OVERVIEW COMBINED

Contents

Background

Interspecies interactions and their influence on the community

Types of interactions

- Competition
- Cooperation
- Accidental effects

Detoxification of the environment - public mechanisms

- Beta-lactamases: excreted --> destroy Ab --> reduce Ab concentration in the environment
- CAT: intracellular enzymatic degradation --> take up Ab & destroy --> reduce Ab concentration in the environment
- Public resistance mechanisms = commensalism because not driven by the presence of the sensitive surrounding bacteria, thus they are not evolutionary adaptations

Why would provide protection to species that compete for same resources?

- Accidental

Mechanisms that ensure maintenance of public resistance mechanisms

- Partial privatization of the public resistance mechanism
  Public resistance mechanisms can be maintained if the advantage of production and privatization is greater than the disadvantage of the competition (no selection against sharing the protection-

==> How does cross-protection, either in one direction or in both directions, impacts the social interaction between competitors and the stability of the interspecies community?

- Aim 1: explore whether the competitive interactions can be compromised by a dependency for protection in a toxic environment
  - *Salmonella* – *E. coli* Nissle model community

- Aim 2: elucidate importance of interspecies competition on the stability of communities relying on mutual cross-protection in the presence of two antibiotics.
  - Compare stability of intra- and interspecies communities over time

Can dependency on cross-protection neutralize competition?

Confirmation of the stronger competitor

Overview

E. coli Nissle outcompetes *Salmonella* in the absence of antibiotics.

Short-term co-culture experiment

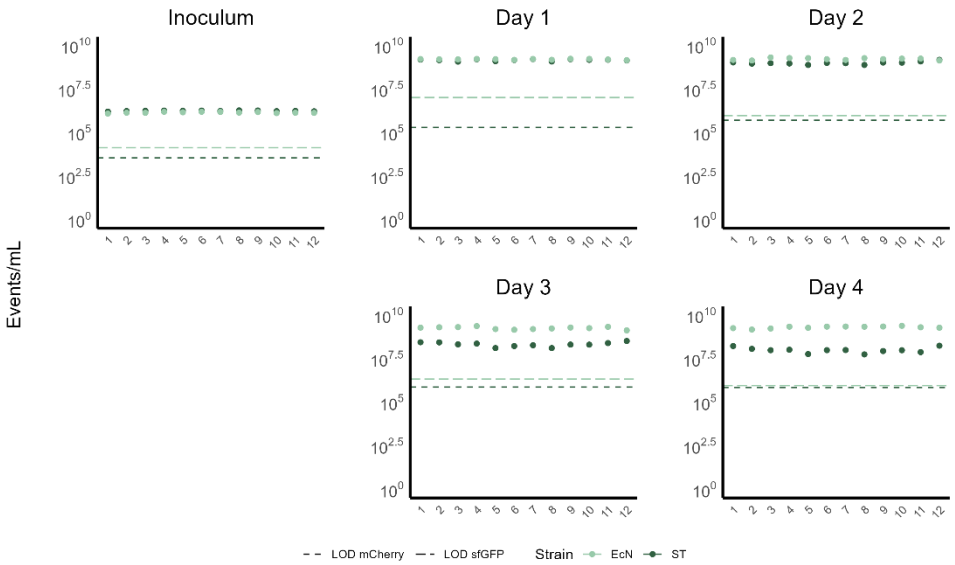


Figure 1: **Competition experiment between *E. coli* Nissle and *Salmonella* Typhimurium in the absence of antibiotics.** The cell counts of twelve biological repeats, originating from different overnight cultures, were followed over the course of four days. The co-cultures were incubated at 37°C with 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.

Extensive explanation

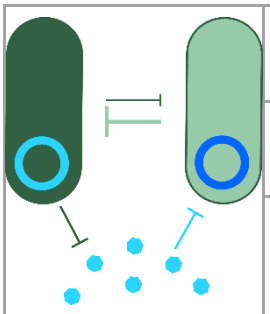
The characterization of the baseline interspecies interaction was done in the growth medium without the addition of a stressor (i.e. antibiotics). When the probiotic *E. coli* Nissle and the pathogen *Salmonella* Typhimurium are put together in co-culture conditions in which both strains can grow and 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. By extrapolating these results over time, we hypothesize that competitive exclusion will ultimately occur.

One-sided interspecies cross-protection

Thereafter, we studied whether cross-protection can neutralize the previously observed competition and, thus, can increase co-existence of both strains.

The stronger competitor relies on the weaker competitor for detoxification

Overview



Competitive strength	EcN > ST
Cross-protection	EcN relies on ST
Hypothesis	Oscillatory dynamics When EcN dominates too much, cross protection of ST decreases --> competitive releave for ST so that can increase in abundance
Result	ST takes overhand first --> EcN well protected --> because also stronger competitor, gradually takes over again by the end of experiment ==> suggests oscillatory dynamics Using 3x MIC get faster dynamics (less dependency of EcN on ST) --> indeed seems to indicate oscillatory dynamics

Short-term co-culture experiment

4x MIC

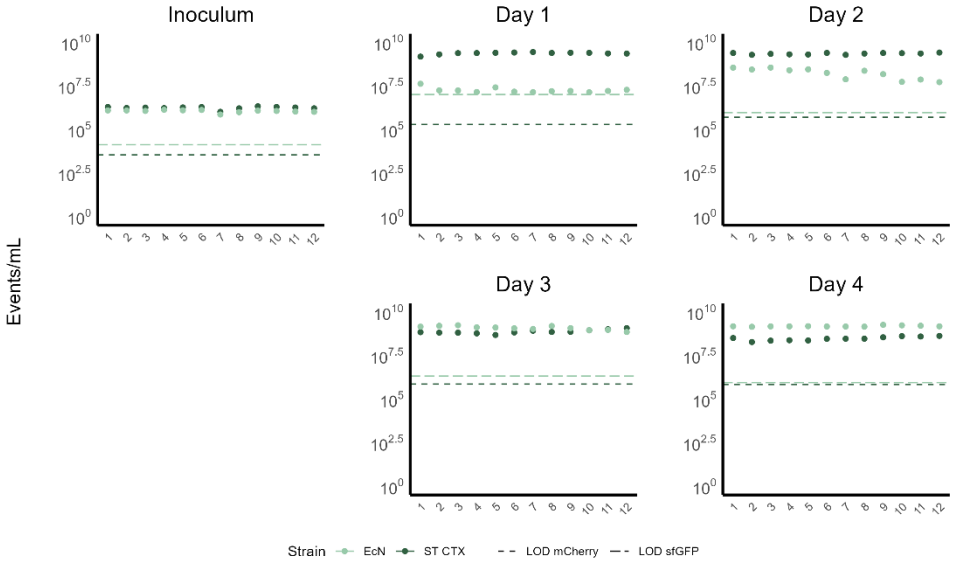


Figure 2: **Interspecies ecological interaction where *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 offers cross-protection to *E. coli* Nissle sfGFP against cefotaxime at a concentration of 4x MIC.** MIC_{EcN} = 0.0625 µg/mL cefotaxime. Twelve biological repeats were followed over the course of four 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.

3x MIC

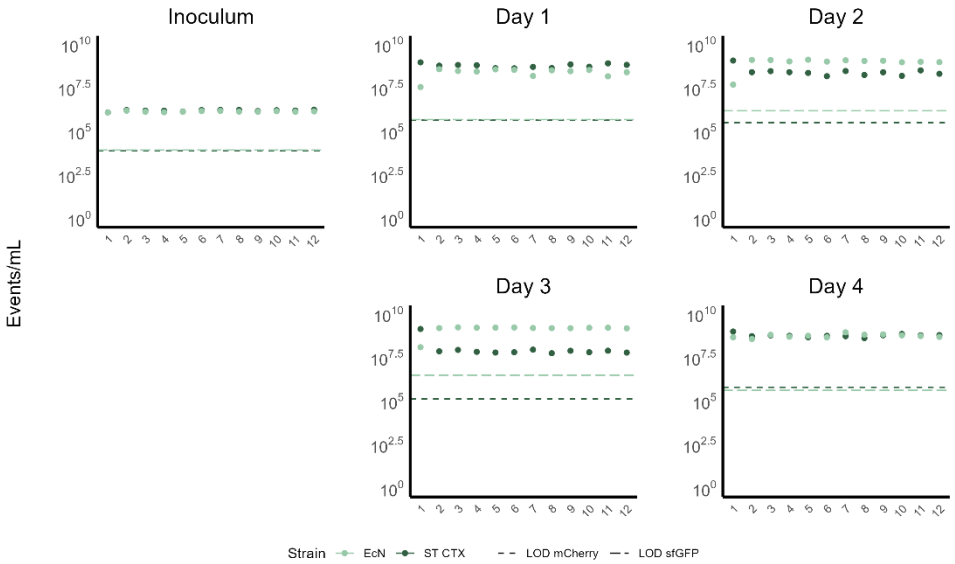


Figure 3: **Interspecies ecological interaction where *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 offers cross-protection to *E. coli* Nissle sfGFP against cefotaxime at a concentration of 3x MIC.** MIC_{EcN} = 0.0625 µg/mL cefotaxime. Twelve biological repeats were followed over the course of four 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.

Extensive explanation

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.

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.

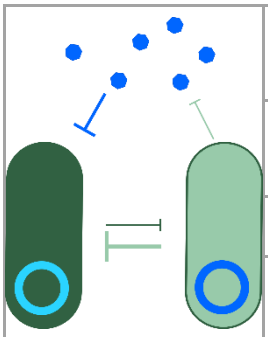
The results show that after one day of incubation in the presence of cefotaxime, *Salmonella* Typhimurium outnumbered the sensitive *E. coli* Nissle, displaying a two-log difference in population size. We hypothesize that, subsequently, the larger resistant *Salmonella* Typhimurium population entails 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. 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.

