co-existence of both strains. Initially, we chose to make the stronger competitor, *E. coli* Nissle, dependent

on the protection offered by *Salmonella*, thereby trying to restore the balance. Hereto, we introduced a plasmid that constitutively expresses a β-lactamase, CTX-M-15, into *Salmonella*. The enzymatic degradation of β-lactam-antibiotics by β-lactamases is known to share its protection with surrounding cells through reduction of the antibiotic concentration in the medium[5,50]. The antibiotic concentration in the medium was set to four times the minimum inhibitory concentration (MIC) of the sensitive strain, as this is commonly used in clinical treatment. In addition, multiple studies have reported that the duration of treatment above four times the MIC is important for the successful therapeutic outcome in critically ill patients[334–338].

As part of the adjustment of the number of cells of each strain for the inoculum, overnight cultures were centrifuged and resuspended in sterile PBS. As a result, any secreted β-lactamases were removed. Since cefotaxime has a rapid and bactericidal activity, we administered cefotaxime two hours after inoculation in order to give the resistant *Salmonella* Typhimurium the opportunity to produce and secrete novel β-lactamases and thus provide immediate protection to *E. coli* Nissle upon administration of the antibiotic. Although the washing step only occurred during the preparation of the inoculum on day 0 and not during the daily dilution in fresh medium, the time of addition of the antibiotic was kept constant for the entire duration of the experiment.

The results, depicted in Figure 4.3, show that after one day of incubation in the presence of cefotaxime, *Salmonella* Typhimurium outnumbered the sensitive *E. coli* Nissle, displaying a two-log difference in population size. We hypothesize that, subsequently, the larger resistant *Salmonella* Typhimurium population entails faster degradation of the antibiotic in the medium the following day, limiting the cell death of *E. coli* Nissle and allowing rapid growth of the probiotic strain. *E. coli* Nissle does not bear any costs for the degradation of the antibiotic, but can enjoy the benefits of the shared protection, a phenomenon termed cheating[339,340]. Because of the combination of cheating and higher competitive strength, the probiotic strain gained the upper hand by day 4 and reduced the *Salmonella* Typhimurium cell numbers. However, we expect that when the protective *Salmonella* Typhimurium population becomes too small, the degradation of the antibiotic will decrease or partly privatize, causing the number of sensitive *E. coli* Nissle cells to

decrease. This creates a balance between dependence of protection by *Salmonella* Typhimurium and cheating or competition, which may lead to oscillatory dynamics or an equilibrium ratio[50,341–344]. Nonetheless, due to the limited length of the co-culture experiment, this could not be observed.

However, when we decreased the antibiotic concentration to three times the MIC, thereby reducing the dependency of *E. coli* Nissle on *Salmonella* Typhimurium and accelerating the degradation of the antibiotic in the medium, we accelerated the dynamics of the interspecies interactions in the antibiotic depleted medium (Figure 4.4). As a result, the competitive advantage of *E. coli* Nissle manifests quicker, which gives *E. coli* Nissle the upper hand at day 2 and even allows it to outcompete *Salmonella* on day 3. Nevertheless, on day 4, the cell number of the sensitive *E. coli* Nissle decreases and the resistant *Salmonella* population can recover until the same population density is reached for both strains. This might be explained by the lower *Salmonella* cell count, leading to higher initial privatization of the antibiotic degradation[345], giving *Salmonella* a growth advantage and causing the cell count of *E. coli* Nissle to slightly decrease. Ultimately, the dependency of *E. coli* Nissle on *Salmonella* did increase the co-existence based on toxicity. In addition, the setback of the *E. coli* cell count and the recovery of the resistant *Salmonella* possibly indicates the onset of oscillatory dynamics.

In addition, we studied the impact of making the weaker competitor, *Salmonella* Typhimurium, rely on *E. coli* Nissle to provide protection against chloramphenicol at a concentration of four times the MIC of the sensitive strain (Figure 4.5). Hereto, a plasmid containing chloramphenicol acetyl transferase (CAT) under the control of a constitutive promoter was introduced in *E. coli* Nissle. Given that chloramphenicol, in contrast to cefotaxime, functions as a bacteriostatic, we opted to add it already at the start of the experiment as immediate protection is not required for the survival of the sensitive population. The sensitive *Salmonella* Typhimurium showed a strong decrease in population size over the course of the four-day experiment, ultimately leading to cell counts below the limit of detection. Some cross-protection was, however, offered during the first days of the experiment, as evidenced by the growth on day 1, compared to the inoculum density, and the almost complete recovery of the cell number after the 1/100 dilution towards day 2. Nevertheless, no stable co-culture was formed in this setup. Moreover,

the competitive exclusion of *Salmonella* occurred faster within this community, than in the absence of antibiotics, which might indicate that the dependency of *Salmonella* on *E. coli* provided an additional advantage to *E. coli* Nissle, thereby increasing the imbalance between both strains.

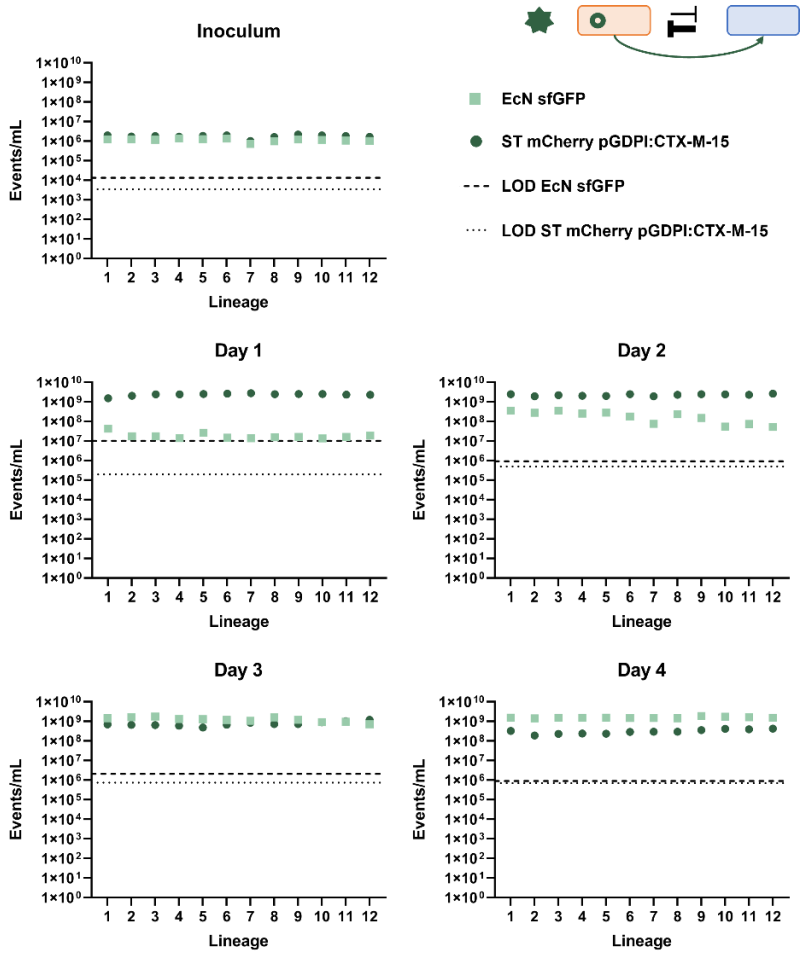


Figure 4.3: Interspecies ecological interaction where *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 offers cross-protection to *E. coli* Nissle sfGFP against cefotaxime at a concentration of 4x MIC. MIC_{EcN} = 0.0625 µg/mL cefotaxime. Twelve biological repeats were followed over the course of four days. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, cefotaxime was added to the medium 2 h after dilution at a concentration of 0.25 µg/mL. The strong initial growth of *Salmonella* Typhimurium allows faster degradation of the antibiotic in the growth medium the subsequent days, allowing *E. coli* Nissle to gradually increase its cell number, until eventually taking the upper hand. Cell counts were measured each cycle using the CytoFLEX S.

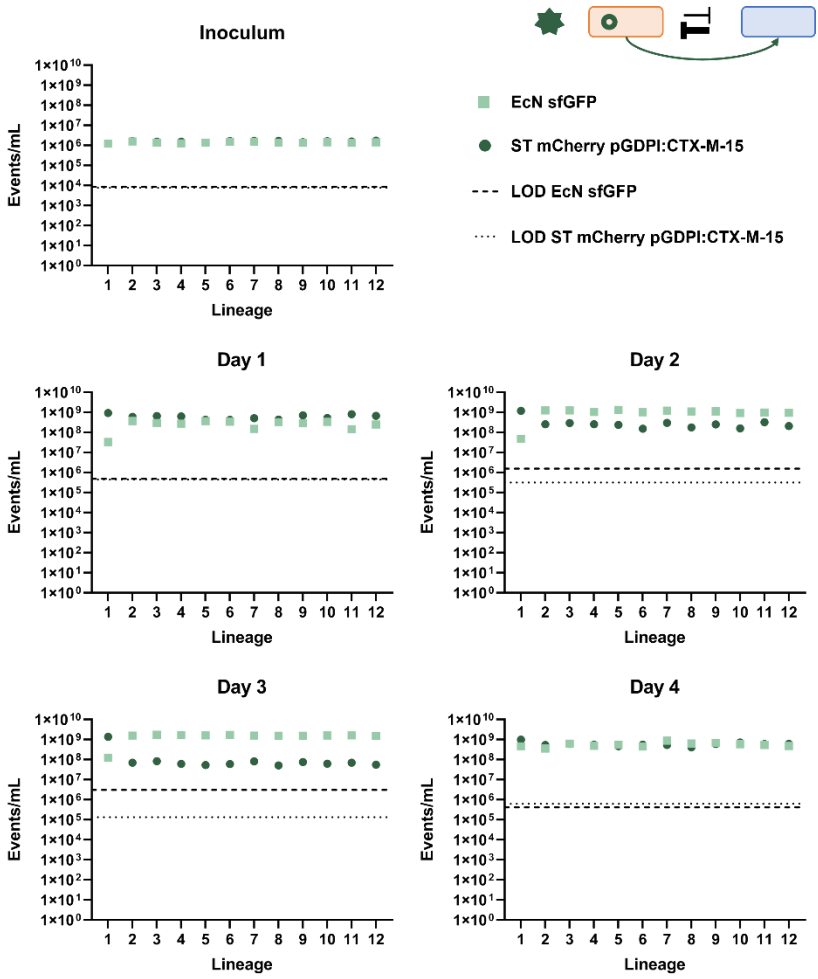


Figure 4.4: Interspecies ecological interaction where *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 offers cross-protection to *E. coli* Nissle sfGFP against cefotaxime at a concentration of 3x MIC. MIC_{EcN} = 0.0625 µg/mL cefotaxime. Twelve biological repeats were followed over the course of four days. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, cefotaxime was added to the medium 2 h after dilution at a concentration of 0.1875 µg/mL. Because of the lower antibiotic concentration, the dependency of *E. coli* Nissle on *Salmonella* Typhimurium is reduced and the interspecies interactions as well as cheating are more outspoken. Subsequently, *E. coli* Nissle gains the upper hand by day 2 and slightly outcompetes *Salmonella* Typhimurium by day 3. However, the lower cell counts of the protective *Salmonella* on day 3, decreases the cross-protection offered to *E. coli* Nissle, thereby restoring the equilibrium. Cell counts were measured each cycle using the CytoFLEX S.

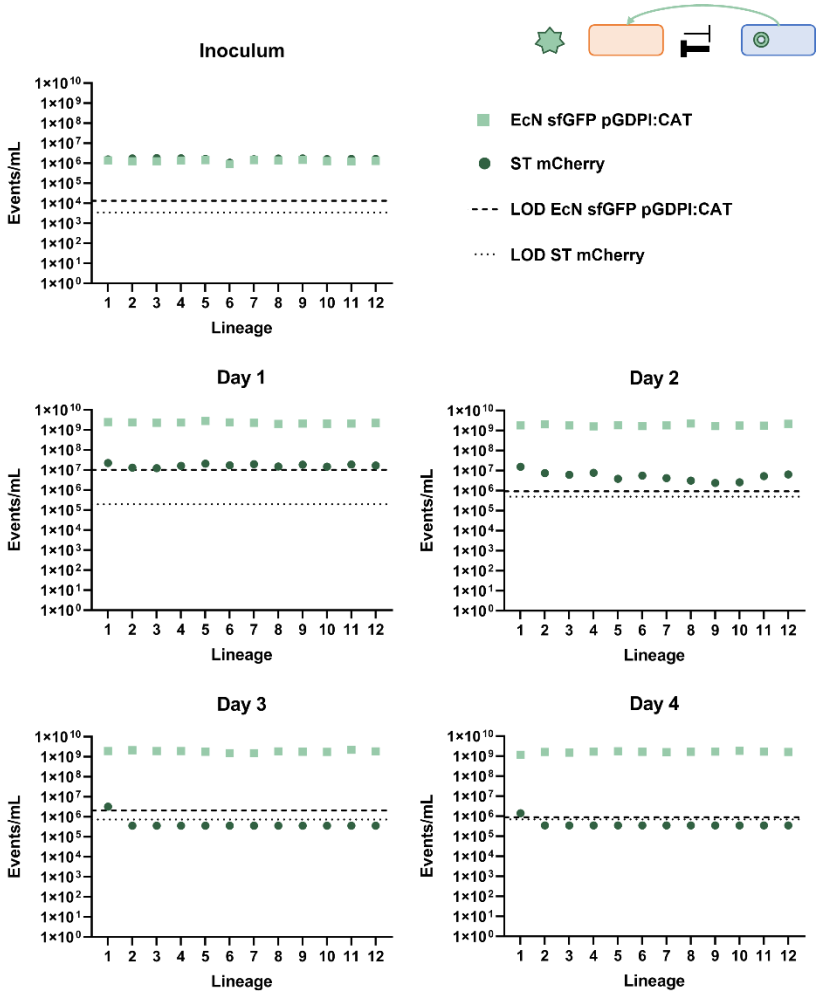


Figure 4.5: Interspecies ecological interaction in medium supplemented with chloramphenicol at a concentration of 4x MIC of *Salmonella* Typhimurium mCherry, making it dependent on the cross-protection of *E. coli* Nissle sfGFP pGDPI:CAT. MIC_{ST} = 4 µg/mL chloramphenicol. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol was added to the medium during the dilution at a concentration of 16 µg/mL. Although cross-protection was offered to *Salmonella* during the first days of the co-culture experiment, the private advantage of producing the resistance mechanism for *E. coli* Nissle exceeded the shared benefits, despite the associated cost, thereby limiting cheating. Moreover, *E. coli* Nissle has the competitive advantage, causing *Salmonella* to disappear from the community. Cell counts were measured each cycle using the CytoFLEX S.

1.3.3 Interspecies co-dependency in a toxic environment cannot stabilize competitive communities at high antibiotic concentrations

One sided beneficial interspecies interactions can occur in natural ecosystems, but are likely accidental as there is no selection pressure to protect another species that does not influence the donor's fitness. However, these interactions can develop in true interspecies cooperative interactions based on natural selection, if the strain that receives the benefit adapts to provide a benefit to the donor strain[2]. Moreover, it cannot be ruled out that mutually positive accidental interactions do occur. Here we explore whether such interactions can be maintained in initially competitive communities by looking at the stability of the *Salmonella-E. coli* community with reciprocal cross-protection.

Hereto, we first validated the mutual interspecies cross-protection in the presence of both antibiotics after 24 hours of co-culture. Similar to the previous co-culture experiments, a washing step was introduced during the preparation of the strains. Consequently, chloramphenicol was mixed in the medium from the start, while cefotaxime was added after two hours, to allow a β-lactamase buffer built-up. Several control communities, such as combinations of natural, sensitive strains as well as strains expressing private resistance mechanisms, were included in this setup.

Increased efflux was chosen as private resistance mechanism for both strains. For *E. coli* Nissle, we used a mutant strain that constitutively overexpresses FloR, a chloramphenicol- and florfenicol-specific efflux pump. In *Salmonella* Typhimurium, we knocked-out the genomic *ramR* gene that expresses a repressor of the AcrAB efflux pump, thereby increasing efflux. Thereafter, cross-resistance offered by the public and private resistance mechanisms was studied in a MIC-test (Figure S. 4.1). In addition, strains containing the empty non-conjugating pGDPI:/ plasmid were considered to verify that the kanamycin resistance, that serves as a selection marker, did not influence the MIC.

Although the *ramR* knock-out mutant doubled the MIC of *Salmonella* Typhimurium towards chloramphenicol, thus offering some cross-resistance, the concentrations used during our experiment still twofold exceed this

concentration. As this strain is solely used during the control experiment, no significant impact is expected. Furthermore, all other private and public resistance mechanisms exhibit no cross-resistance. In addition, the kanamycin resistance selection marker in the pGDPI plasmid also did not offer any cross-resistance to the used antibiotics.

In the 24-hour cross-protection experiment with two antibiotics, both *Salmonella* Typhimurium and *E. coli* Nissle performed better in each other's presence than in monoculture ($p = 0.0558$ and $p = 0.0009$, respectively) (Figure 4.6, Figure S. 4.2), if both strains expressed a public resistance mechanism that degraded one of both antibiotics. The strains with a private resistance mechanism only had a minor, non-statistically significant, effect on the antibiotic susceptibility of the other strain in duo-culture conditions, confirming that the improved growth of both strains can be attributed to the protection provided by the public resistance mechanism and was not due to an inoculum effect provided by the resistant strain or the activation of stress responses due to competition[46]. Competition, however, might explain the decreases in survival.

After validation of the mutual cross-protection in the presence of both antibiotics, a short co-culture experiment was set up to further study the interspecies interactions (Figure 4.7). On the first day, *Salmonella* Typhimurium displays strong and constant growth in all lineages, while the population density of *E. coli* Nissle varies per repeat and a number of the *E. coli* populations even fall below the limit of detection. A first hypothesis states that the low inoculum density of the cefotaxime-resistant *Salmonella* offers limited protection to *E. coli*, as was also observed in the one-sided interspecies experiment on the first day. Second, this could be rationalized by the initial unlimited two-hour growth of *E. coli* Nissle pGDPI:CAT, accompanied by degradation of chloramphenicol, protecting the *Salmonella*. After the addition of cefotaxime and subsequent partial killing of the cefotaxime-sensitive *E. coli* Nissle population, recovery can take place once sufficient cefotaxime is degraded.

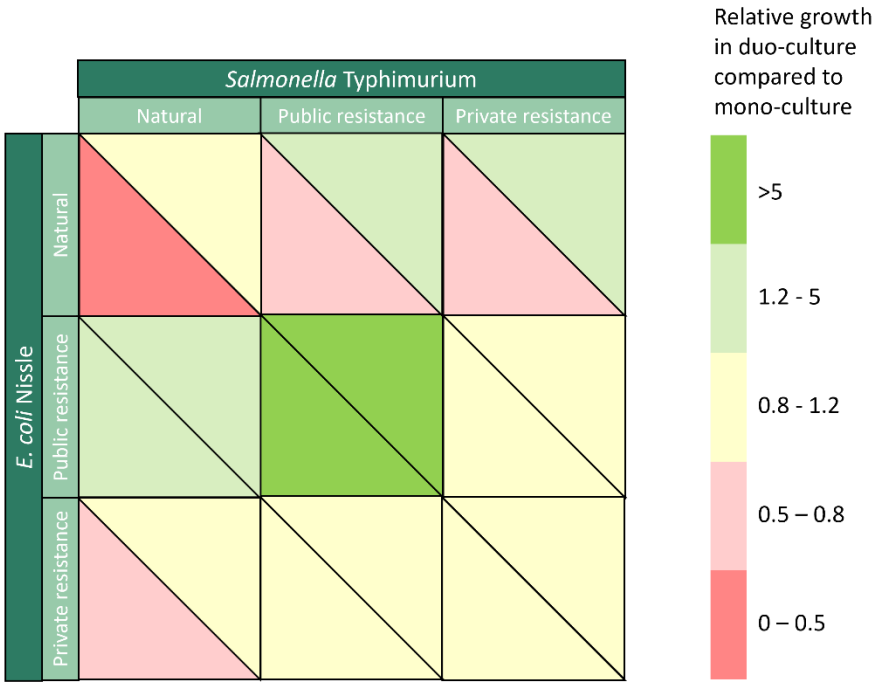


Figure 4.6: In the presence of both chloramphenicol, added from the start, and cefotaxime, added after 2 hours, *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 and *E. coli* Nissle sfGFP pGDPI:CAT offer mutual cross-protection. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm for 24 h. The color scale indicates the relative growth of *Salmonella* Typhimurium, upper triangle, and *E. coli* Nissle, lower triangle, in duo-culture compared to monoculture, calculated as the CFU/mL in duo-culture divided by the CFU/mL of the same strain in monoculture. Green colors indicate stimulated growth in duo-culture, while red colors indicate inhibition. Cross-protection is observed when both strains perform better in co-culture than in monoculture, which only occurred when both strains express a public resistance mechanism. Limited protection or inhibition was found in all other combinations. Cell counts were obtained via spot-plating. Selective agar was used where possible. The absolute cell counts and statistical analysis are shown in Figure S. 4.2.

After the establishment of dominance by the *Salmonella* strain, we anticipated the initiation of oscillatory dynamics. In our setup, the growth potential of one strain depends on the population density and antibiotic degradation of the other, thereby creating the potential for negative frequency-dependent selection. This anticipated shift in dominance to the chloramphenicol-resistant *E. coli* Nissle was indeed found to occur on day 2. Thereafter, however, oscillation does not occur and *E. coli* remains dominant. One possible explanation for the absence of oscillatory dynamics, is that the effect

of the enhanced protection conferred by the larger *E. coli* Nissle population on *Salmonella* is smaller than the increased competitive pressure of *E. coli* Nissle towards *Salmonella*. In addition, as also suspected in the experiments with one-sided protection, the cross-protection of CAT might be rather limited compared to the cefotaxime degradation by the β-lactamase. After day 2, the total cell density gradually decreases, suggesting a lack of protection of *E. coli* Nissle against the bactericidal cefotaxime by the smaller β-lactamase-producing *Salmonella* population. Ultimately the community is headed for collapse, as evidenced by the strong reduction in population size towards day 4. Hereto, it is important to note that the cell counts via the CytoFLEX S are based on fluorescence, meaning that dead but intact cells are still counted. For example, the two-log reduction in cell number towards day 4 most likely indicates that the cells are no longer active or growing, but that we are observing the diluted population from the day before. Consequently, we assume that the community has completely collapsed on day 4 and will not recover. Moreover, the absolute cell number appears to be important for rapid degradation of the antibiotics and, thus, to provide cross-protection to the sensitive strain. The low cell numbers on day 4 are, therefore, an additional reason to assume that the community is no longer able to recover. Conclusively, the nutrient and time limitations in combination with the interspecies interactions and growth rates upon antibiotic degradation most probably determine the exact outcome of the community. However, to unravel the exact dynamics of the community over the course of 24 hours, additional measurements at different timepoints are advised.

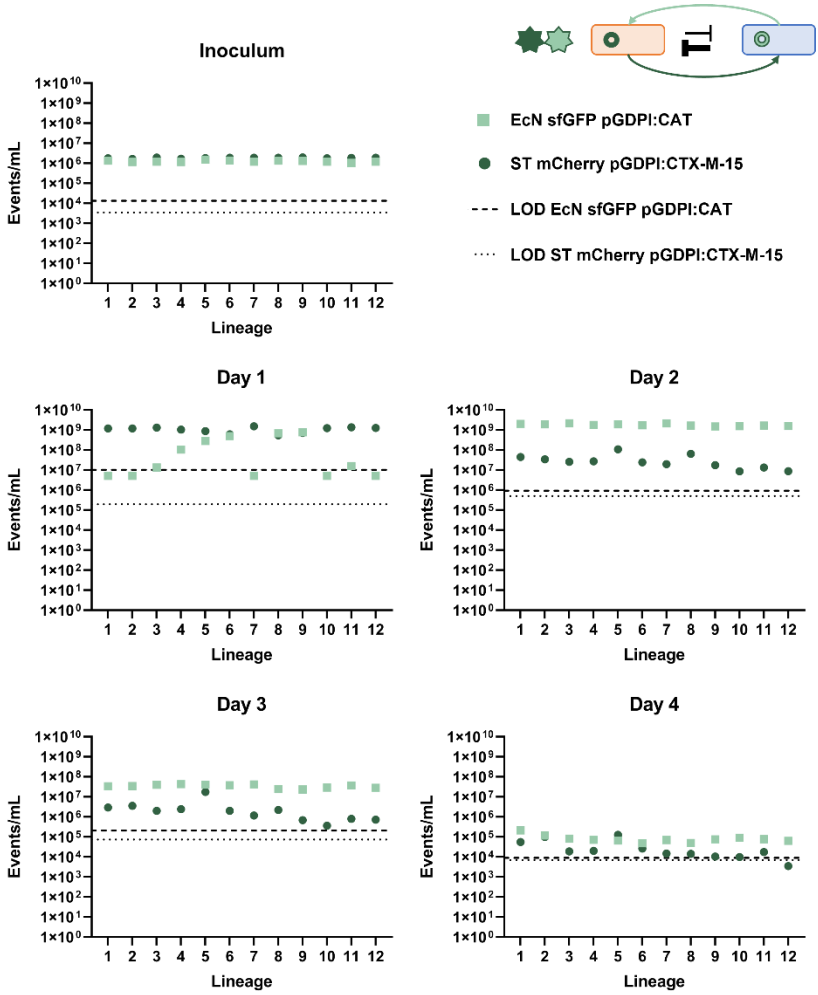


Figure 4.7: Interspecies mutual cross-protection between *E. coli* Nissle sfGFP pGDPI:CAT and *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 did not generate a stable community at concentrations of 4x MIC. $MIC_{EcN,CTX}$ = 0.0625 µg/mL cefotaxime, $MIC_{ST,Cm}$ = 4 µg/mL chloramphenicol. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol was added to the medium during the dilution at a concentration of 16 µg/mL, while cefotaxime was supplemented after 2 h at a concentration of 0.25 µg/mL. After 1 day, *Salmonella* gains the upper hand. Although oscillatory dynamics were expected after the establishment of dominance, this was only observed from day 1 to day 2, as the large *Salmonella* population offered protection to *E. coli* Nissle, while the chloramphenicol was broken down slower, limiting the *Salmonella* growth. However, once *E. coli* Nissle gained the upper hand, *Salmonella* was unable to recover, possibly due to the competitive strength of *E. coli* Nissle in combination with the limited cross-protection offered by CAT. Ultimately, this disbalance in cell count led to the collapse of the community. Cell counts were measured each cycle using the CytoFLEX S.

1.3.4 Forced mutualism based on cross-protection appears more stable within species than between species

Despite the collapse of our interspecies community (Figure 4.7), Yurtsev et al. (2016) showed that intraspecies communities can form stably oscillating co-cultures based on similar public resistance mechanisms and antibiotics[50]. Their model system consisted of two *E. coli* strains that each carry a public resistance mechanism to a different antibiotic on a plasmid. More specifically, the first strain offered resistance against ampicillin via a β-lactamase enzyme, while the second strain deactivated chloramphenicol through a chloramphenicol acetyltransferase type I. The intraspecies co-culture of both publicly resistant *E. coli* strains exhibited stable oscillations across a broad range of antibiotic concentrations, even exceeding the MIC value, when subject to daily 100× dilutions. According to their results, the daily dilutions were the cause of the oscillatory dynamics, as the survival and growth of one strain was dependent on the degradation of the antibiotic by the other strain and, thus, the abundance of the second strain. By replacing the stringent daily dilution by a more frequent, smaller dilution, the co-culture could reach a stable equilibrium ratio without oscillations. In addition, it was shown that these oscillations could destabilize the forced mutualism. Especially at high chloramphenicol concentrations, the probability of collapse increased rapidly. The cross-protection offered by the chloramphenicol acetyl transferase appeared to be lower than the cross-protection offered by the β-lactamase. At high chloramphenicol concentrations, insufficient protection was offered to the ampicillin resistant strain, reducing its relative abundance. Consequently, the protection offered to the bactericidal ampicillin in the next cycle was limited, ultimately leading to collapse[50].

As intraspecies communities relying on mutual cross-protection against two antibiotics can form stable oscillatory co-cultures, we were interested in the stability of interspecies communities. We compared the intra- and interspecies community stabilities to evaluate the influence of interspecies interactions. More specifically, we aimed to explore whether interspecies competition destabilizes cross-protection. Therefore, we conducted additional short-term co-culture experiments in an intraspecies framework, including both a co-culture consisting of two *E. coli* Nissle strains and a co-culture consisting of two *Salmonella* Typhimurium strains. In both intraspecies communities, the strains are isogenic, except for a differential fluorescent

genomic label that allows distinction and the plasmid carrying the public resistance mechanism. The genomic, fluorescent labels did not significantly influence the growth or competitive abilities of the strains, based on an intraspecies co-culture experiment in the absence of antibiotics (Figure S. 4.3 and Figure S. 4.4).

Intraspecies *Salmonella* Typhimurium community

Although *Salmonella* Typhimurium is intrinsically more tolerant to cefotaxime, the same cefotaxime concentration was used as in sections 4.3.2 and 4.3.3, i.e. four times the MIC of the sensitive *E. coli* Nissle.