The weaker competitor relies on the stronger competitor for detoxification

Overview



Competitive strength	EcN > ST
Cross-protection	ST relies on EcN
Hypothesis	Collapse of ST
Result	Competitive exclusion of ST Faster than in absence of antibiotics Initial protection observed (growth on day 1 compared to inoculum & maintenance of abundance on day 2 despite dilution)

Short-term co-culture experiment

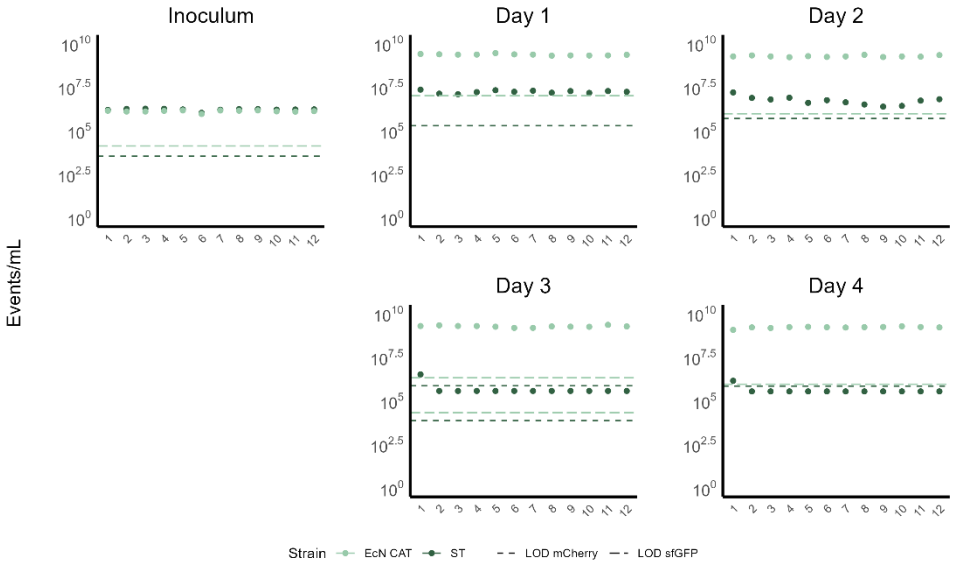


Figure 4: **Interspecies ecological interaction in medium supplemented with chloramphenicol at a concentration of 4x MIC of *Salmonella* Typhimurium mCherry, making it dependent on the cross-protection of *E. coli* Nissle sfGFP pGDPI:CAT.** MIC_{ST} = 4 µg/mL chloramphenicol. The co-cultures were incubated 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.

Extensive explanation

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. 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.

Importance of interspecies competition on stability of mutual cross-protective communities

Two-sided interspecies communities

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.

Controls

Overview

MIC assay	No significant cross-resistance
24h cross-protection	ST & EcN performed better in duo culture than in mono culture only when both had the public resistance mechanism => cross-protection of public resistance mechanisms

24h cross-protection

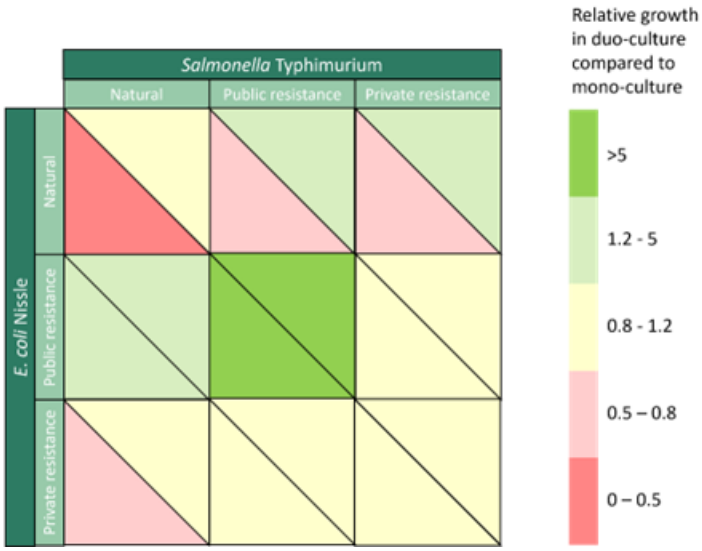


Figure 5: **In the presence of both chloramphenicol, added from the start, and cefotaxime, added after 2 hours, *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 and *E. coli* Nissle sfGFP pGDPI:CAT offer mutual cross-protection.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm 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 colours 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.

Extensive explanation

Include strains with private resistance mechanisms

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.

MIC assay

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.

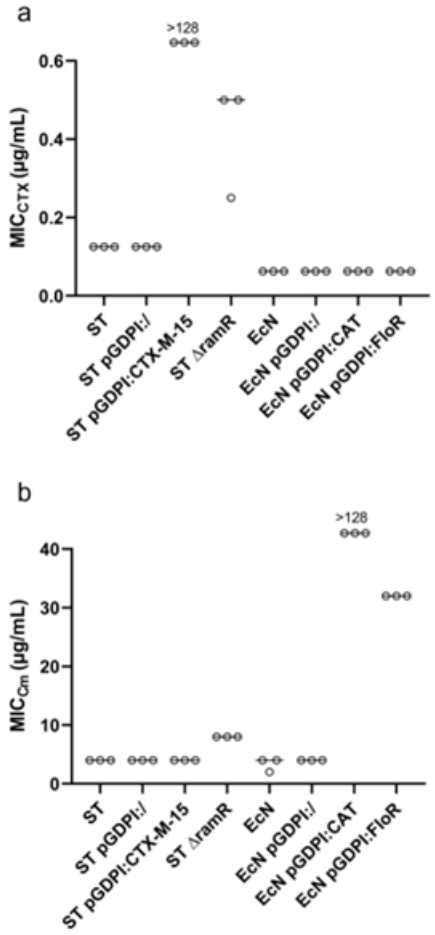


Figure 6: **Minimum inhibitory concentrations against a) cefotaxime and b) chloramphenicol of different *Salmonella* Typhimurium and *E. coli* Nissle strains.**

24h cross-protection

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), 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.

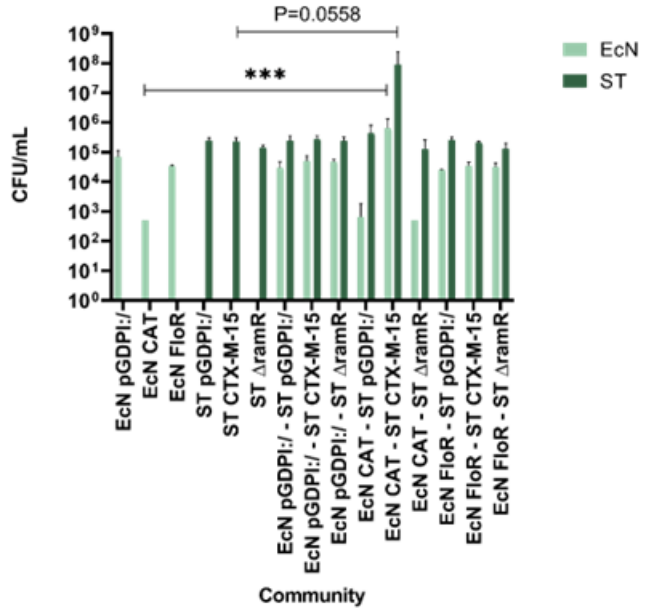
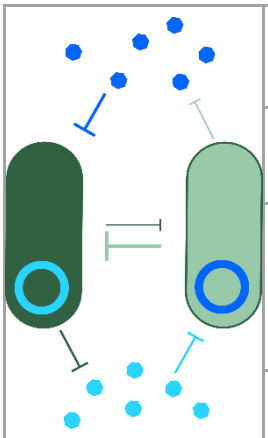


Figure 7: **Absolute cell counts of mono- and duo-culture *Salmonella* Typhimurium and *E. coli* Nissle after 24 hours treatment with chloramphenicol and cefotaxime.** *E. coli* Nissle (sensitive: EcN pGDPI:/, public resistance against chloramphenicol: EcN CAT or private resistance against chloramphenicol: 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, α = 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.

Both strains depend on each other for the detoxification of two antibiotics

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.

Overview



Competitive strength	EcN > ST
Cross-protection	EcN relies on ST ST relies on EcN
Hypothesis	Oscillatory dynamics • Both strains rely on the detoxification for one of the antibiotics on the other strain
Result	On first day ST takes the overhand --> by day 2, EcN takes over --> instead of oscillatory dynamics, we observe that EcN remains dominant once it is dominant --> leading to collapse of the community • Is the advantage of enhanced protection of a larger EcN population on ST not bigger than the disadvantage of increased competitive pressure on ST? • Is chloramphenicol degradation not sufficient? (more private mechanism)

Short-term co-culture experiment

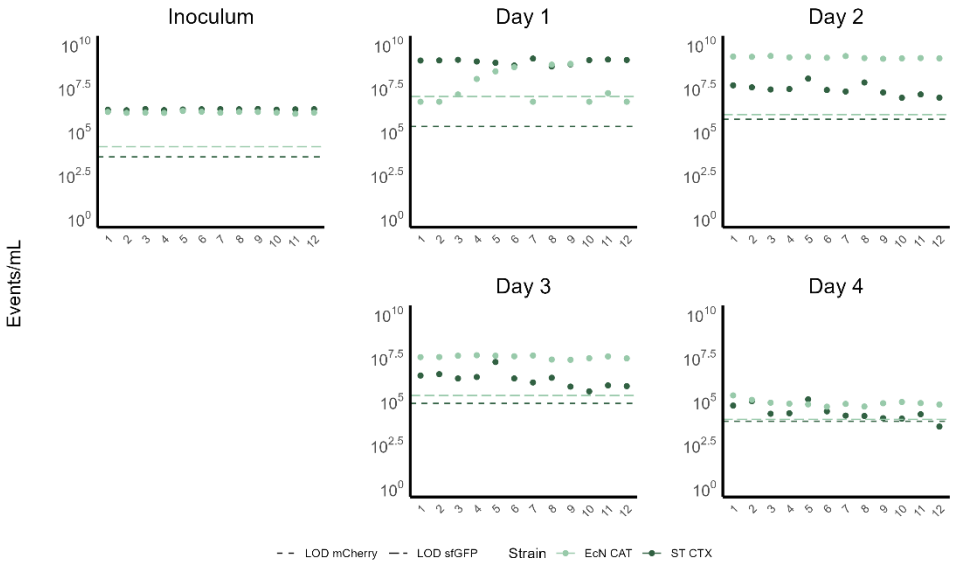


Figure 8: **Interspecies mutual cross-protection between *E. coli* Nissle sfGFP pGDPI:CAT and *Salmonella* Typhimurium mCherry pGDPI:CTX-M-15 did not generate a stable community at concentrations of 4x MIC.** $MIC_{EcN,CTX}$ = 0.0625 µg/mL cefotaxime, $MIC_{ST,Cm}$ = 4 µg/mL chloramphenicol. The co-cultures 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.

Extensive explanation
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.

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.

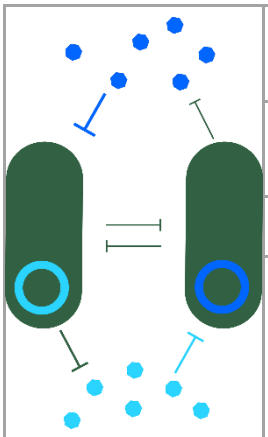
Forced mutualism based on cross-protection appears more stable within species than between species

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.

In the 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.

Intraspecies *Salmonella* Typhimurium community

Overview



Competitive strength	ST CAT = ST CTX
Cross-protection	ST CAT relies on ST CTX ST CTX relies on ST CAT
Hypothesis	Oscillatory dynamics
Result	On day ST CAT takes the upper hand, switching to ST CTX on day 2, and then switching back to ST CAT which remains dominant • No immediate collapse of the community -> more stable than the interspecies community • Either collapse takes longer or the concentration of ST CTX at which the community is stable is not yet reached

Short-term co-culture experiment

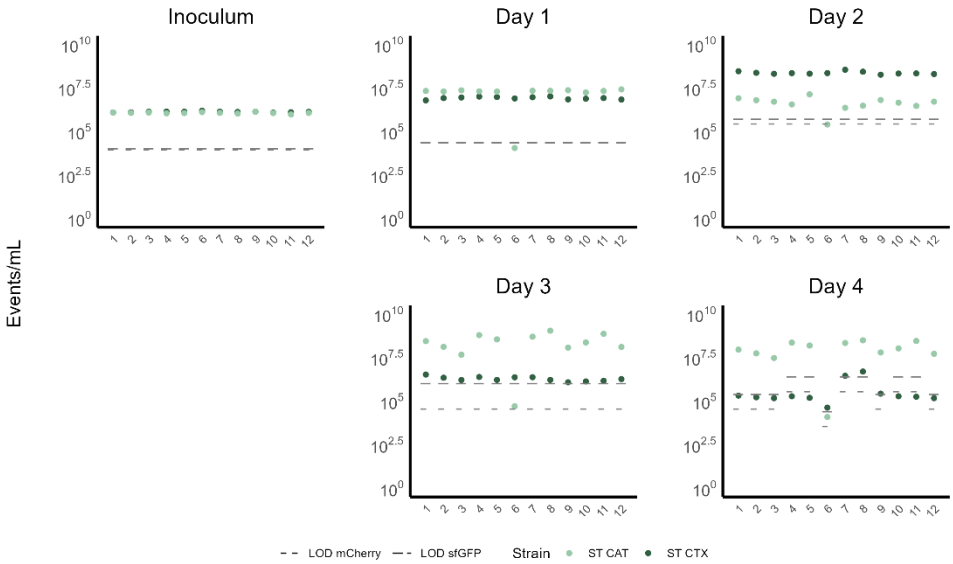


Figure 9: **The mutual cross-protection in the intraspecies *Salmonella* Typhimurium community creates a stable co-culture for at least four days.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol 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.

Extensive explanation

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

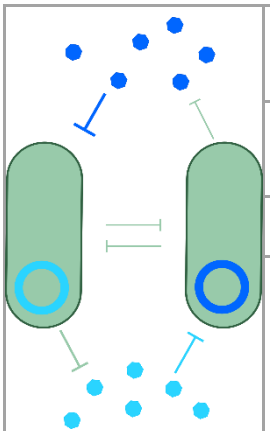
In contrast to the interspecies community, where the cefotaxime-resistant *Salmonella* took the upper hand, the chloramphenicol-resistant *Salmonella* dominated the intraspecies community after the first day. Possibly, the difference between the inter- and intraspecies community can be explained by the 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.

Intraspecies *E. coli* Nissle community

Overview

	**Competitive strength**	EcN CAT = EcN CTX
	Cross-protection	EcN CAT relies on EcN CTX EcN CTX relies on EcN CAT
	Hypothesis	Oscillatory dynamics
	Result	More stochastic differences, but more stable than interspecies community • Delay in manifestation of first major differences in this community • Some of the repeats still maintain a stable and high cell number on the last day

Short-term co-culture experiment

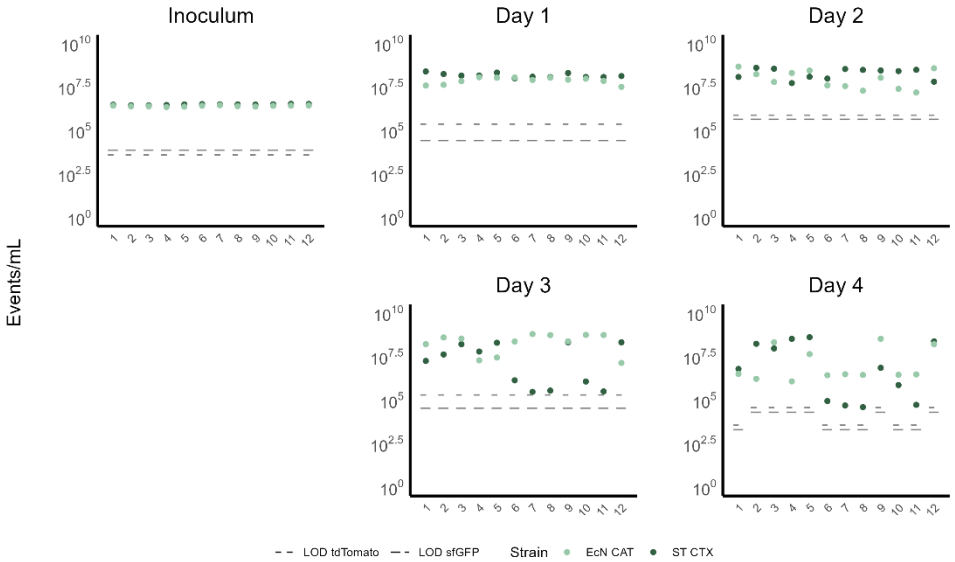


Figure 10: **The stability of the intraspecies *E. coli* Nissle community relying on mutual cross-protection is determined by the creation of strong differences in population size by stochastic effects.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 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.

Extensive explanation

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

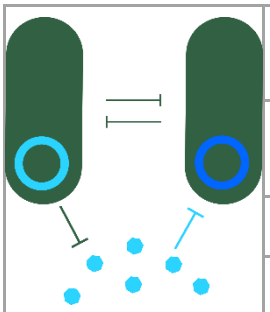
interspecies combination of *E. coli* Nissle and *Salmonella* Typhimurium. On the one hand, there is a delay in the manifestation of the first major differences in this community. On the other hand, some of the repeats still maintain a stable and high cell number on the last day.

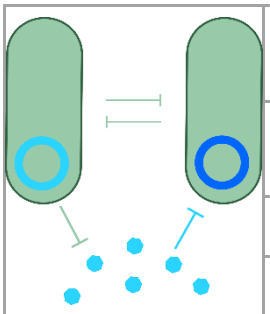
Differences in antibiotic degradation and sensitivity between strains appear more important to determine community stability than interspecies competition

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

Cefotaxime

Overview

	**Competitive strength**	Assume ST = ST CTX
	Cross-protection	ST relies on ST CTX
	Hypothesis	
	Result	Stable co-culture with [ST CTX] = 10x [ST] • Limited cost associated with production of resistance mechanism Or • Costly production is compensated by partial privatization of the protection during the initial stage of degradation • Sensitive strain may grow faster once enough cefotaxime is degraded to maintain stable co-culture

	**Competitive strength**	Assume EcN = EcN CTX
	Cross-protection	EcN relies on EcN CTX
	Hypothesis	
	Result	No stable co-culture • Some cross-protection offered • Hypothesis 1: EcN breaks down cefotaxime slower than ST • Hypothesis 2: EcN less efficiently reduces the cefotaxime concentration in surrounding medium, lowering the cross-protection • Once sufficient cefotaxime broken down, still enough nutrients for sensitive strain to grow?