In contrast to the interspecies community, where the cefotaxime-resistant *Salmonella* took the upper hand, the chloramphenicol-resistant *Salmonella* dominated the intraspecies community after the first day (Figure 4.8). Possibly, the difference between the inter- and intraspecies community can be explained by the lower or slower cross-protection offered by *Salmonella* Typhimurium sfGFP pGDP:CAT compared to *E. coli* Nissle sfGFP:CAT. In addition, *Salmonella* Typhimurium is inherently more tolerant to cefotaxime than *E. coli* Nissle and, therefore, relied less on the cross-protection by the cefotaxime resistant strain. Similar to interspecies community, oscillatory dynamics were expected once a difference in cell density is created. Indeed, the small difference in population size on the first day was flipped towards the second. Thereafter, towards day 3, the chloramphenicol resistant *Salmonella* regained the upper hand, presumably due to the extensive protection offered by the abundant cefotaxime-resistant population. However, once the chloramphenicol-resistant strain prevailed and the difference is several orders of magnitude, a switch of dominance could no longer be observed.

In contrast to the interspecies community, in the intraspecies *Salmonella* community, there is no immediate collapse after the chloramphenicol-resistant strain becomes dominant, but stabilization occurs. On the one hand, this could be explained by the inherent higher tolerance of *Salmonella* to cefotaxime, making the cefotaxime-sensitive (i.e. chloramphenicol-resistant) strain less dependent on the protection of the cefotaxime-resistant strain. On the other hand, the increased stability of the intraspecies community could indicate that in the interspecies community competition was indeed at the basis of the collapse.

In addition, it can be noted that the cell number of the cefotaxime-resistant strain decreases from day 3 to day 4. However, the decrease is less than two log, which indicates that the daily 1/100 dilution is overcome and both strains still display growth. In a first hypothesis, this decline is caused by *Salmonella* Typhimurium sfGFP pGDPI:CAT offering less protection against chloramphenicol. In this scenario, if this downward trend were to continue, the intraspecies *Salmonella* community would ultimately collapse. Nevertheless, as the collapse of the system proceeds more slowly than the interspecies community, the intraspecies community would be regarded as more stable. In our second hypothesis, because of the inherently higher tolerance of *Salmonella*, the cefotaxime-sensitive strain relies less on the protection of the cefotaxime-resistant strains. Hence, the lower cell density of the cefotaxime-resistant strain may still provide sufficient protection and the community has not yet reached its stable point. Alternatively, the oscillatory dynamics we expected to see have a period that spans multiple dilution cycles and the balance between both strains will still shift in the following cycles[50]. However, to be able to determine the stability of the system with certainty, the experiment should be run longer.

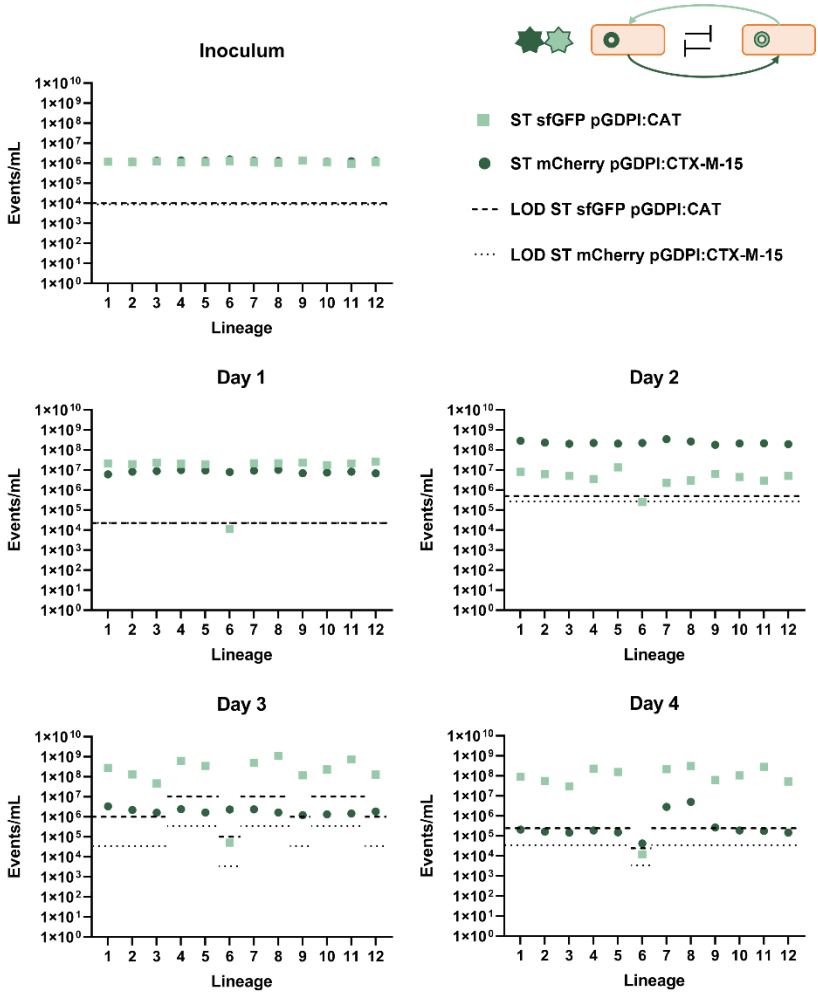


Figure 4.8: The mutual cross-protection in the intraspecies *Salmonella* Typhimurium community creates a stable co-culture for at least four days. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol was added to the medium during the dilution at a concentration of 16 µg/mL, while cefotaxime was supplemented after 2 h at a concentration of 0.25 µg/mL. The limited degradation of chloramphenicol by *Salmonella* Typhimurium sfGFP pGDPI:CAT delays the overshoot of the cefotaxime-resistant strain from day 1 to day 2. Thereafter, the switch in dominance towards the chloramphenicol-resistant strain, based on strong protection and subsequent growth in combination with negative frequency-dependent selection of the cefotaxime-resistant strain, can be observed. Although the reciprocal switch does not occur by day 4, the total community density remains approximately constant, indicating the increased stability of the co-culture. Cell counts were measured each cycle using the CytoFLEX S.

Intraspecies *E. coli* Nissle community

The *E. coli* Nissle intraspecies community is more variable and seems to depend on stochastic effects that create differences in population densities for the initialization of instability (Figure 4.9). Only small differences in population size were detected after one day, but consistently when a difference occurred the cefotaxime-resistant strain was dominant in all lineages with a difference in population size. These minor growth differences can be attributed to differences in cross-protection efficiency between the strains. However, the bigger differences created towards day two do not appear to be directly correlated to the ratio on the first day. Since no trend can be detected and the different populations still show fairly similar cell numbers on day 1, the outcome towards day 2 seems to be based on stochastic effects, which are probably due to the dilution, which creates a non-homogeneous influencing factor. Before dilution, the medium containing the co-cultures were thoroughly mixed by pipetting up-and-down five times to achieve optimal homogenization. However, inadvertent selection of one strain over the other might have still occurred. Additionally, while the 96-well plates were incubated with shaking, the possibility of biofilm formation on the bottom of the wells cannot be disregarded, particularly given that competition can induce biofilm formation[46]. This limitation of the setup may have also resulted in inaccurate transfer of populations from one day to the next. The bigger differences in population size on day 2, however, appear to determine the final outcome of the co-culture. Most repeats where the cefotaxime-resistant strain has taken the upper hand on day 2, display a strong shift in dominance on day 3 and ultimately collapse towards the end of the experiment. The small population size of the cefotaxime-resistant strain could cause a lack of protection, followed by a sharp decrease in total population size, which can induce the collapse of the co-culture. In contrast, communities that, on day 2, have a more equal distribution of both strains, and co-cultures where the chloramphenicol-resistant *E. coli* Nissle has the upper hand, remain more balanced over the course of the experiment. Still, accidental effects created by non-homogeneous selection during dilution or small imbalances in cross-protection can, also in these communities, create a large difference in population size, thereby inducing collapse. Nevertheless, the intraspecies *E. coli* Nissle community can be regarded as more stable than the interspecies combination of *E. coli* Nissle and *Salmonella* Typhimurium. On the one hand,

there is a delay in the manifestation of the first major differences in this community. On the other hand, some of the repeats still maintain a stable and high cell number on the last day.

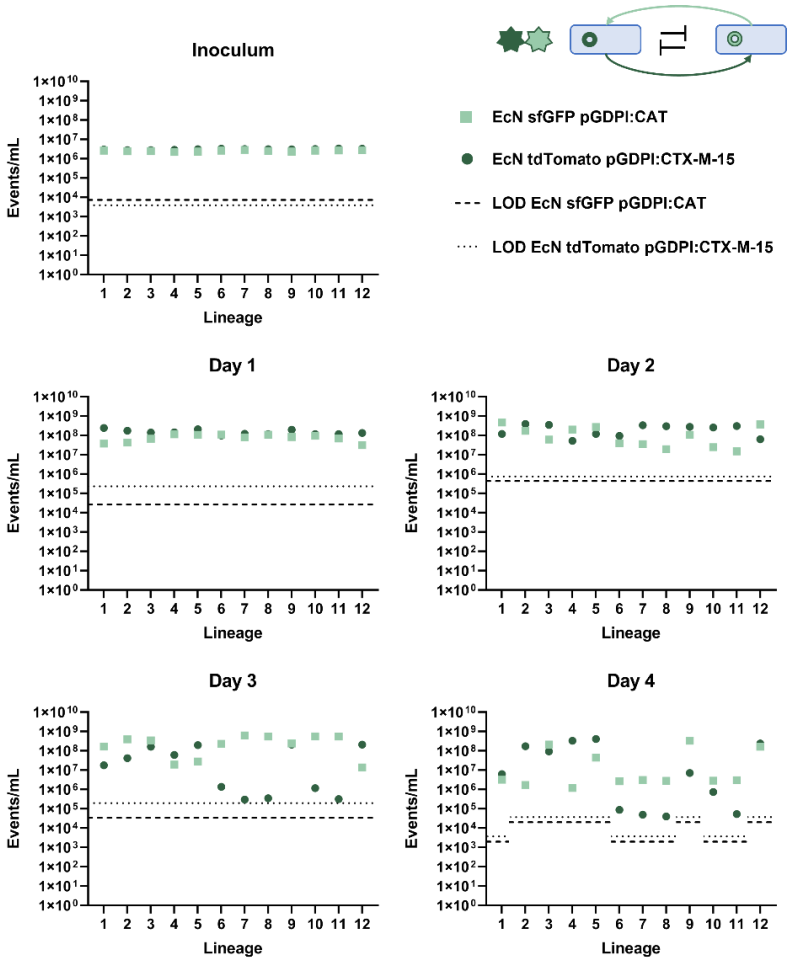


Figure 4.9: The stability of the intraspecies *E. coli* Nissle community relying on mutual cross-protection is determined by the creation of strong differences in population size by stochastic effects. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol was added to the medium during the dilution at a concentration of 16 µg/mL, while cefotaxime was supplemented after 2 h at a concentration of 0.25 µg/mL. Over the course of the experiment, the creation of larger differences in population size, most probably by stochastic effects, seems to induce the collapse of the system, as insufficient protection is offered by the smaller population. Nevertheless, co-cultures that remain approximately in equilibrium, appear to be more stable and even display oscillatory dynamics, for example repeat 3 and 12. Cell counts were measured each cycle using the CytoFLEX S.

As both intraspecies communities are more stable on the short term than the interspecies community, we performed some additional experiments to gain more insights into the interaction dynamics and possible causes of collapse in the different communities. In doing so, we will gain a better understanding of the importance of interspecies competition on the stability of the co-culture.

1.3.5 Differences in antibiotic degradation and sensitivity between strains appear more important to determine community stability than interspecies competition

Intraspecies community stability with one-sided protection is defined by degradation efficiency and sensitivity

Cefotaxime

To elucidate the underlying dynamics and identify the determining factor of community stability, we first studied the one-sided intraspecies cross-protection against cefotaxime. Hereto, novel short-term intraspecies co-culture experiments were performed for *Salmonella* Typhimurium as well as *E. coli* Nissle. As in sections 4.3.3 and 4.3.4, cefotaxime was added to the medium daily after two hours. Based on the results shown in Figure 4.10, the intraspecies *Salmonella* cross-protection does create a stable duo-species community in which the prevalence of the resistant strain is ten times greater than that of the sensitive strain. The sensitive *Salmonella* strain can grow, indicating it is able to enjoy the benefits offered by the public resistance, but doesn't outcompete the resistant strain. This suggests that either there is a limited cost associated with the production of the resistance mechanism or that the costly production is compensated by a partial privatization of the protection during the initial stage of degradation. Besides, in this intraspecies setup, the sensitive strain has no additional competitive advantage over the resistant strain. As a result, the resistant strain remains dominant in this intraspecies community, as opposed to the interspecies community. Nevertheless, the extent of protection is sufficient to establish and maintain a stable co-culture, which may suggest that the sensitive strain grows faster once enough cefotaxime is broken down.

In contrast, no stable intraspecies *E. coli* Nissle duo-community is formed (Figure 4.11). Although some cross-protection is offered, as evidenced by the

growth of the sensitive strain on day 1, the population size of the sensitive strain strongly decreases by day 4. Importantly, *E. coli* Nissle is more sensitive to cefotaxime than *Salmonella* Typhimurium, as evidenced by the MIC. Nevertheless, the sensitive *E. coli* Nissle was able to survive and establish itself in the population when protected by *Salmonella* Typhimurium in the interspecies experiment (Figure 4.3). Hence, a first hypothesis states that *E. coli* Nissle breaks down the antibiotic slower than *Salmonella* Typhimurium. Consequently, this lower degradation rate may result in a significant reduction in the cell number of the sensitive strain due to cell mortality preceding recovery. Second, we hypothesized that *E. coli* Nissle less efficiently reduces the antibiotic concentration in the surrounding medium, thus lowering the cross-protection. Besides, other factors might still influence the growth of the sensitive strain once sufficient cefotaxime is degraded. For example, the combination of the higher *E. coli* Nissle growth rate and complete niche overlap can create a lack of sufficient nutrients and might explain the limited growth[346,347]. Ultimately, this can create an imbalance, that might be enhanced every dilution, and can lead to the eradication of the sensitive strain, visualized by population sizes below the limit of detection on day 4 for half the lineages.

These differences between the intra- and interspecies communities imply that differences in sensitivity to and degradation of cefotaxime, as well as interspecies competition can play a role in determining community stability.

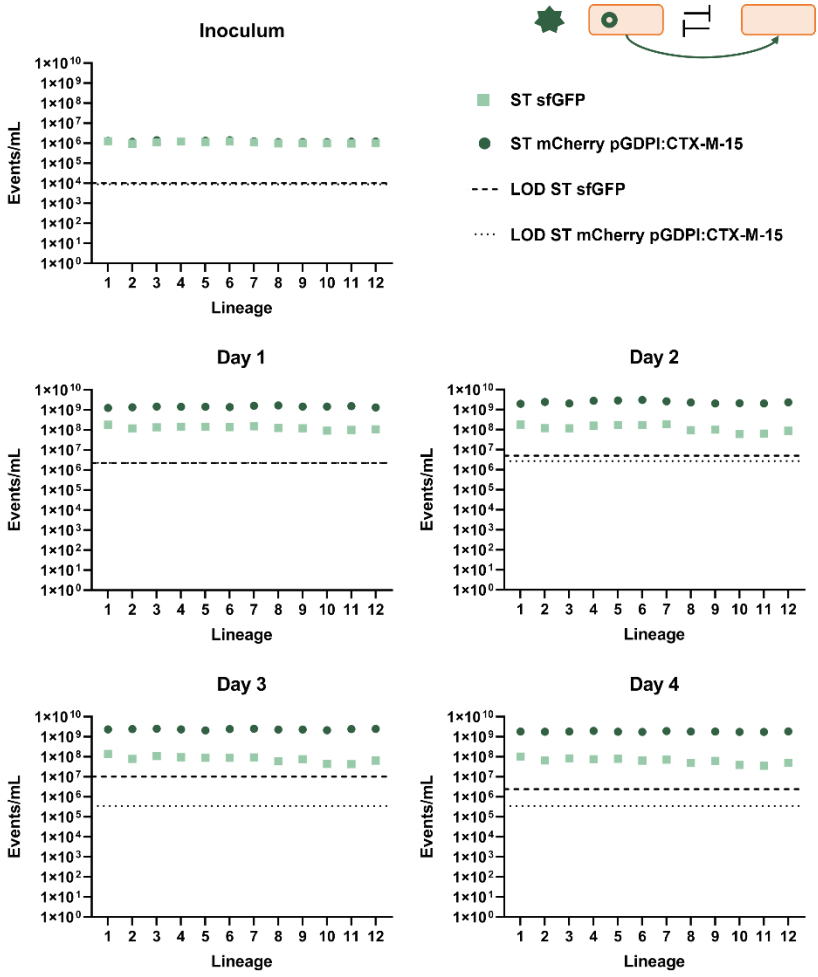


Figure 4.10: Intraspecies *Salmonella-Salmonella* cross-protection against cefotaxime creates a stable co-culture. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, cefotaxime was supplemented to the medium 2 h after dilution at a concentration of 0.25 µg/mL. Although the resistant strain seems to profit from the initial privatization of cefotaxime degradation, the sensitive strain, that bears no production cost, can establish itself within the population following the substantial degradation of cefotaxime. Cell counts were measured each cycle using the CytoFLEX S.

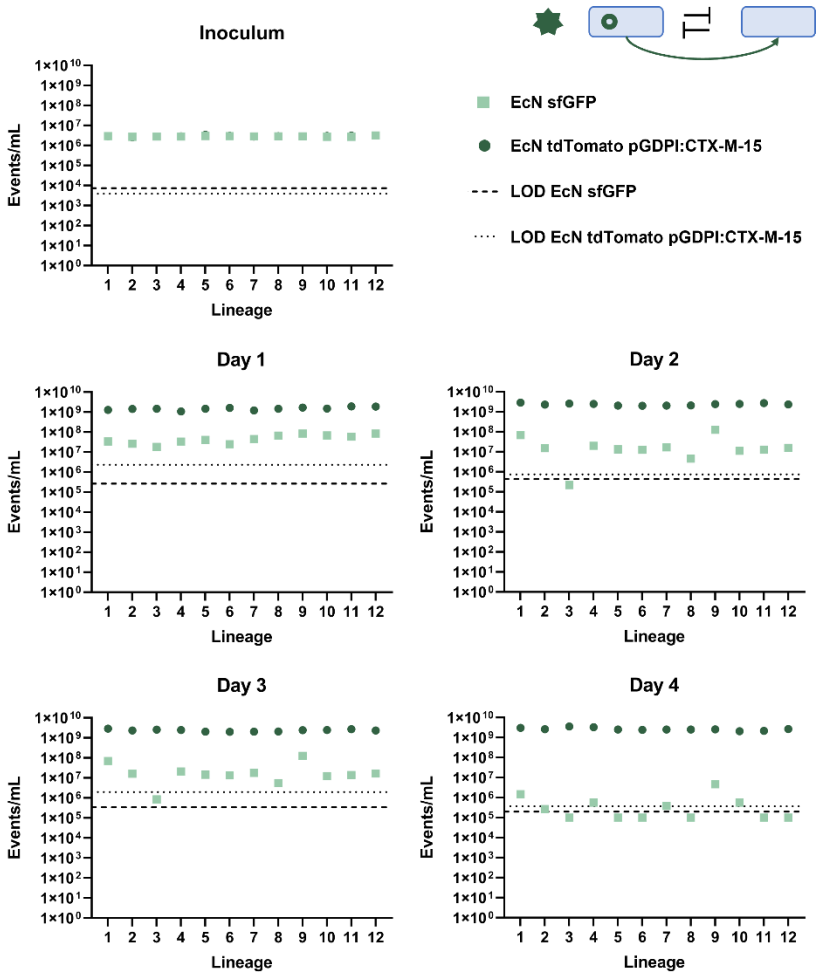


Figure 4.11: *E. coli* Nissle tdTomato pGDPI:CTX-M-15 does not offer sufficient cross-protection against cefotaxime to the sensitive *E. coli* Nissle to stabilize the intraspecies co-culture. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, cefotaxime was supplemented to the medium 2 h after dilution at a concentration of 0.25 µg/mL. The higher susceptibility of *E. coli* Nissle, compared to *Salmonella* Typhimurium, in combination with the possible slower or less efficient degradation of the antibiotic leads to the gradual eradication of the sensitive strain. Cell counts were measured each cycle using the CytoFLEX S.

Chloramphenicol

Intraspecies *Salmonella* cross-protection against the bacteriostatic antibiotic chloramphenicol does not allow growth of the sensitive strain on day 1, leading to cell counts below the LOD (Figure 4.12). These results, therefore, suggest that the protection provided by the intracellular degradation of chloramphenicol by the chloramphenicol acetyl transferase was insufficient to provide protection to a sensitive *Salmonella* strain. Possibly, the inoculum density of the resistant *Salmonella* was too low, requiring a long period to reach the critical density necessary to confer adequate cross-protection. Once the antibiotic was sufficiently degraded, the medium might have already been too depleted to show visible growth. Protection could, however, have been offered during the following days, where the resistant population is several orders of magnitude larger. Nevertheless, because of the daily dilution, the sensitive strain does not recover to cell numbers above the limit of detection. Accordingly, no definite conclusion can be drawn about the presence of sensitive *Salmonella* in the community or the stability of this sensitive sub-population. In contrast, in the one-sided interspecies experiment, the sensitive *Salmonella*, protected by chloramphenicol-resistant *E. coli* Nissle, was able to maintain cell numbers above the limit of detection, as displayed in Figure 4.5. Although the chloramphenicol acetyl transferase is constitutively expressed from the pGDPI plasmid in both species, this does not automatically ensure the same amount of protein is present inside the cell. For example, the copy number of the plasmid can vary between cells and species[348]. Besides, chloramphenicol is degraded intracellularly, making it dependent on diffusion of the antibiotic by the resistant strain[50,316]. Therefore, a different degradation rate or efficiency can be achieved by distinct antibiotic-resistant bacterial species, consequently offering varying levels of cross-protection.

Similar reasoning can be followed for the intraspecies *E. coli* Nissle community (Figure 4.13). The low inoculum density of the resistant strain does not offer sufficient cross-protection to allow the sensitive strain to start growing, leading to cell counts below the limit of detection on day 1 and day 2. Nevertheless, the sensitive population is able to recover and can even establish itself within the population by day 4 in seven out of twelve repeats. Moreover, the population density of the sensitive strain in these repeats recovers to $\pm 10^6$ cells/mL, similar to the inoculum density, which indicates

that the population grows at least 2 logs once sufficient protection is offered, thereby overcoming the reduction in cell number caused by the daily dilutions. We hypothesize that the larger resistant population after day 1 degrades the antibiotic faster and, thus, offers more cross-protection. These results suggest that the chloramphenicol degradation and subsequent cross-protection strongly depend on the population size of the resistant strain.

Since *Salmonella* Typhimurium and *E. coli* Nissle share the same MIC value, we do not expect major differences in sensitivity to chloramphenicol. Nevertheless, *Salmonella* was able to grow immediately from day 1 under the degradation and protection of *E. coli* Nissle (Figure 4.5), while in the intraspecies *E. coli* Nissle community, a larger resistant population is required before the sensitive strain can recover (Figure 4.13). This might indicate that the intraspecies competition based on complete niche overlap may have initially been stronger than the interspecies competition between *Salmonella* Typhimurium and *E. coli* Nissle. However, if the antibiotic is degraded faster due to the higher cell densities of the resistant population in subsequent days, we hypothesize that the sensitive *E. coli* Nissle strain can start to grow faster and, therefore, compete for nutrients with the resistant strain. Conversely, we hypothesize that the competition between *Salmonella* Typhimurium and *E. coli* Nissle increases with higher *E. coli* Nissle cell numbers, even if this provides more protection, causing the *Salmonella* Typhimurium population to disappear from the community. As a result, the interspecies competition would, in these conditions, destabilize the cross-protection community.

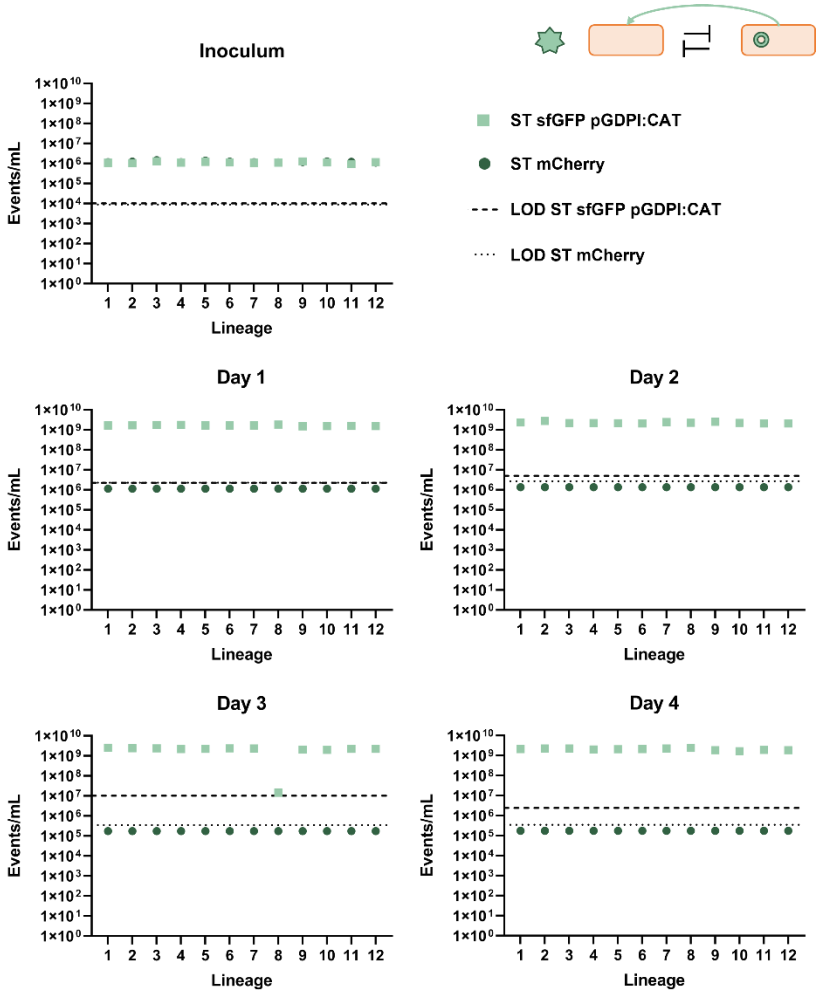


Figure 4.12: *Salmonella* Typhimurium sfGFP pGDPI:CAT does not offer sufficient intraspecies cross-protection against chloramphenicol. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol was added to the medium during the dilution at a concentration of 16 µg/mL. The number of resistant cells in the inoculum seems insufficient to protect the sensitive population on day 1. Thereafter, the sensitive strain cannot recover to a cell number above the limit of detection under the protection offered by *Salmonella* Typhimurium sfGFP pGDPI:CAT. Nevertheless, this is not an indication of complete absence of a sensitive population in the community. The sharp drop in cell number of repeat 8 of the resistant *Salmonella* Typhimurium on day 3 most probably originates from an error during the dilution or measurement of this sample. Cell counts were measured each cycle using the CytoFLEX S.

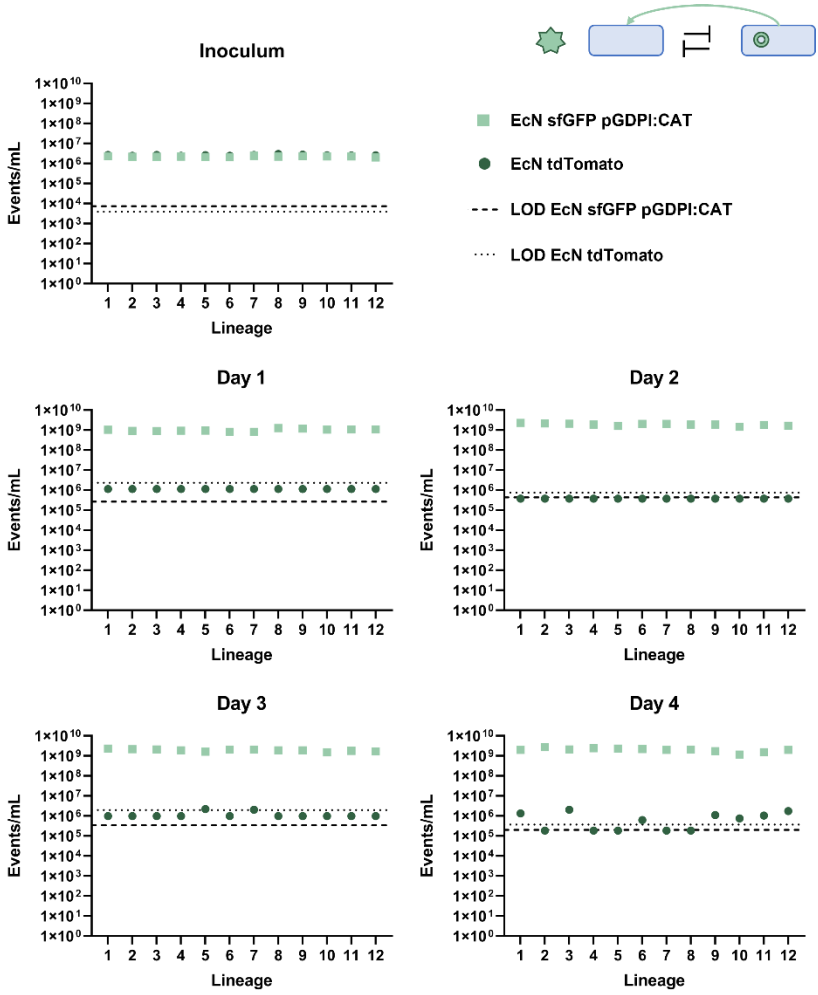


Figure 4.13: The *E. coli* Nissle sfGFP pGDPI:CAT population size determines the protection against chloramphenicol and stability of the co-culture. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol was added to the medium during the dilution at a concentration of 16 µg/mL. Although the inoculum density of *E. coli* Nissle sfGFP pGDPI:CAT is too low to offer sufficient cross-protection to the sensitive *E. coli* Nissle strain on day 1, the latter is able to recover once the degradation rate and protection increase with the higher cell number of the resistant strain the following days. Cell counts were measured each cycle using the CytoFLEX S.