Short-term cross-protection experiments

ST

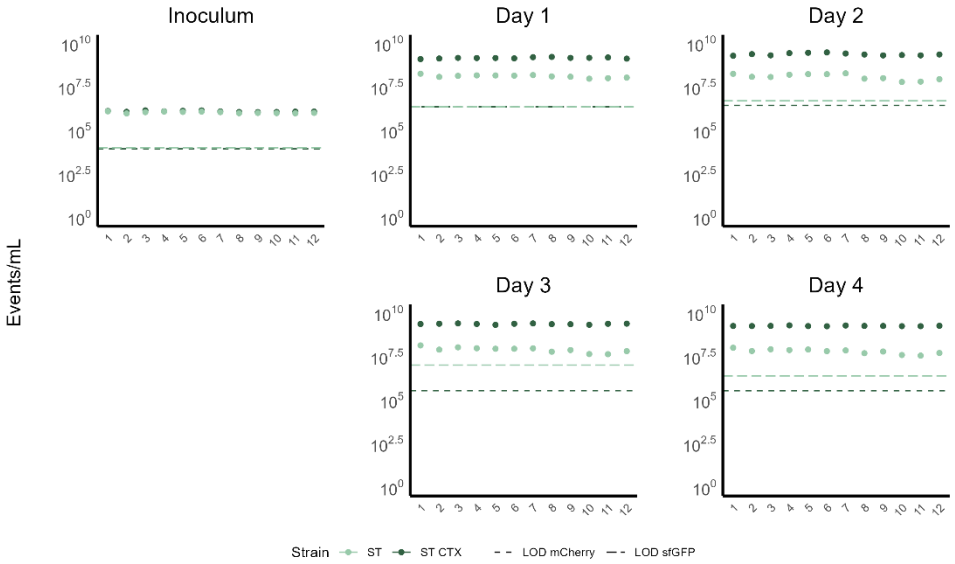


Figure 11: **Intraspecies *Salmonella*-*Salmonella* cross-protection against cefotaxime creates a stable co-culture.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, cefotaxime was supplemented to the medium 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.

EcN

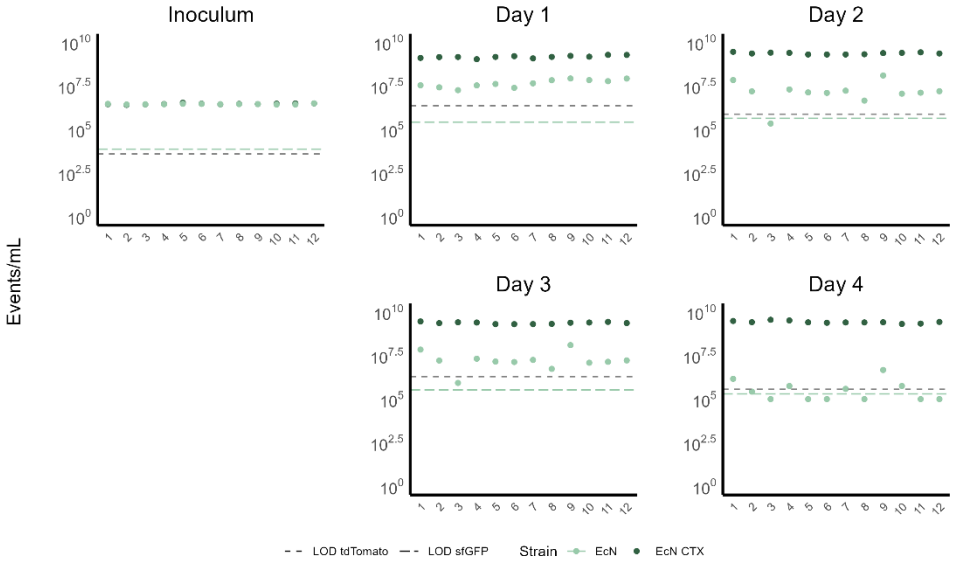


Figure 12: ***E. coli* Nissle tdTomato pGDPI:CTX-M-15 does not offer sufficient cross-protection against cefotaxime to the sensitive *E. coli* Nissle to stabilize the intraspecies co-culture.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into 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.

Extensive explanation

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. Cefotaxime was added to the medium daily after two hours. Based on the results, the

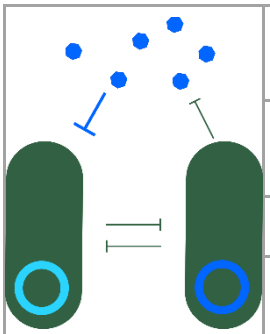
intraspecies *Salmonella* cross-protection does create a stable duo-species community in which the prevalence of the resistant strain is ten times greater than that of the sensitive strain. The sensitive *Salmonella* strain can grow, indicating it is able to enjoy the benefits offered by the public resistance, 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.

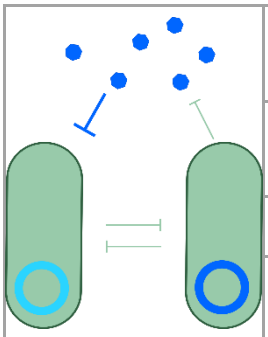
In contrast, no stable intraspecies *E. coli* Nissle duo-community is formed. Although some cross-protection is offered, as evidenced by the growth of the sensitive strain on day 1, the population size of the sensitive strain strongly decreases by day 4. Importantly, *E. coli* Nissle is more sensitive 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. 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.

Chloramphenicol

Overview

	**Competitive strength**	Assume ST = ST CAT
	Cross-protection	ST relies on ST CAT
	Hypothesis	
	Result	Insufficient protection of sensitive strain by intracellular degradation of chloramphenicol acetyltransferase • Too low inoculum density resulting in long period to reach critical density necessary to confer adequate cross-protection? • Daily dilution: diluted out sensitive strain before it could recover (higher concentrations of resistant strains at later days)

	**Competitive strength**	Assume EcN = EcN CAT
	Cross-protection	EcN relies on EcN CAT
	Hypothesis	
	Result	Once sufficient protection is offered, sensitive strain can recover (overcoming the daily dilution) • Too low inoculum density resulting in long period to reach critical density necessary to confer adequate cross-protection at the start • Larger resistant population after day 2 degrades chloramphenicol faster and, thus, offers more cross-protection

==> chloramphenicol degradation and subsequent cross-protection strongly depends on the population size of resistant strain

Short-term cross-protection experiments

ST

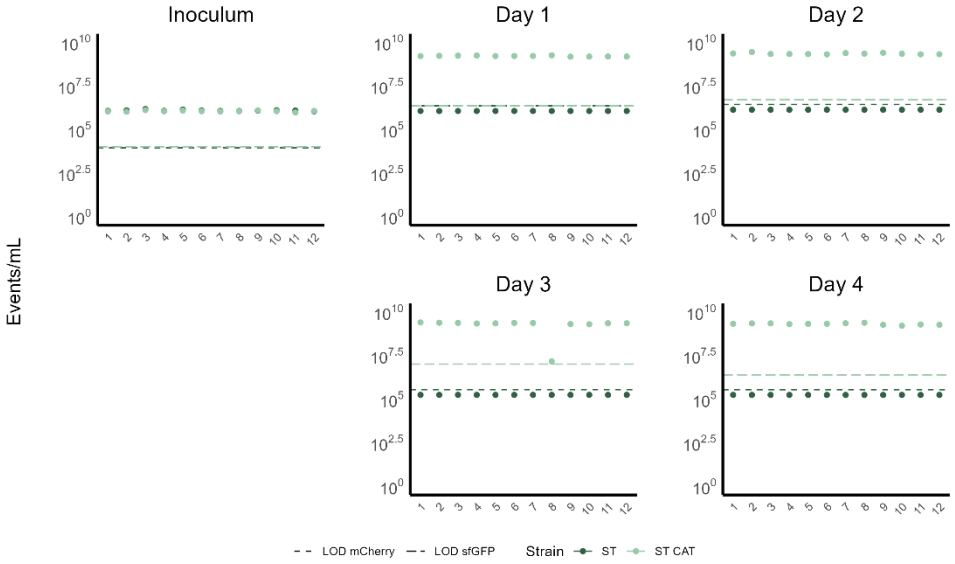


Figure 13: ***Salmonella*** **Typhimurium sfGFP pGDPI:CAT does not offer sufficient intraspecies cross-protection against chloramphenicol.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol was 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.

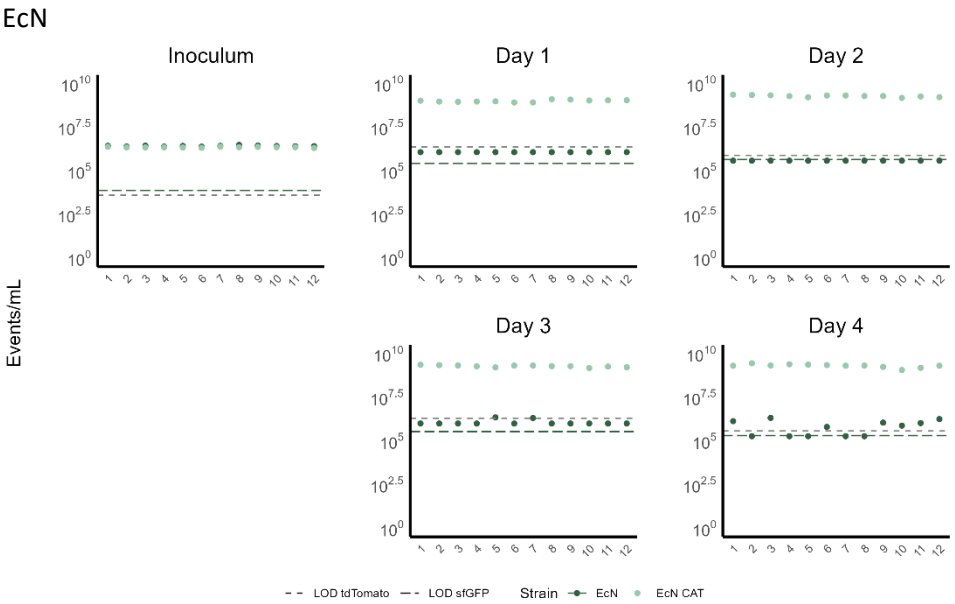


Figure 14: **The *E. coli* Nissle sfGFP pGDPI:CAT population size determines the protection against chloramphenicol and stability of the co-culture.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm and were diluted every 24 h into fresh CFA medium (1/100). Daily, chloramphenicol 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.

Extensive explanation

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. 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. 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. 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 ± 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, while in the intraspecies *E. coli* Nissle community, a larger resistant population is required before the sensitive strain can recover. 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.