The protective profile of the intraspecies *E. coli* Nissle community resembles that of the interspecies community more than the intraspecies *Salmonella* community

To investigate the switch in dominant strain observed in the intra- and interspecies communities exposed to both antibiotics, we performed 24-hour co-culture experiments starting from different initial frequencies. While keeping the total inoculum density constant at 3 10^6 events/mL, the ratios varied between 1:999 and 999:1 (chloramphenicol-resistant strain : cefotaxime-resistant strain), as these reflect the differences in population size encountered during the short-term co-culture experiments. Besides, the theoretical, exponential growth of the chloramphenicol-resistant strain after two hours was included in all profiles. Although this does not take into account the lag time, thereby overestimating the growth, it gives an indication of the population size of the chloramphenicol-resistant strain at the moment cefotaxime is added. Besides, we should take into account that the CytoFLEX S measurements based on fluorescence might also detect dead cells.

Interspecies community

In the interspecies community (Figure 4.14a), when the chloramphenicol-resistant *E. coli* Nissle was added in 100 or 1000 fold lower inoculum densities compared to the *Salmonella* density and the inoculum densities in the short-term co-culture experiments in previous sections, the *Salmonella* cell count remained at the level of the inoculum density, while the *E. coli* Nissle cell numbers did not exceed the theoretic growth. We hypothesize that the growth and subsequent chloramphenicol degradation by *E. coli* in the first two hours was insufficient to protect *Salmonella*. As *Salmonella* was, thus, not metabolically active at the moment of cefotaxime addition, no cross-protection was offered to *E. coli* Nissle, which was consequently killed by the bactericidal effect of cefotaxime and induced collapse of the system. However, when the *E. coli* Nissle density was 3 10^5 (1:9), only tenfold lower than *Salmonella*, additional growth of the *E. coli* strain could be observed after the addition of cefotaxime. Nevertheless, the *Salmonella* cell count did not indicate any growth, as the final density was even lower than the inoculum density. Therefore, we hypothesize that this higher inoculum density of *E. coli* Nissle did offer sufficient cross-protection to make *Salmonella* metabolically active and break down cefotaxime, but insufficient to actually allow growth.

Based on the 1:9 results, adequate degradation of chloramphenicol by *E. coli* Nissle after two hours was expected to allow metabolic activity of *Salmonella* on the right side of the graph, where the inoculum density of *E. coli* Nissle was increased to 3 10^6 events/mL (9:1 - 999:1). Nevertheless, no growth of *Salmonella* could be observed. Besides, the density of 3 10^5 events/mL or lower of the cefotaxime-resistant *Salmonella* also appeared to be insufficient to provide cross-protection to *E. coli*, as evidenced by an absence of additional growth by this strain. A lack of cefotaxime-resistant *Salmonella* would, consequently, cause a collapse of the community, as observed before. When studying the condition with equal inoculum density (1:1, 1.5 10^6 events/mL) as used in the short-term co-culture experiments, *E. coli* Nissle's chloramphenicol degradation allowed *Salmonella* to become metabolically active, based on the 1:9 condition, but did not allow actual growth yet after two hours, based on the 9:1 results. Nevertheless, we hypothesize that the metabolically active *Salmonella* was able to sufficiently degrade cefotaxime, preventing *E. coli* Nissle's cell death, keeping it metabolically active or even allowing additional growth. The consequent additional degradation of chloramphenicol enabled *Salmonella* to start growing and overtake *E. coli* Nissle, that also displayed additional growth. Nevertheless, the exact dynamics over the course of 24 hours remain elusive, as it is not possible to determine which strain started growing first and what the growth rate of each of the strains was, based on these results. Therefore, additional measurements at different time-points might be interesting in future experiments.

Intraspecies *Salmonella* Typhimurium community

The intraspecies *Salmonella* 1:999 and 1:99 ratios produced similar results as the interspecies interactions (Figure 4.14b). However, the 1:9 condition differed, as both strains converged to the same population density. This might be explained by the before hypothesized more privatized or slower degradation of chloramphenicol by *Salmonella* compared to *E. coli* Nissle, resulting in less cross-protection towards the cefotaxime-resistant strain. Consequently, less cefotaxime was degraded and the chloramphenicol-resistant strain could not significantly grow additionally. Nevertheless, *Salmonella* is inherently less sensitive to cefotaxime and a minimal increase in

cell count could be noted for both strains compared to the inoculum density. Therefore, it is uncertain whether live or dead cells were measured.

For the condition of equal inoculum density, the results suggest a scenario similar to the interspecies community. Chloramphenicol degradation during the first two hours led to a metabolically active cefotaxime-resistant strain, which subsequently degraded cefotaxime. However, as mentioned earlier, *Salmonella* is less sensitive to cefotaxime, possibly resulting in reduced dependency. Consequently, we suspect that the chloramphenicol-resistant strain may restart growing faster or is killed less and, therefore, has to recover less than the *E. coli* Nissle in the interspecies community. Consequently, the chloramphenicol-resistant *Salmonella* became dominant in this community, rather than the cefotaxime-resistant strain. The latter could, ultimately, still grow, but appeared to suffer from the lesser protection of *Salmonella* against chloramphenicol.

On the right side of the figure, the inoculum density of the chloramphenicol-resistant strain remained approximately the same to the condition of equal inoculum density. Consequently, it is also assumed, here, that chloramphenicol was sufficiently degraded after two hours to make the cefotaxime-resistant strain metabolically active. If this initial assumption of metabolic activity without growth of the cefotaxime-resistant strain after two hours, which was based on the results of equal inoculum density, is correct, the eventual growth of the cefotaxime-resistant strain indicates that chloramphenicol degradation continued even after the addition of cefotaxime. The additional degradation of chloramphenicol, without visible additional growth, might imply that part of the chloramphenicol-resistant population was killed, but that recovery took place once sufficient cefotaxime had been broken down. The hypothesized recovery of the chloramphenicol-resistant population and the subsequent growth of the cefotaxime-resistant strain in the intraspecies *Salmonella* Typhimurium community, compared to the interspecies collapse, could, on the one hand, be explained by the reduced sensitivity to cefotaxime. On the other hand, the stronger competition of *E. coli* Nissle in the interspecies community may have been the cause of the collapse. In general, the range of mutual cross-protection in the intraspecies *Salmonella* community appears to be broader than in the interspecies community.

Intraspecies *E. coli* Nissle community

Finally, in the intraspecies *E. coli* Nissle community, two out of three repeats showed enrichment of the chloramphenicol-resistant strain in the 1:99 condition, without growth of the cefotaxime-resistant strain. Once again, this can indicate a greater degradation potential of chloramphenicol from *E. coli* Nissle, causing the cefotaxime-resistant strain to become metabolically active earlier compared to the intraspecies *Salmonella* community. However, when compared with the interspecies dynamics, where chloramphenicol was also degraded by *E. coli* Nissle, it is striking that the chloramphenicol-resistant strain could already grow here in the 1:99 case, which could indicate that *E. coli* Nissle degrades cefotaxime more quickly than *Salmonella*, despite the higher sensitivity based on the MIC value. By accelerating the cefotaxime degradation, the cell death of the chloramphenicol-resistant strain was limited and recovery growth became more visible. Similar reasoning can be followed for the 1:9 condition.

In contrast, in the communities where the cefotaxime-resistant strain started with low inoculum density (9:1 – 999:1), no growth could be observed. This indication of collapse of the system might be explained by the higher sensitivity of *E. coli* Nissle to cefotaxime. We hypothesize that the lower inoculum density of the cefotaxime-resistant strain and subsequent lower cross-protection prevented the recovery of the chloramphenicol-resistant strain, so that no additional degradation of chloramphenicol took place. Consequently, the cefotaxime-resistant strain could not initiate growth.

Based on the results of the 1:9 and 1:99 conditions, with a reduced number of chloramphenicol-resistant cells, it was expected that in the equal inoculum density condition sufficient chloramphenicol had been broken down after two hours for metabolic activity, but not for growth of the cefotaxime-resistant strain. We hypothesize that similar reasoning can be followed as before. Namely, following sufficient cefotaxime degradation, the chloramphenicol-resistant strain will restart to grow and degrade chloramphenicol, ultimately leading to growth of the cefotaxime-resistant strain.

Although the ratio experiments correspond well with and provide additional insights into the four-day co-culture experiments, the extrapolation is not always straightforward. Sometimes, a shift between the ratio experiments and

the co-culture experiments can be noted. For example, in the interspecies co-culture experiment with two-sided protection, a large proportion of lineages had a cefotaxime-resistant population that is two orders of magnitude larger than the chloramphenicol population on day 1 (Figure 4.7). Towards day 2, a strong shift in dominance took place, giving the chloramphenicol-resistant population the upper hand. In the ratio experiment, however, such a shift in dominance was only observed in the 1:9 condition and not in the 1:99 condition as for the co-culture experiment. This could be explained, on the one hand, by the washing step, which was present in the ratio experiment, but not during the daily dilution in the co-culture experiment. On the other hand, it could be that the absolute cell number of the strains is more important than the ratio between the different strains. More specifically, the cell density of the smallest population seems decisive. On day 1 of the interspecies experiment, the smaller chloramphenicol population had a density of approximately 10^7 cells/mL. After the 1/100 daily dilution into fresh medium, the cell number fell to 10^5 cells/mL, which corresponds to the 1:9 condition in the ratio experiment. Moreover, Yurtsev et al. (2016) also pointed out that the cell density immediately after dilution appeared to be crucial for the stability of the system[50]. More specifically, the swift, initial degradation of the antibiotic by pre-existing β-lactamases, either within the periplasmic space of the cells or in the medium transferred during the dilution step, appeared to play an important role in their co-culture experiments. This observation underscores our rationale for introducing cefotaxime after a two-hour interval, taking into account the washing step applied during inoculum preparation.

Besides providing novel insights in the dynamics of the co-culture experiments, the ratio experiments allowed us to compare the protective profiles of the different combinations, thereby gaining better insight in the stability of the co-cultures.

Comparison of the protective profiles

To more easily compare the protective profiles, a simplified, alternative representation was constructed. Figure 4.15 shows the change in the relative abundance of chloramphenicol-resistant cells (number of chloramphenicol-resistant cells divided by the total amount of cells in the co-culture) compared to the inoculum ratio. Since the interspecies community was the only one to

show a clear collapse in the short-term co-culture experiments, it was assumed that protection in this community would differ strongly from the intraspecies controls, due to competition or differences in growth rate that disrupt the balance. However, the bundled data show that the profile of the *E. coli* Nissle intraspecies community is much more similar to the interspecies community than to the intraspecies *Salmonella* Typhimurium one. For example, both the interspecies community and the intraspecies *E. coli* Nissle community show a strong enrichment of the chloramphenicol-resistant population in the 1:9 condition. In addition, the stable point of the interspecies and intraspecies *E. coli* Nissle communities approximately coincides, while that of the intraspecies *Salmonella* community is shifted to the right. This strong similarity points to a rather limited influence of competition on the cross-protection and subsequent stability of the system. Larger differences appear to arise from intrinsic properties of the bacterial species, such as sensitivity, degradation capacity and rate. Therefore, a supernatants experiment was finally performed to validate these hypotheses.