The protection profile of intraspecies EcN community resembles that of the interspecies community more than the intraspecies ST 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

Overview

Ratio (EcN CAT : ST CTX)	Outcome
999:1, 99:1, 9:1	• Still no growth of ST CTX despite higher EcN CAT concentrations • (999:1, 99:1) insufficient cross-protection of EcN CAT against cefotaxime
1:1	• Hypothesis: metabolically active ST CTX was able to sufficiently degrade cefotaxime, preventing EcN CAT's cell death, keeping it metabolically active or even allowing additional growth --> additional degradation of chloramphenicol enabled ST CTX to grow and overtake EcN CAT
1:9, 1:99, 1:999	• (1:999 and 1:99) insufficient protection by EcN CAT --> ST CTX not metabolically active and so no cross-protection against cefotaxime --> EcN CAT killed upon addition of cefotaxime • (1:9) EcN CAT offers sufficient cross-protection for ST CTX to be metabolically active and thus break down cefotaxime, but insufficient for growth of ST CTX

24 h co-culture experiment

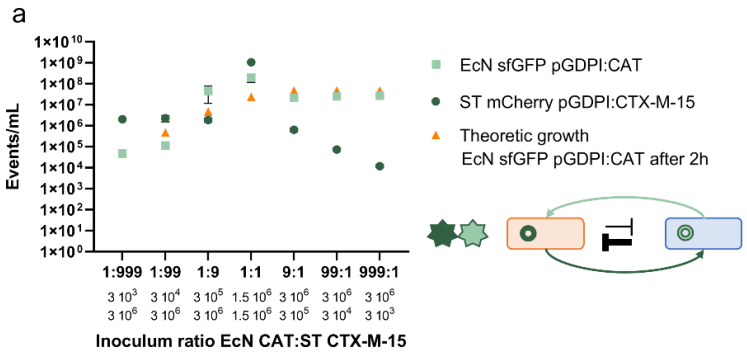


Figure 15: **Protective profiles of inter- and intraspecies communities starting from different inoculum ratios in media supplemented with both antibiotics.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm for 20 h. Chloramphenicol was added to the growth medium from the start, 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.

Extensive explanation

In the interspecies community, 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 EcN community

Overview

Ratio (EcN CAT : EcN CTX)	Outcome
999:1, 99:1, 9:1	• No growth observed: lower inoculum density of cefotaxime-resistant strain and subsequent lower cross-protection prevented recover of chloramphenicol resistant strain --> no additional degradation of chloramphenicol and thus no growth of cefotaxime resistant strain
1:1	• Metabolically active EcN CTX was able to sufficiently degrade cefotaxime, preventing EcN CAT's cell death --> EcN CAT becomes dominant, while ST CTX seems to suffer from the lesser protection of ST against chloramphenicol
1:9, 1:99, 1:999	• (1:99, 1:9) greater degradation potential of chloramphenicol from EcN causing the cefotaxime resistant strain to become metabolically active at lower ratio & EcN might also degrade cefotaxime quicker than ST, allowing growth of chloramphenicol resistant strain already in these ratio's

24 h co-culture experiment

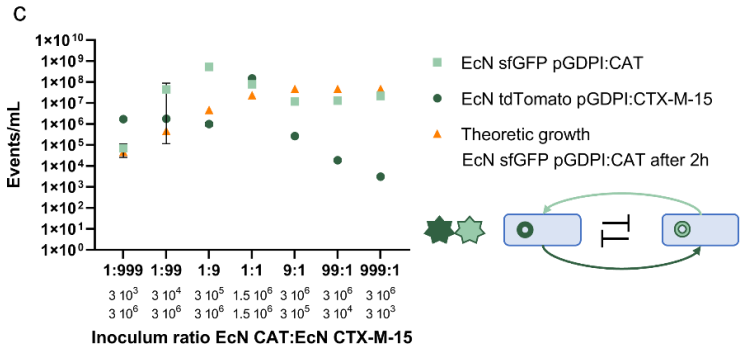


Figure 16: **Protective profiles of inter- and intraspecies communities starting from different inoculum ratios in media supplemented with both antibiotics.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm for 20 h. Chloramphenicol was added to the growth medium from the start, 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. **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.

Extensive explanation

The intraspecies *Salmonella* 1:999 and 1:99 ratios produced similar results as the interspecies interactions. 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 ST community

Overview

Ratio (ST CAT : ST CTX)	Outcome
999:1, 99:1, 9:1	• Still no growth of ST despite higher EcN concentrations • (999:1, 99:1) insufficient cross-protection of Ecn against cefotaxime
1:1	• Hypothesis: metabolically active EcN CTX was able to sufficiently degrade cefotaxime, preventing EcN CAT's cell death, keeping it metabolically active or even allowing additional growth --> additional degradation of chloramphenicol enabled EcN CTX to grow
1:9, 1:99, 1:999	• (1:999 and 1:99) insufficient protection by ST CAT --> ST CTX not metabolically active and so no cross-protection --> ST CAT killed upon addition of cefotaxime • (1:9) both strains converged to same population density (more privatized or slower degradation of chloramphenicol by ST CAT --> ST CTX degrades less cefotaxime --> minimal growth of both strains)

24 h co-culture experiment

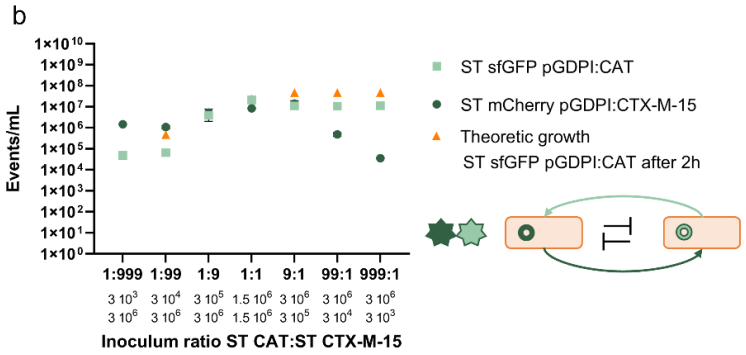


Figure 17: **Protective profiles of inter- and intraspecies communities starting from different inoculum ratios in media supplemented with both antibiotics.** The co-cultures were incubated at 37°C with continuous shaking at 200 rpm for 20 h. Chloramphenicol was added to the growth medium from the start, 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. **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.

Extensive explanation

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.

Notes

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. 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.

Comparison of the protective profiles

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.

To more easily compare the protective profiles, a simplified, alternative representation was constructed: 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.

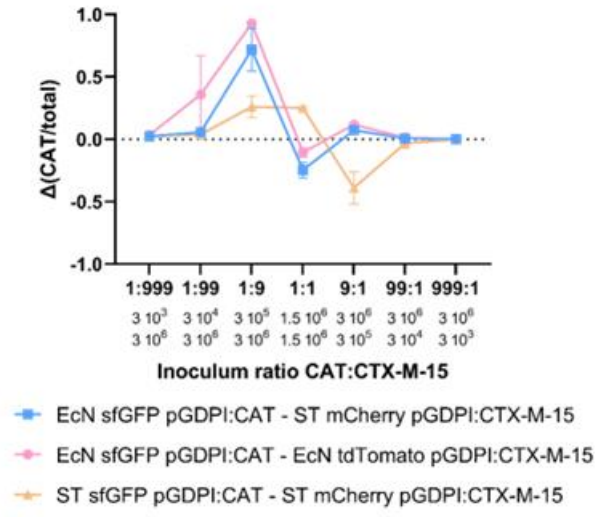


Figure 18: **Comparison of the inter- and intraspecies cross-protection dynamics at different inoculum ratios.** The x-axis describes the tested inoculum ratios ranging from 1:999 to 999:1, annotated as the chloramphenicol-resistant strain (indicated as CAT), compared to the cefotaxime-resistant strain (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.

EcN and ST 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

Cefotaxime

Overview

Cefotaxime	• ST more thoroughly degraded cefotaxime, thereby offering more cross-protection, although EcN more rapidly decreased the cefotaxime concentration in the medium • ST is less susceptible to cefotaxime than EcN

Supernatans experiment

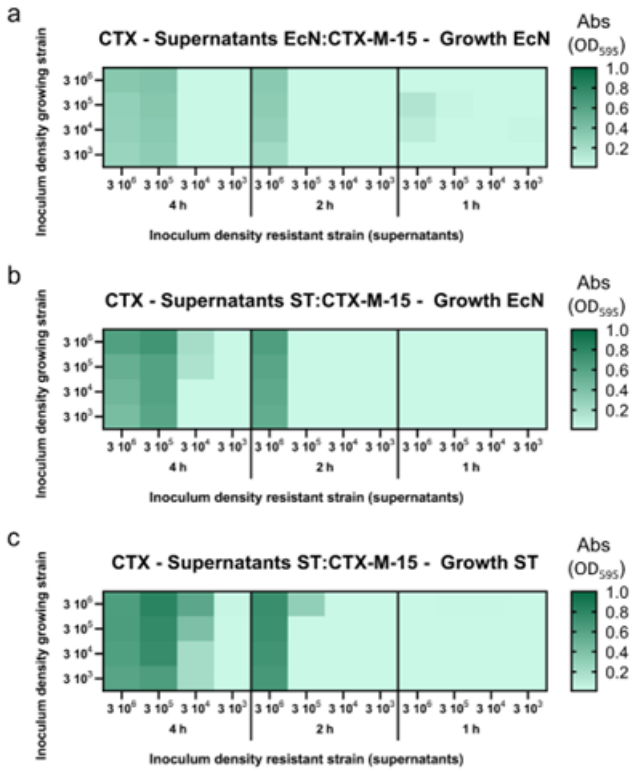


Figure 19: **Cross-protection offered against cefotaxime after one, two and four hours based on growth in supernatants.** Growth of the sensitive strain after 20 hours, depicted as the absorbance at 595 nm, in the supernatants of cefotaxime-resistant strains. Supernatants was taken after one, two or 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.