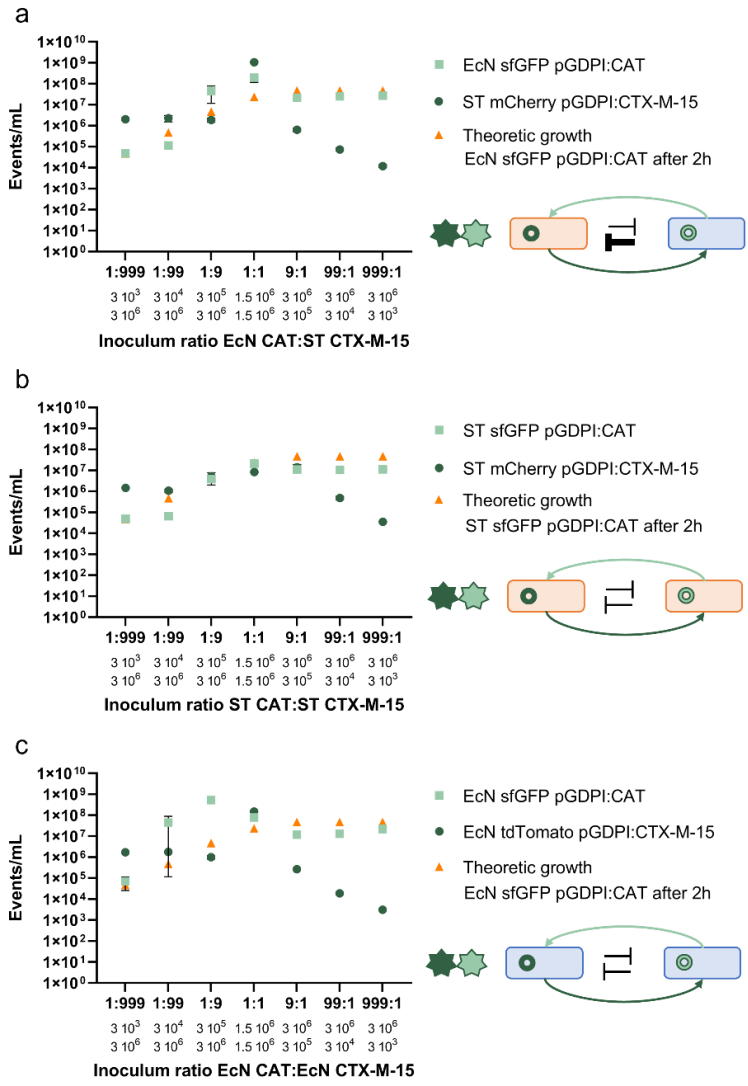


Figure 4.14: Protective profiles of inter- and intraspecies communities starting from different inoculum ratios in media supplemented with both antibiotics. The co-cultures were incubated at 37°C with continuous shaking at 200 rpm for 20 h. Chloramphenicol was added to the growth medium from the start, while cefotaxime was administered after two hours. The x-axis describes the tested inoculum ratios ranging from 1:999 to 999:1, annotated as the chloramphenicol-resistant strain (indicated as CAT), compared to the cefotaxime-resistant strain (indicated as CTX-M-15), supplemented with the inoculum densities. The y-axis indicates the events/mL as measured after 24 hours by the CytoFLEX S, representing the CFU/mL. For all conditions, three biological repeats were performed, except for 1:1, where the twelve datapoints of the short-term co-culture experiment on day 1 were used. The orange triangles

indicate the theoretical, exponential growth of the chloramphenicol-resistant strain after two hours. **a)** Interspecies cross-protection appears to allow growth of either or both strains only in the 1:9 and 1:1 conditions. **b)** In the intraspecies *Salmonella* Typhimurium community, both strains display growth in the 1:9 and 1:1 condition. Although the chloramphenicol-resistant strain did not surpass the predicted growth after two hours, the tenfold increase in cell counts of the cefotaxime-resistant strain in the 9:1, 99:1 and 999:1 conditions on the right, also indicate regrowth of the chloramphenicol-resistant population and subsequent additional degradation of chloramphenicol. **c)** Mutual cross-protection leading to growth of both strains can only be observed in the 1:1 condition in the intraspecies *E. coli* Nissle community. Besides, the degradation of chloramphenicol by 10^4 *E. coli* Nissle sfGFP pGDPI:CAT cells or more appears to be sufficient to make the cefotaxime-resistant *E. coli* Nissle metabolically active. The consequent degradation of cefotaxime allows the growth of the chloramphenicol-resistant strain.

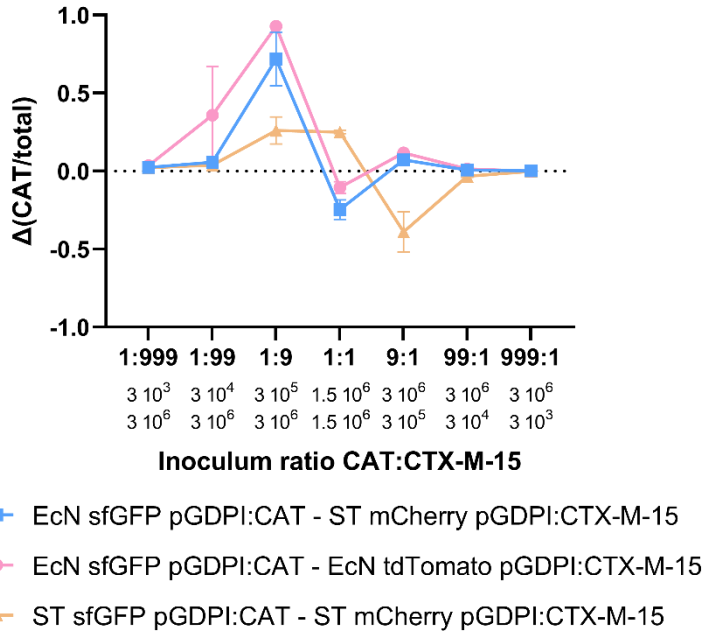


Figure 4.15: Comparison of the inter- and intraspecies cross-protection dynamics at different inoculum ratios. The x-axis describes the tested inoculum ratios ranging from 1:999 to 999:1, annotated as the chloramphenicol-resistant strain (indicated as CAT), compared to the cefotaxime-resistant strain (indicated as CTX-M-15), supplemented with the inoculum densities. The y-axis depicts the change in relative abundance of the chloramphenicol-resistant strain after 24 hours compared to the inoculum ratio.

***E. coli* Nissle and *Salmonella* Typhimurium differ in sensitivity, degradation-capacity and -rate for both antibiotics.**

Next to differences in competition between intra- and interspecies communities, *Salmonella* and *E. coli* Nissle can also differ in the antibiotic susceptibility of the sensitive strain and the rate and efficiency at which the publicly resistant strain detoxifies the environment. To determine both the susceptibility towards either antibiotic of the sensitive *Salmonella* and *E. coli* strains and the degradation rate of the publicly resistant strains. we performed supernatants experiment.

During the supernatants experiment of cefotaxime, the resistant strains, starting from different inoculum densities, were allowed to grow in the medium for one, two and four hours, before being spun down and separated from the supernatants. After filter sterilization, the supernatants were used as growth medium for the sensitive strain, without addition of nutrients.

Already after 1 hour, the effect of cefotaxime degradation by *E. coli* Nissle is evident in Figure 4.16a, as some growth took place in the supernatant of the highest density of the resistant strain. Nevertheless, even after several hours, the growth remained limited to an absorbance of 0.4. In contrast, the sensitive *E. coli* Nissle could only start growing in the supernatants of the resistant *Salmonella* strain after two hours of degradation, but the growth was more outspoken (Figure 4.16b). Besides, sensitive *Salmonella* could grow in the supernatants of resistant *Salmonella* starting from lower inoculum densities than *E. coli* Nissle, which might indicate it relied less on the inoculum size (Figure 4.16c). These results indicate that *Salmonella* more thoroughly degraded cefotaxime, thereby offering more cross-protection, although *E. coli* Nissle more rapidly decreased the antibiotic concentration in the medium. Besides, as expected based on the MIC test, *Salmonella* was less susceptible to cefotaxime.

Remarkably, less growth could be observed in the supernatants of 10^6 resistant CFU/mL compared to 10^5 CFU/mL for all strains after four hours. On the one hand, this might indicate consumption of part of the nutrients. On the other hand, other factors, such as waste production or communication via quorum sensing, can influence further growth. As this limited growth could also be observed in supernatants originating from media without antibiotics,

the influence of degradation products of cefotaxime seems to be limited (Figure S. 4.5).

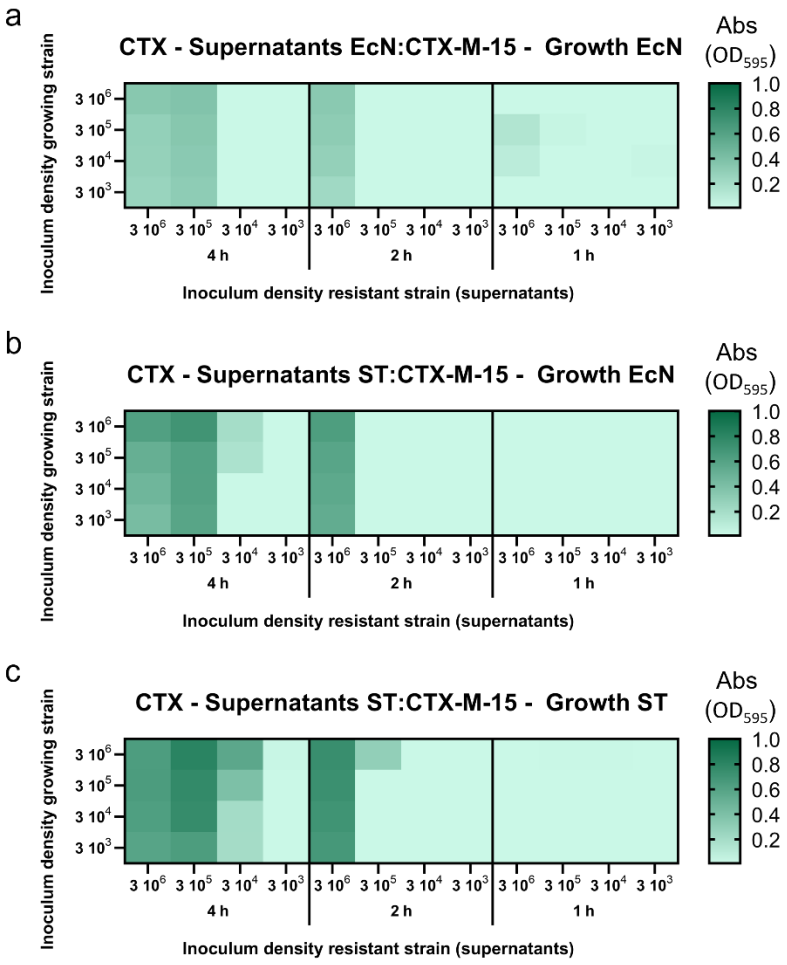


Figure 4.16: Cross-protection offered against cefotaxime after one, two and four hours based on growth in supernatants. Growth of the sensitive strain after 20 hours, depicted as the absorbance at 595 nm, in the supernatants of cefotaxime-resistant strains. Supernatants was taken after one, two or four hours from cultures starting from different inoculum densities. **a)** Growth of sensitive *E. coli* Nissle sfGFP in the supernatants of *E. coli* Nissle tdTomato pGDPI:CTX-M-15. **b)** Growth of sensitive *E. coli* Nissle sfGFP in the supernatants of *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15. **c)** Growth of sensitive *Salmonella* Typhimurium sfGFP in the supernatants of *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15.

For the supernatants experiment of chloramphenicol, the resistant strains, starting from different inoculum densities, were allowed to grow in the medium for two or six hours. The selection of the two-hour time point was based on its alignment with the moment of cefotaxime introduction in the four-day co-culture experiments. In addition, we hypothesized that the degradation of chloramphenicol was slower than the cefotaxime degradation. First, chloramphenicol is degraded intracellularly and, therefore, depends on the uptake and release of the antibiotic, while β-lactamases are believed to be periplasmic or even secreted in the medium[9,50,316–318]. Second, the results of the ratio experiments suggest the cefotaxime-resistant strain was unable to grow after two hours, even if the chloramphenicol-resistant strain was present in abundance. In contrast, growth could be noted on the left side of the graphs in the ratio experiments, indicating rapid degradation of cefotaxime, thereby limiting the bactericidal effect. As a result, we decided to study a later time-point for chloramphenicol.

In general, the supernatants experiments with chloramphenicol degradation (Figure 4.17) confirmed the hypothesis that after two hours, neither strain had broken down chloramphenicol sufficiently to allow the protected strain to grow. After 6 hours, the sensitive *E. coli* Nissle could grow in the supernatants of the chloramphenicol-resistant *E. coli* Nissle, although this growth was hindered in the supernatant of communities starting from higher densities (Figure 4.17a). This inhibition could also be noted in the growth of sensitive *Salmonella* in the *E. coli* Nissle supernatants, although it was less pronounced (Figure 4.17b). On the one hand, this can indicate some niche separation between both strains, if the inhibition is caused by nutrient depletion. On the other hand, intraspecies communication, may be greater than interspecies communication. In addition, the resistant *Salmonella* needed higher cell-densities to sufficiently degrade chloramphenicol and allow growth of the sensitive *Salmonella (*Figure 4.17c). However, reduced growth could also be observed within this combination and in the absence of the antibiotic (Figure S. 4.6) for high inoculum densities of the resistant strain that provided the supernatants. Finally, the obtained results hint at higher sensitivity of *E. coli* Nissle to chloramphenicol than *Salmonella* Typhimurium, even though both strains have the same MIC. Taken together, these results suggest that the resistant *Salmonella* less efficiently degraded the chloramphenicol in the surrounding medium than *E. coli* Nissle. Besides, several other factors, such as

consumption of nutrients, built up of waste products or inter- and intraspecies communication, limited the growth of the sensitive strain in the supernatants of high-density populations.

The differences in sensitivity, degradation-rate and -capacity were consistent with the hypotheses made in the ratio experiment and supported the importance of these species-specific properties in determining the stability of the system. However, it cannot be ruled out that, in addition to cross-protection, other inter- and intraspecies interactions, such as competition, are important.

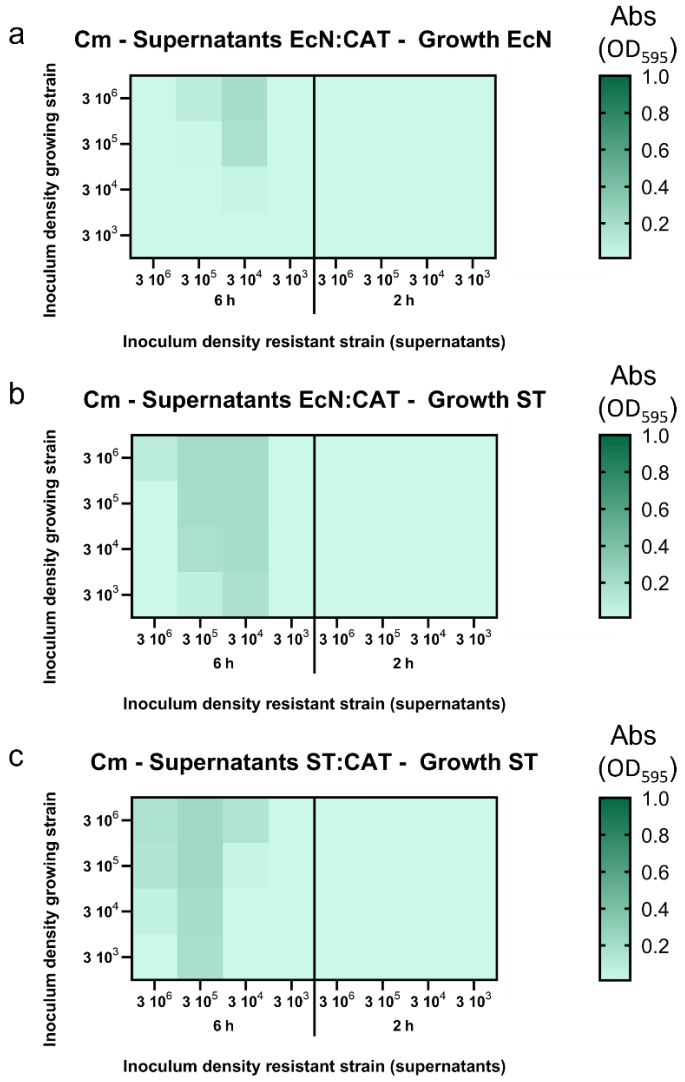


Figure 4.17: Cross-protection offered against chloramphenicol after two and six hours based on growth in supernatants. Growth of the sensitive strain after 20 hours, depicted as the absorbance at 595 nm, in the supernatants of chloramphenicol-resistant strains. Supernatants was taken after two and six hours from cultures starting from different inoculum densities. **a)** Growth of sensitive *E. coli* Nissle tdTomato in the supernatants of *E. coli* Nissle sfGFP pGDPI:CAT. **b)** Growth of sensitive *Salmonella* Typhimurium mCherry in the supernatants of *E. coli* Nissle sfGFP pGDPI:CAT. **c)** Growth of sensitive *Salmonella* Typhimurium mCherry in the supernatants of *Salmonella* Typhimurium sfGFP pGDPI:CAT.