Extensive explanation

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, 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. 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. 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.

Chloramphenicol

Overview

Chloramphenicol	• After 2 h insufficient degradation to allow growth of sensitive strain after 6 h growth possible (although limited in supernatants of high-density populations) • EcN higher sensitivity to chloramphenicol than ST despite an identical MIC • ST less efficiently degrades chloramphenicol in the surrounding medium than EcN

Supernatans experiment

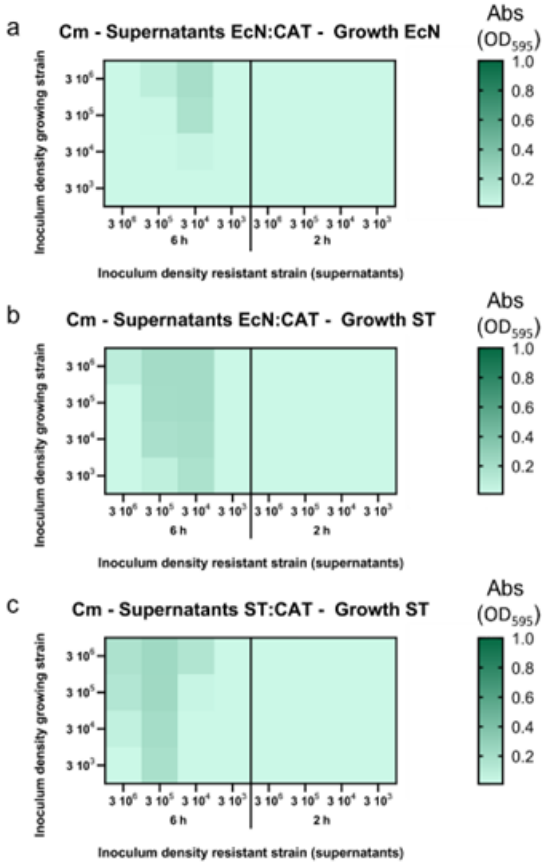


Figure 20: **Cross-protection offered against chloramphenicol after two and six hours based on growth in supernatants.** Growth of the sensitive strain after 20 hours, depicted as the absorbance at 595 nm, in the supernatants of chloramphenicol-resistant strains. Supernatants was taken after two and 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.

Extensive explanation

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 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. This inhibition could also be noted in the growth of sensitive *Salmonella* in the *E. coli* Nissle supernatants, although it was less pronounced. 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*. However, reduced growth could also be observed within this combination and in the absence of the antibiotic 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.

Which parameters determine the outcome of co-existence or exclusion?

Goal: can we reproduce the outcomes of the short-term co-culture experiments and can we use this model to explore which parameters influence the outcome of the co-culture experiments?
Hypothesis: antibiotic sensitivity and degradation rate are the major contributors to the stability of the co-culture, while the resource competition has a minor effect?

Model choice

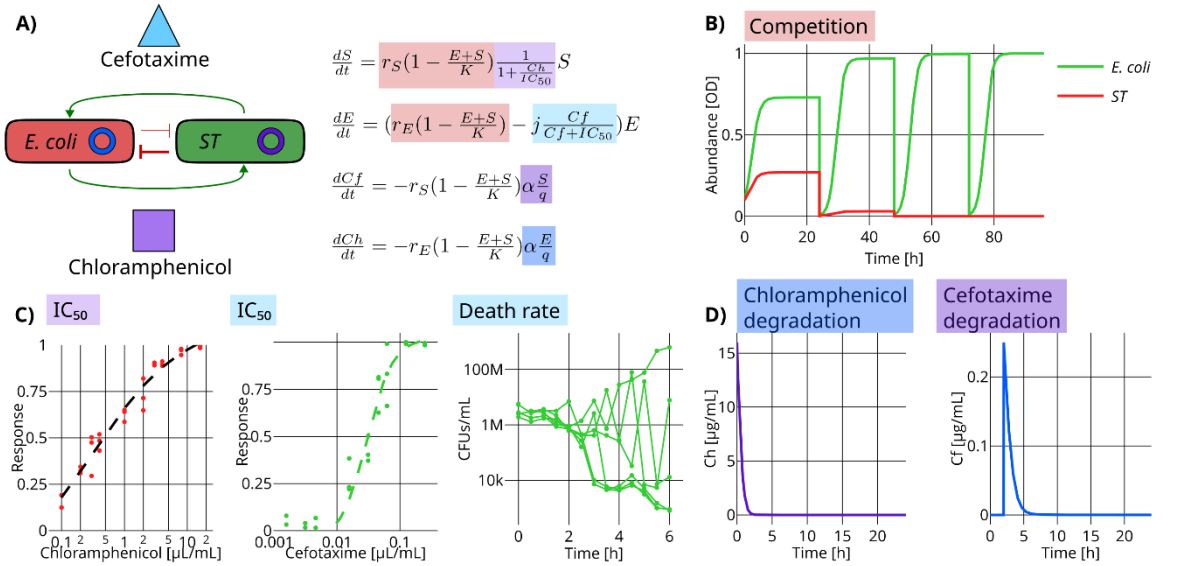


Fig. 1. Description of the model and parameters influencing community dynamics. Community dynamics depend on the strength of the negative and positive interaction, its direction as well as the mechanistics of the antibiotics. **(A)** Schematic description of the system and equations used for modeling. The negative interaction is modeled via competition (red). The species with the higher maximum growth rate (r) negatively influences the other species growth. Cefotaxime acts as a bactericidal and is modeled as a function of its concentration (Cf), maximum death rate (j) and its IC50 (light blue). Chloramphenicol acts bacteriostatic and is modeled as a function of the concentration (Ch) and the IC50 (light purple) of chloramphenicol. Each species can interact positively with the other species by degrading the antibiotic which only affects the partner species. Degradation depends on growth, a degradation rate alpha and a scaling factor q, relating species density with molecular concentration of the antibiotic (blue). **(B)** In absence of antibiotics the system is governed only by the direction and strength of the negative interaction. Because E. coli has the higher maximum growth rate, it excludes ST.**(C)** Mechanistics of the two antibiotics. First two panels: IC50 measuring with response curves for chloramphenicol and cefotaxime with susceptible species (dashed line is obtained fit). Chloramphenicol still causes a response at very low concentrations and has thus a very low IC50. Third panel: The acting of cefotaxime additionally depends on a maximum death rate j. Time-kill curves showed great variability. **(D)** Simulated maximum degradation. Cefotaxime is added after 2 hours. For both species, q was chosen that the antibiotic is degraded completely after 2 hours at a degradation rate α 1 1/h. This is the upper boundary of possible degradation and can be tuned by lowering α.

Parameterization

Experimental parameters

Transfers, dilution factor and interval

In the short-term co-culture experiment, 4 transfers are performed. The transfers are performed by a dilution factor of 100 after 24 h (interval).

Starting concentrations

The inoculation density of each strain is equal. The antibiotics starting concentration are 16 µg/mL (chloramphenicol) and 0.25 µg/mL (cefotaxime; after 2 hours).

Bacterial parameters

Maximal growth rate

Experimentally, the OD of the different strains was measured over a period of 24 h to obtain the growth curves. From the log-transformed exponential phase, the maximal growth rate was deducted as the slope of the linear regression.

Antibiotics parameters

Detoxification rate

The detoxification rate can be estimated, but we will vary in this parameter as we expect that this is an important parameter to explain the outcome.

Death rate of cefotaxime

Attempt to determine it experimentally (a lot of variation), but will still vary in the concentration to explain the outcomes we obtained.

IC50 (K)

Determined the concentration at which 50% of the community is inhibited (half-maximal inhibitory concentration). This value can be extracted from a MIC assay, fitting a four-parameter sigmoid curve on the inhibition curves.

Parameter exploration

Hypothesis: species that is chloramphenicol resistant will always take over, regardless of the initial resource competition

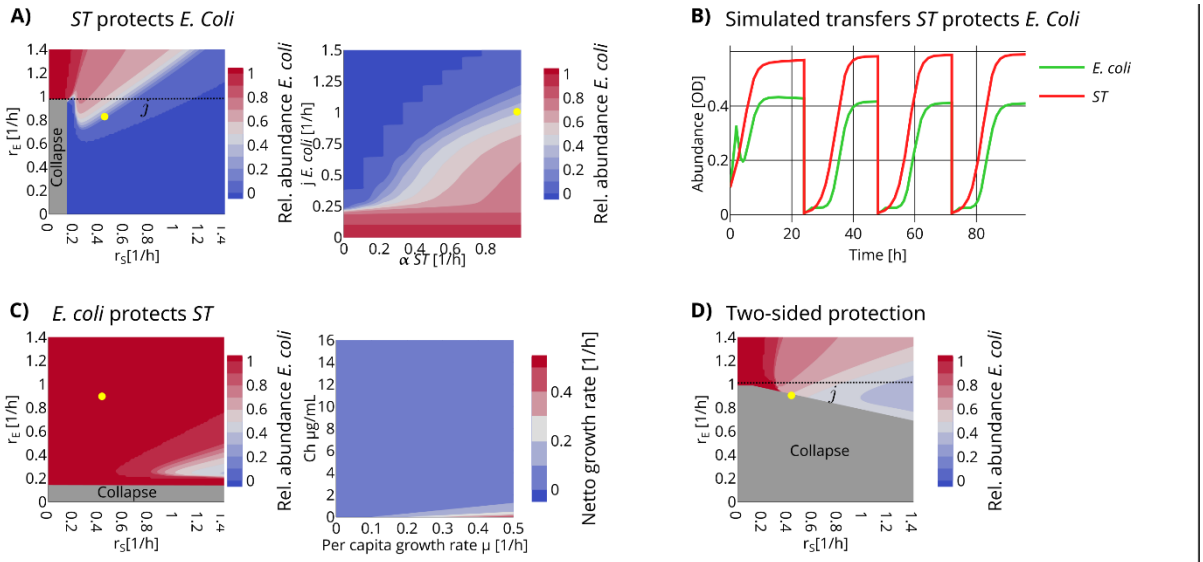


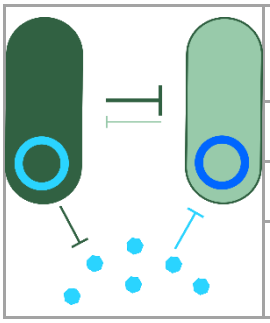
Fig. 2. Influence of the nature of interaction on coexistence. **(A)** One sided protection going from ST to E. Coli allows for coexistence for a large parameter space. Left panel: Relative abundance of E. coli after 10 simulated transfers as a function of the maximum growth rate (r) of the two species. The two species coexist if the maximum growth rate of E. coli is bigger than the maximum growth rate of ST. Yellow dot represents the estimated maximum growth rates for the experiments. Dashed line shows the death rate used for the simulations (1 1/h). Right panel: Coexistence is observed in simulations also at lower degradation rates (α). Lower death rates greatly favors coexistence. Yellow dot represents the estimated parameters based on experiments. **(B)** Simulated transfer experiments with parameters estimated from experiments shows the coexistence of the two species. **(C)** One sided protection going from E. coli to ST mostly leads to exclusion of ST. Left panel: Relative abundance of E. coli after 10 simulated transfers shows that ST is going extinct in most simulations, expect when ST has a much greater maximum growth rate than E. coli. Yellow dot shows estimated parameters from experiments. Right panel: Netto growth rate (Observed growth considering available resources (μ) and inhibition at a certain chloramphenicol concentration). Chloramphenicol acts very effectively at very low concentrations (see Fig. 1 C), therefore even at extremely high degradation, growth is still inhibited making coexistence very unlikely for all directions and strength of the negative interaction. **(D)** Two-sided protection. At maximum degradation, coexistence is possible at high maximum growth rates and high degradation at both directions of the negative interaction. The estimated parameters are right at the border of coexistence and collapse assuming maximum degradation (yellow dot). If one of the species degrades just slightly less, the system collapses.

If we inverse the stronger (resource) competitor, what is the outcome then?

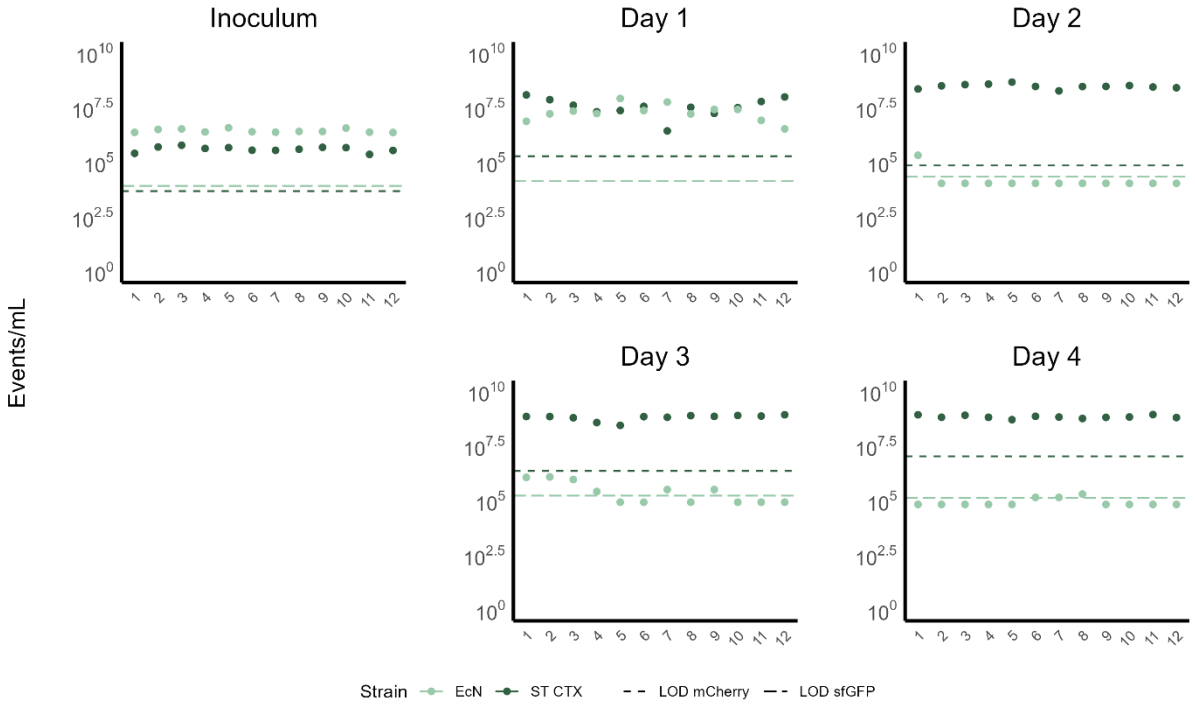
In order to hold up the hypothesis that the influence of the resource competition is submissive to the effect of the degradation mechanism in determining the stability of the community, we wanted to repeat the experiment, but now in a situation where *Salmonella* is the stronger competitor.
If the strain with the CAT resistance mechanism takes the overhand despite being the weaker competitor, this indicates that indeed the degradation mechanism is the main contributor.
To this end, we used the 20 % CFA medium (as indicated of the screening from other project because in this medium *Salmonella* is the stronger competitor) with the same strains (EcN CAT and ST CTX). This minimizes the changes needed to be done to the system.

The weaker competitor is dependent on the stronger competitor for the detoxification of the environment

Overview

	**Competitive strength**	ST > EcN CAT
	Cross-protection	EcN relies on ST CTX
	Hypothesis	ST will outcompete EcN
	Result	ST CTX provides cross-protection to EcN, allowing it to grow at first, but it is quickly outcompeted by ST

Short-term competition experiment

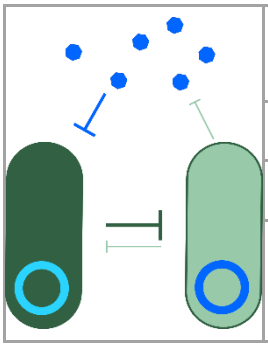


More details

As expected, when the stronger competitor has the advantage of also detoxifying the environment, it can quickly outcompete the weaker competitor.

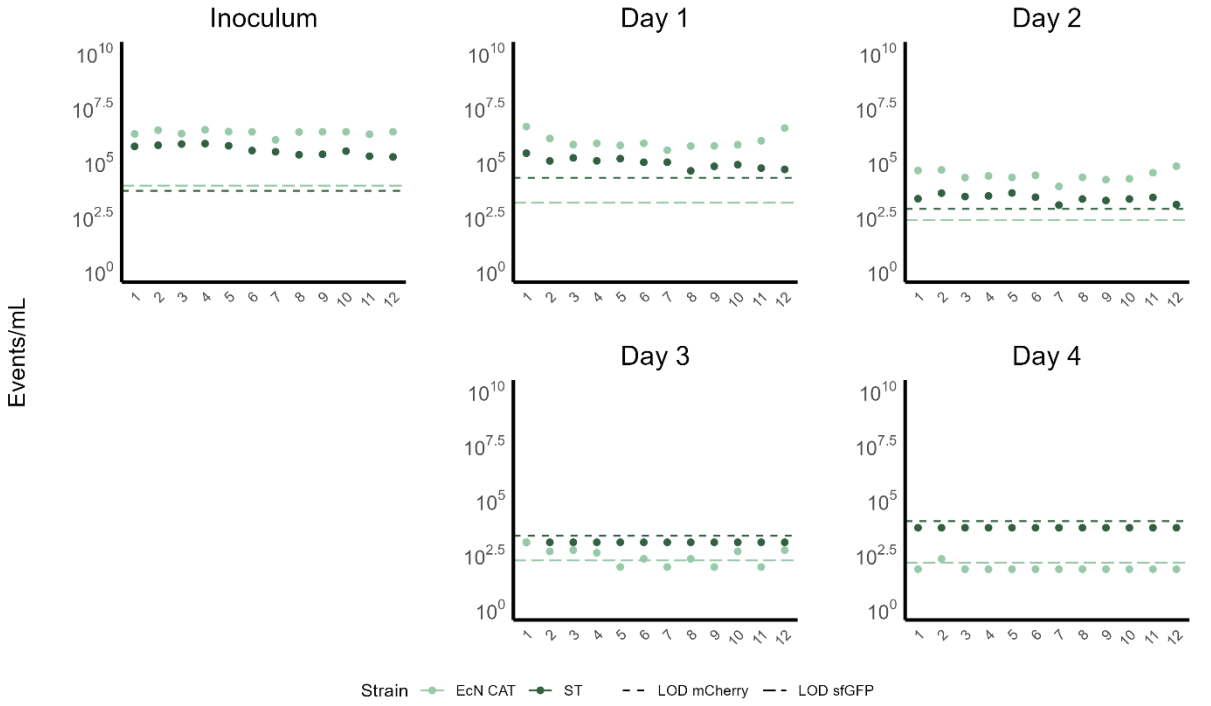
The stronger competitor is dependent on the weaker competitor for the detoxification of the environment

Overview



Competitive strength	ST > EcN CAT
Cross-protection	ST relies on EcN CAT
Hypothesis	Oscillatory dynamics
Result	CAT resistance mechanism does not seem to work in this situation, causing a collapse of the system

Short-term competition experiment



More details

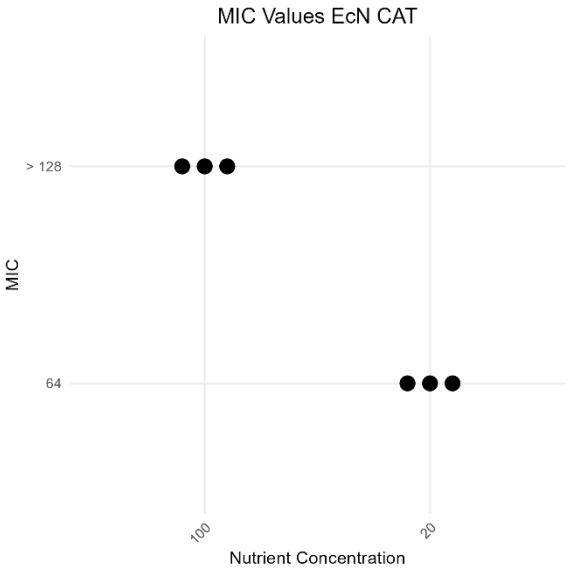
If indeed the detoxification mechanism is the most important, rather than the resource competition, we would expect here that EcN CAT becomes the dominant strain and remains dominant. However, it seems that EcN CAT is not able to grow in this condition. The concentration remains stable until the first dilution, after which it is diluted out over the following transfers. The results suggest that there is a problem with the chloramphenicol degradation as ST is also not able to grow.