1.4 Discussion

1.4.1 Cross-protection against a toxic environment can increase co-existence

Bacteria are social organisms that live in dense and diverse communities[130,303,304,349]. These interspecies communities are most often dominated by competitive interactions, as the involved strains have different evolutionary interests[1,2,47]. It has, however, previously been shown that the presence of a stressor in the environment can enhance positive interactions, in otherwise competitive communities[12,48,49]. Therefore, we wanted to study whether dependencies, in the form of cross-protection against antibiotics as a stressor, can enhance the interspecies co-existence, thereby neutralizing competition.

Hereto, we chose to focus on the interspecies interactions between the probiotic *E. coli* Nissle and the enteropathogen *Salmonella* Typhimurium. The relevance of this model community is evidenced by the well-documented competitive abilities of *E. coli* Nissle and its frequent use as a probiotic[200,204], consequently leading to its co-occurrence with the pathogen in ecological niches[204,328–330]. The interspecies dynamics were studied in absence of antibiotics, as well as in the presence of one or two antibiotics, creating one-sided and two-side dependencies, respectively. By limiting the co-culture experiments to four days in high antibiotic concentrations, we could focus on the short-term ecological interactions, without having to take into account evolutionary interactions. MIC tests with cefotaxime and chloramphenicol for the surviving strains after the short-term co-culture experiment did not indicate a change in resistance for any strain. This suggests a lack of plasmid conjugation between the strains or the evolution of resistance mechanisms. However, no additional experiments to study the evolution of social interactions were performed. In future research, prolonging the experimental time might be interesting, so that the combination of ecological and evolutional interactions can be studied.

After the validation of the competitive dominance of the probiotic *E. coli* Nissle over *Salmonella* Typhimurium, the influence of the addition of a toxic compound, an antibiotic, was studied. More specifically, we evaluated the impact of cross-protection against cefotaxime offered by the weaker *Salmonella* to the stronger competitive *E. coli* Nissle. In our setup, a

combination of competitive dominance and cheating allowed *E. coli* to gain the upper hand after a few days. However, when the competitive *E. coli* Nissle population became too dominant and the population size of the resistant *Salmonella* too small, the provided cross-protection became insufficient. Subsequently, the balance between both populations was restored. Although the length of our experiment was too short to make conclusions about the stability of the community, we hypothesize that the recovery of the weaker *Salmonella* population indicates the first cycle of a neutrally stable oscillatory system[50]. As a result, the addition of a toxic substance in the medium can neutralize the competition and increase co-existence between both strains. Furthermore, our findings imply that a co-evolution period is not required to provide cross-protection, as long as public resistance mechanisms are produced. This highlights the importance for applications in the field, as public resistance mechanisms are common in nature[9,316,317,319,350,351], including in *Salmonella*[352]. By focusing on the *E. coli* Nissle-*Salmonella* interactions, our study can provide novel insights in the efficacy of combinatorial therapy of antibiotics, bactericidal as well as bacteriostatic, and probiotics against antibiotic-resistant pathogens. Our results suggest that probiotic competition can become less efficient or can even be neutralized when the probiotic relies on the pathogen for survival, thereby pointing out the importance of further studying social interspecies interactions when developing antimicrobial applications. For example, to better mimic the *in vivo* environment of the GIT, the batch system with serial transfer should be replaced by a chemostat[353,354]. In addition, this change of model system might greatly influence the stability of the community, as daily dilutions are predicted to determine the oscillatory dynamics, while working with a chemostat could lead to convergence towards an equilibrium ratio in stable communities[50,219].

Nevertheless, the results of our one-sided cross-protection experiments were very condition dependent. The differences in species growth rate, competitive strength, antibiotic degradation rate and capacity as well as inherent sensitivity greatly influenced the outcome of the systems. Therefore, it could be interesting to conduct studies in the future in which the resistance mechanisms are exchanged between the two strains, making *E. coli* Nissle carry the public resistance mechanism against cefotaxime, while *Salmonella* Typhimurium provides resistance to chloramphenicol.

1.4.2 Differences in antibiotic degradation and sensitivity between strains are more important to determine community stability than interspecies competition

In addition to one-sided cross-protection, we also studied the impact of interspecies competition on the stability of communities relying on mutual cross-protection, by directly comparing the outcome of intra- and interspecies systems. Previous research by Yurtsev et al. (2016) showed that stable oscillatory systems can be obtained in intraspecies communities that depend on public resistance mechanisms for mutual cross-protection, similar to the mechanisms used in our research[50]. Also in our setup, oscillatory dynamics were expected. The growth potential of one strain depends on the population density and antibiotic degradation of the other, thus creating the potential for negative frequency-dependent selection. Consequently, following the formation of dominance by one strain, we expect the onset of oscillatory dynamics.

In all intra- and interspecies communities, the first difference with substantive relevance was created on day 1 or day 2, when the cefotaxime-resistant strain gained the upper hand. Initially, we assumed that the unhindered growth of the chloramphenicol-resistant strains in the two hours before cefotaxime addition allowed sufficient modification of chloramphenicol to enable the growth of the cefotaxime-resistant strain. As the chloramphenicol-resistant strain is, thereafter, partially killed by cefotaxime while the cefotaxime-resistant strain can grow, this would explain the dominance of the cefotaxime-resistant strain. However, this assumption was contradicted by the results obtained in the supernatants test that indicated the cefotaxime-resistant strain could not grow after two hours. Therefore, a novel hypothesis is formulated: During the initial two hour growth of the chloramphenicol-resistant strain, accompanied by degradation of chloramphenicol, the cefotaxime-resistant strain can become metabolically active and starts degrading cefotaxime upon administration, although the cross-protection against chloramphenicol is insufficient to allow actual growth[355–357]. Following partial killing of the chloramphenicol-resistant strain upon cefotaxime addition, recovery can take place once sufficient cefotaxime is degraded. During this recovery, additional chloramphenicol is broken down, eventually leading to strong growth of the cefotaxime-resistant strain. This novel theory

is also supported by the results obtained in the ratio experiment. Nevertheless, the nutrient and time limitations in combination with the interspecies interactions and growth rates upon antibiotic degradation most probably determine the exact outcome of the community.

The day after the cefotaxime-resistant population gains the upper hand, the expected shift in dominance occurs, establishing the initiation of the predicted oscillatory dynamics. However, once the chloramphenicol-resistant strain becomes dominant, the oscillations cease. In the interspecies community, this might be explained by the stronger competitive ability of *E. coli* Nissle over *Salmonella*. Possibly, the increased protection offered by the larger *E. coli* Nissle population to *Salmonella* is outweighed by the intensified competitive pressure exerted by *E. coli* Nissle against *Salmonella*. However, this trend can also be observed in the intraspecies communities. Based on the validation experiments, the limited cross-protection offered by CAT compared to the cefotaxime degradation by the β-lactamase is probably decisive. Nevertheless, even without oscillations and with differences in cross-protection, a stable co-culture could emerge. It would, however, have to consist of more chloramphenicol-resistant cells than cefotaxime-resistant cells, which is confirmed in the ratio experiments. Though, this difference in population size can make the system less robust to perturbations, such as the accidental non-homogeneous selection during daily dilution, especially since the smaller population provides the resistance against the bactericidal cefotaxime[50].

Despite that the different communities show similar trends, the interspecies community appears to be less stable than both intraspecies communities. Although competition may play some role, it probably is not the determining factor. Based on the validation experiments, the differences in sensitivity, degradation rate and capacity between the different strains are more likely to be key determinants for the stability of the communities. Besides, the collapse of the interspecies community seems to contrast with the 24-hour validation experiment of cross-protection. Nevertheless, previous research on mutual cross-protective communities with ampicillin and kanamycin resistance mechanisms also showed collapse of the system, even if the co-culture was viable on the first day. Similar to our results with chloramphenicol, they believed the cross-protection offered by kanamycin deactivation was insufficient[358].

Taking together the main findings of this study, we can conclude that community stability is highly condition dependent. For example, lowering antibiotic concentrations could increase the stability of the system by reducing dependency[50]. However, this lower antibiotic concentration could also enhance the impact of evolutionary interactions compared to ecological interactions. To investigate exclusively ecological interactions over an extended period in a structured framework, mathematical models, based on our dataset, could be used[50,344,359]. Besides antibiotic concentrations, also the choice of medium can have a significant impact on the outcome of the system. For instance, employing a medium with reduced nutrient richness may amplify exploitation competition for resources, conferring an added advantage to the strain initiating growth first[47]. Simultaneously, it can impact interference competition dynamics[144,360,361]. Additionally, low nutrient concentrations can activate biofilm production, potentially resulting in strain segregation and increased antibiotic tolerance among biofilm cells[362]. Finally, the frequency and intensity of dilution is a key determining factor for the stability of the system[50]. Also in this regard, mathematical models can offer a valuable tool for further investigation. One of the main advantages of mathematical models is the flexibility with which different parameters can be varied. As a result, such models could aid in further exploring the differences in outcome between a batch system with periodic dilution and a continuous system, like a chemostat, as proposed before.

1.5 Conclusion

This study investigated whether protection against an antibiotic through a public resistance mechanism can suppress competition and, thus, increase co-existence. In the presence of one antibiotic, a one-sided dependency is created between the otherwise competing strains. When the stronger competitor, *E. coli* Nissle, becomes dependent on the weaker *Salmonella* Typhimurium, a balance between the competitive upper hand and dependency is created, leading to a stable co-culture of both strains. Therefore, toxicity can increase co-existence, which can have a major influence on, for example, the use of combinatorial therapy of probiotics and antibiotics. Yet, creating a stable co-culture in the presence of one or two antibiotics is highly condition dependent. For example, the degradation capacity and speed and the number of resistant cells determine the efficiency of protection. In addition, the sensitivity of the strains and the competitive

interactions, considering both interference and exploitative competition in inter- and intraspecies communities, can play an important role in determining the outcome. The differences in stability observed between inter- and intraspecies communities that exhibit mutual cross-protection in the presence of both antibiotics cannot solely be ascribed to interspecies competition. Consequently, it cannot be concluded that interspecies competition is the decisive factor in destabilizing cross-protection.

1.6 Supplementary Figures & Tables

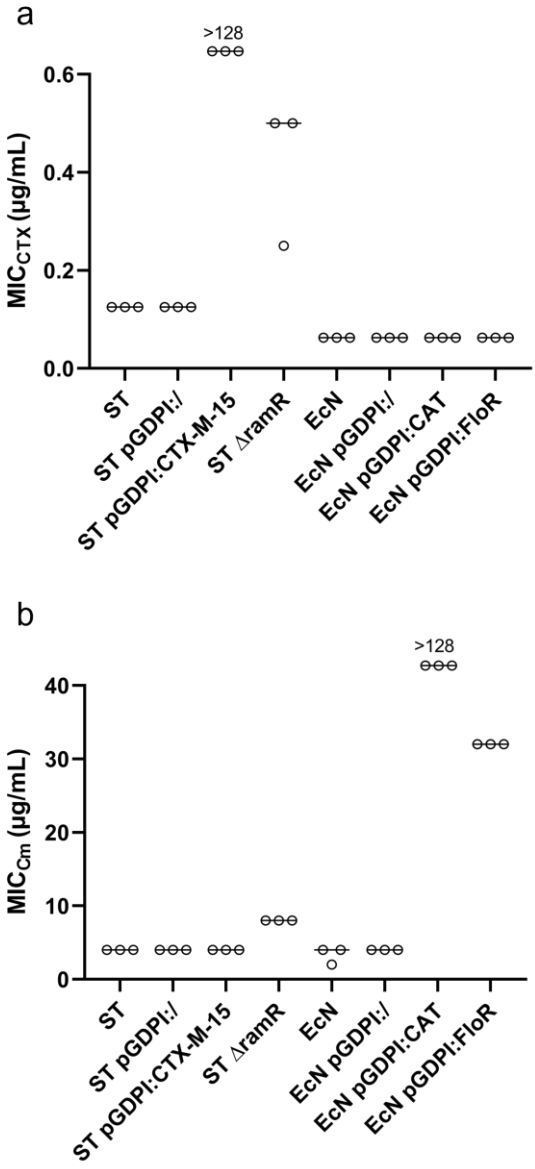


Figure S. 4.1: Minimum inhibitory concentrations against a) cefotaxime and b) chloramphenicol of different *Salmonella* Typhimurium and *E. coli* Nissle strains.

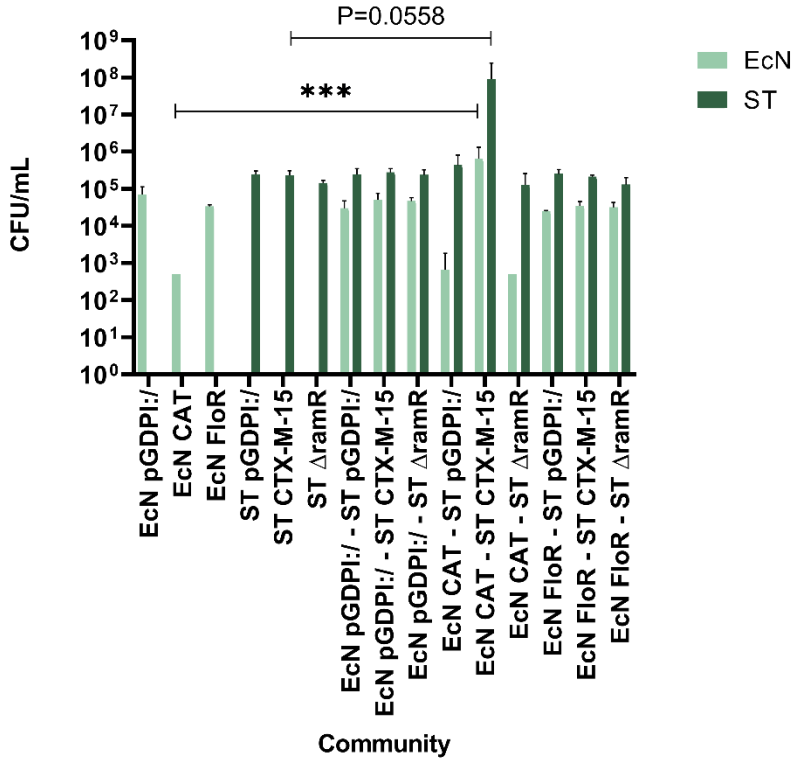


Figure S. 4.2: Absolute cell counts of mono- and duo-culture *Salmonella* Typhimurium and *E. coli* Nissle after 24 hours treatment with chloramphenicol and cefotaxime. *E. coli* Nissle (sensitive: EcN pGDPI:/, public resistance against chloramphenicol: EcN CAT or private resistance against chloramphenicol: EcN FloR) and *Salmonella* Typhimurium (sensitive: ST pGDPI:/, public resistance against cefotaxime: ST CTX-M-15 or private resistance against cefotaxime: ST ΔramR) were grown in mono- or duo-culture in the presence of both chloramphenicol (16 µg/mL), added from the start, and cefotaxime (0.25 µg/mL), added after two hours. After 24 h of incubation at 37°C in shaking conditions, the cell counts were determined using a spot-plating method. Duo-culture growth of each strain was compared to its respective monoculture control using an ordinary one-way ANOVA, with Šídák's multiple comparisons test ($n = 3$, $\alpha = 0.05$). All p-values lower than 0.99 are indicated in the graph. Mutual cross-protection was only offered when both strains expressed a public resistance mechanism.

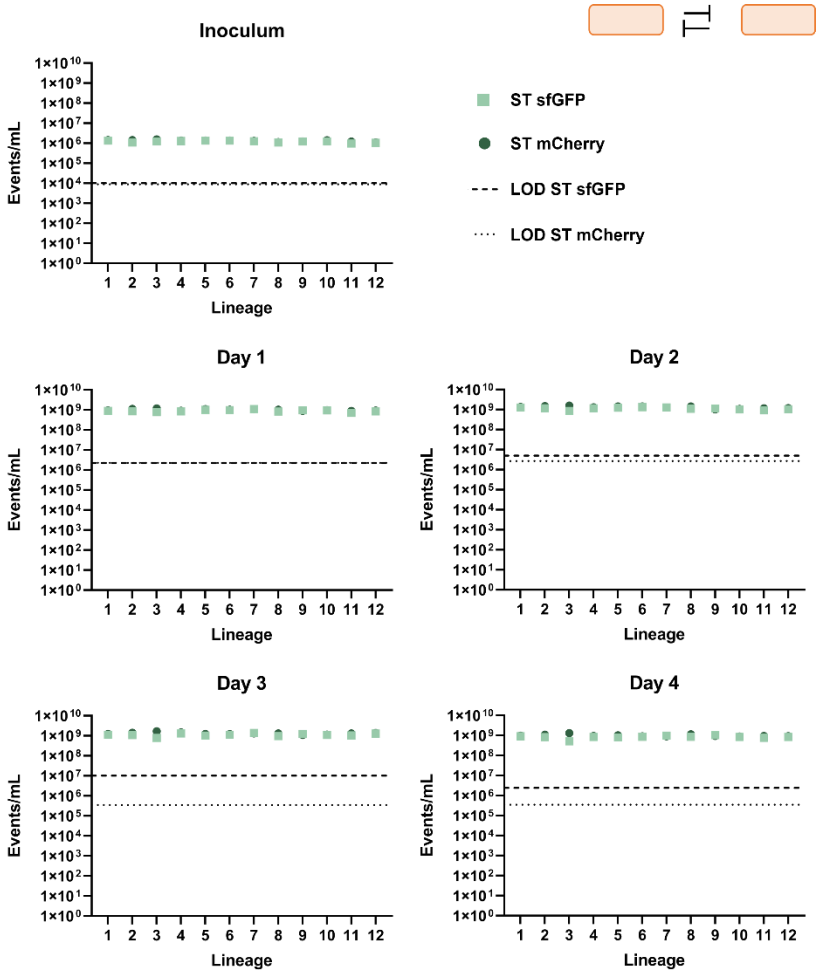


Figure S. 4.3: *Salmonella-Salmonella* growth control in the absence of antibiotics. The genomic fluorescent labels do not influence growth potential or any competitive traits in our setup, as the strains remain in equilibrium over the course of four days.

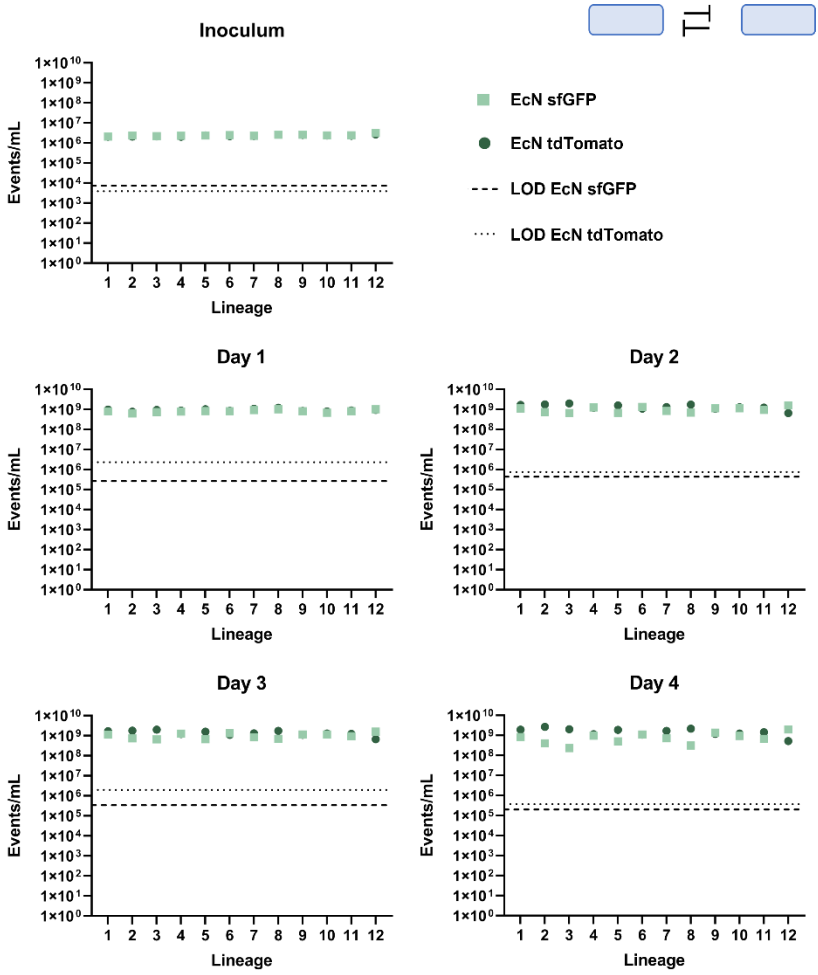


Figure S. 4.4: *E. coli* Nissle- *E. coli* Nissle growth control in the absence of antibiotics. Although *E. coli* Nissle tdTomato performs better than *E. coli* Nissle sfGFP in seven out of twelve repeats by day four, no consistent difference could be observed. As a result, we do not expect the differences to be due to a differential growth rate or competitive strength because of the different fluorescent labels, but rather due to a stochastic effect that is enlarged by the non-homogeneous influencing factor created during repetitive dilution.

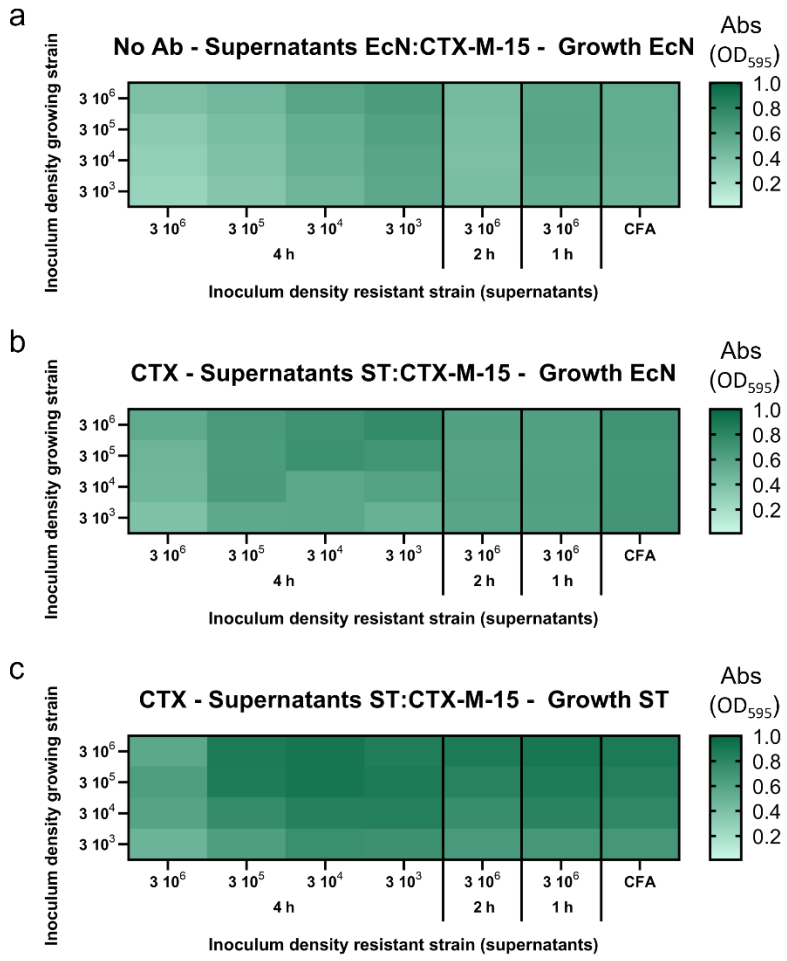


Figure S. 4.5: Growth of sensitive strains in the supernatants of cefotaxime-resistant strains in the absence of antibiotics. Growth of the sensitive strain after 20 hours, depicted as the absorbance at 595 nm, in the supernatants of cefotaxime-resistant strains. Supernatants was taken after one, two or four hours from cultures starting from different inoculum densities. **a)** Growth of sensitive *E. coli* Nissle sfGFP in the supernatants of *E. coli* Nissle tdTomato pGDPI:CTX-M-15. **b)** Growth of sensitive *E. coli* Nissle sfGFP in the supernatants of *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15. **c)** Growth of sensitive *Salmonella* Typhimurium sfGFP in the supernatants of *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15.

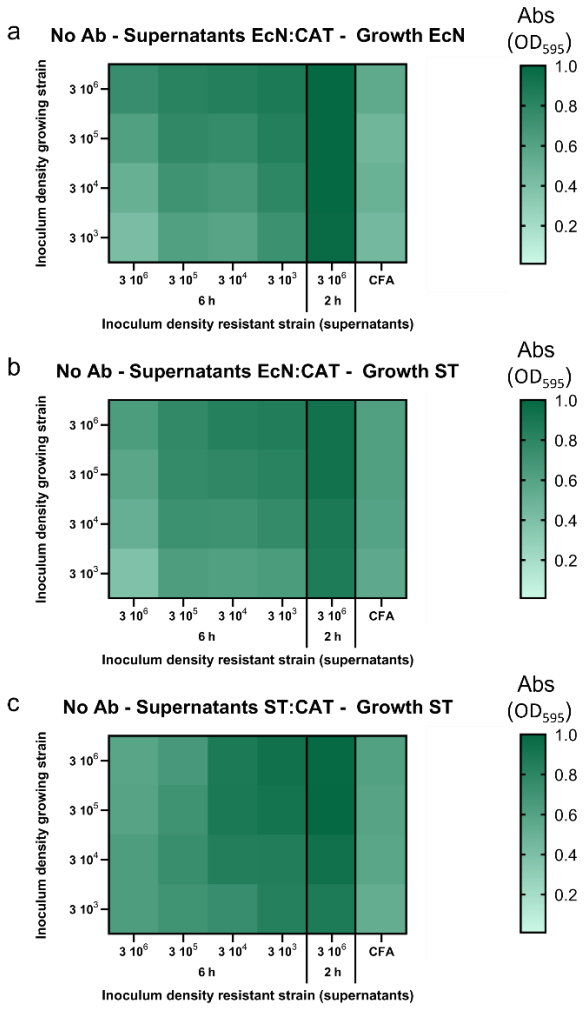


Figure S. 4.6: Growth of sensitive strains in the supernatants of chloramphenicol-resistant strains in the absence of antibiotics. Growth of the sensitive strain after 20 hours, depicted as the absorbance at 595 nm, in the supernatants of chloramphenicol-resistant strains. Supernatants was taken after two and six hours from cultures starting from different inoculum densities. **a)** Growth of sensitive *E. coli* Nissle tdTomato in the supernatants of *E. coli* Nissle sfGFP pGDPI:CAT. **b)** Growth of sensitive *Salmonella* Typhimurium mCherry in the supernatants of *E. coli* Nissle sfGFP pGDPI:CAT. **c)** Growth of sensitive *Salmonella* Typhimurium mCherry in the supernatants of *Salmonella* Typhimurium sfGFP pGDPI:CAT.

Table S. 4.1: Overview of primers used for the construction and validation of the different plasmids used in this chapter. Tails that encode homology arms or tails with restriction sites are indicated in bold. The melting temperature, without tails, was calculated via the IDT oligo analyzer tool. FW = Forward, RV = Reverse

Primer	Sequence 5' → 3'	Description
S&P-02041	CAGCCTAGCCGGGAGATC TG	FW primer pGDPI for sequencing and electroporation check
S&P-02044	CCAACTCAGCTTCCTTTCG GG	RV primer pGDPI for sequencing and electroporation check
S&P-02070	**GATAAATGCTTCTCTAGA AA**GTTGTTGTTATTTCGTA TCTTCCAG	FW primer for extraction CTX-M-15 from pACYC184, tail to create restriction site for XbaI
S&P-02071	**GGTGGTGGTGGTGCTCG AG**GGCGAAAATGAGACG TTGATC	FW primer for extraction CTX-M-15 from pACYC184, tail to create restriction site for XhoI
S&P-02395	**AAACACGGAA**GAAGCAA AAGATAATCGGATAAAAT GTAGCAATTC	FW primer *floR* and promoter region on p39R861-4 with 10 bp tail homologous to the primer region of S&P-02398
S&P-02396	**TTCCAGTTTG**CCTGCTACC AGTCGTCGGTTAG	RV primer *floR* and promoter region on p39R861-4 with 10 bp tail homologous to the primer region of S&P-02397
S&P-02397	**TGGTAGCAGG**CAAACTG GAACTTCATGTCGGC	FW primer backbone pGDPI with 10 bp tail homologous to the primer region of S&P-02396
S&P-02398	**CTTTTGCTTC**TTCCGTGTT TCGTAAAGTCTGG	RV primer backbone pGDPI with 10 bp tail homologous to the primer region of S&P-02395