To explore and try to explain this observation, we determined the MIC value in the 20% CFA medium of EcN CAT, performed a short-term competition experiment with a EcN CAT monoculture and plated out the evolved communities on LB + Cm agar plates.

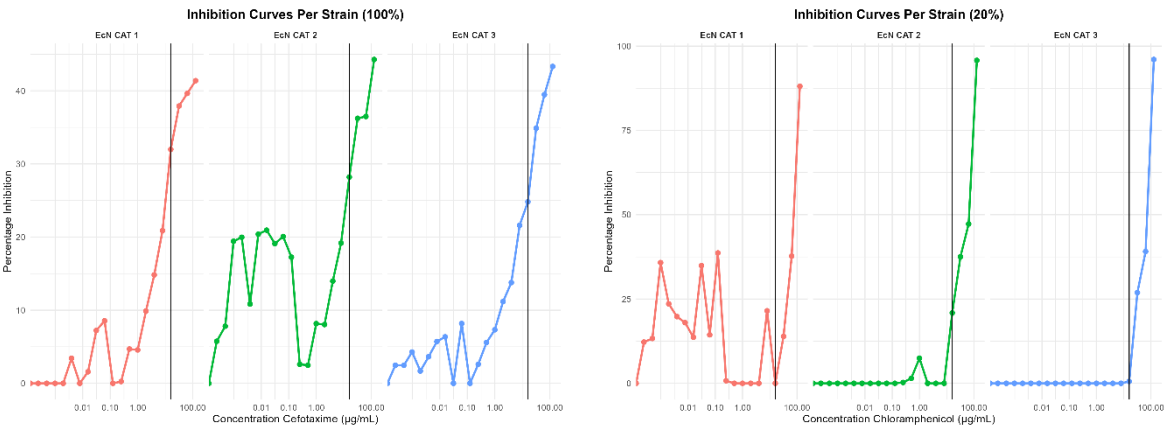
MIC assay

For the MIC assay, the -80 °C stock of EcN CAT was used (ancestor of the inoculum) and the MIC was determined both in 100% and 20% CFA medium. The results show that in a 100%, the MIC value is above 128 µg/mL as expected. In the 20% CFA, the MIC value was 64 µg/mL which is surprising given that it has an antibiotic resistance mechanism and indicates that the nutrient level can influence the

antibiotic resistance. However, during the short-term co-culture experiment, 16 µg/mL chloramphenicol was used, which is well below the MIC value. Therefore, this could not explain the observed collapse of the community.



As there is maybe a stronger inhibition of the strain in 20% CFA at sub-MIC concentrations compared to 100% CFA, we also plotted inhibition curves for both conditions. We then looked at the percentage of inhibition at 16 µg/mL chloramphenicol, but the average inhibition is even lower in 20% CFA compared to 100% CFA. Therefore, this is also not an explanation of the observed collapse of the community.



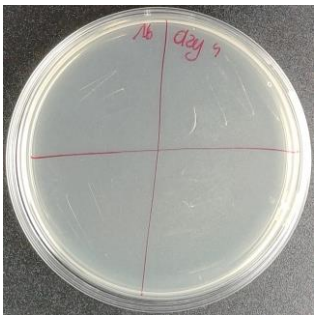
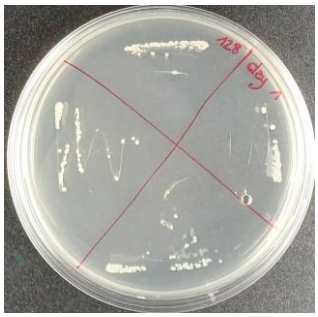
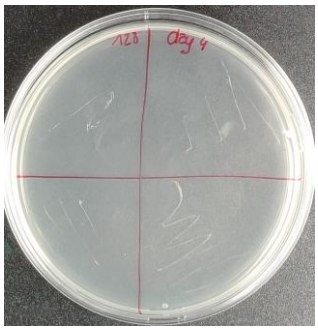
Short-term experiment with mono-culture of EcN CAT

Next, we set up a short-term experiment with only the mono-culture of EcN CAT in the presence and absence of chloramphenicol. This experiment indicates that EcN CAT is able to survive and grow in the presence of chloramphenicol and does not collapse. Therefore, this indicates that EcN CAT is resistant in the conditions used, and that the interaction with ST is causing the lack of chloramphenicol degradation.

Plating out evolved communities

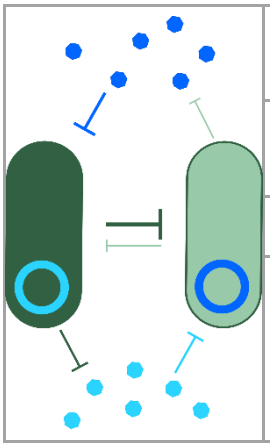
By plating out the ancestor and evolved communities on LB + Cm agar plates, we wanted to determine if EcN CAT lost its resistance mechanism. Two concentrations of Cm were used: 16 µg/mL and 128 µg/mL. This experiment is not ideal to do this as the inoculation density of the different communities is different and therefore, we cannot make any conclusions on the level of resistance.

We can see that the ancestor is clearly resistant. On day 1, there is still some growth on the plates, but we cannot conclude if this is less compared to the ancestor as the EcN CAT concentration from day 1 differs from the -80°C stock of the ancestor. On day 4, we see no growth, but this is probably because the whole EcN CAT population was already diluted out by this time, as can be seen in the short-term competition experiment results above.

	Ancestor	**Day 1**	**Day 4**
16 µg/mL			
128 µg/mL			

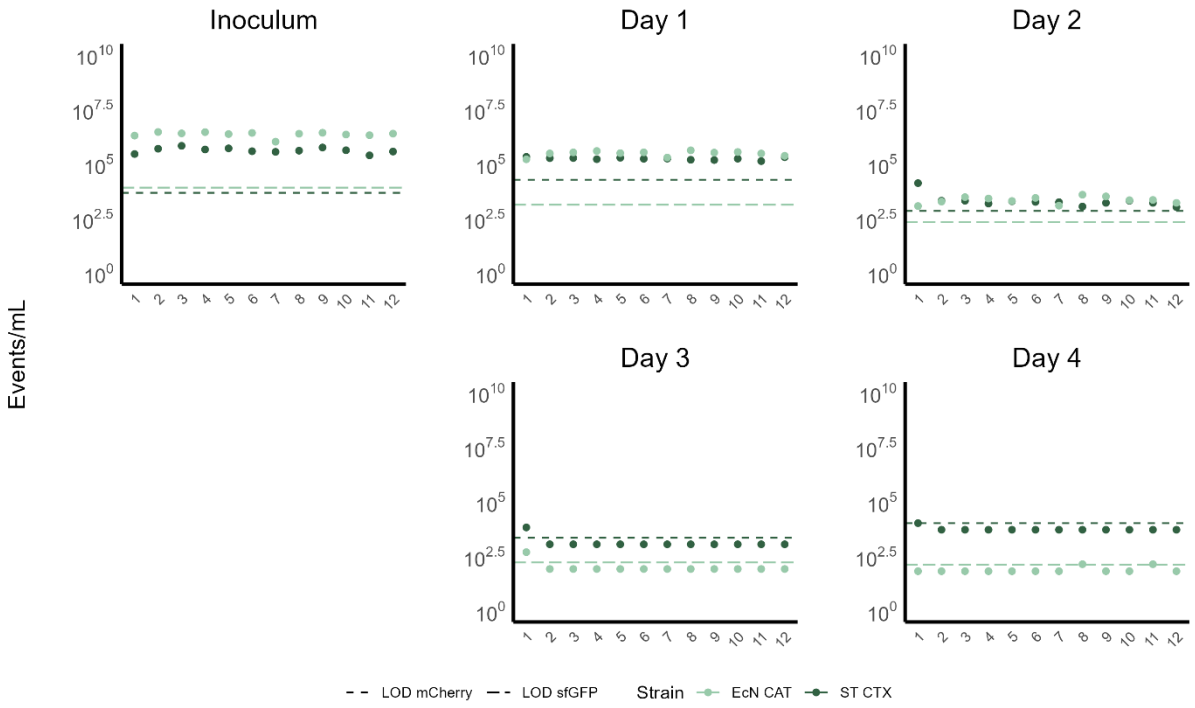
Both strains depend on each other for the detoxification of the environment

Overview



Competitive strength	ST CTX > EcN CAT
Cross-protection	ST CTX relies on EcN CAT EcN CAT relies on ST CTX
Hypothesis	Oscillatory dynamics
Result	CAT resistance mechanism does not seem to work in this situation, causing a collapse of the system

Short-term competition experiment



More details

Both strains of the community are unable to survive in these conditions, as seen by the reduction in concentration of the inoculum to day 1. Afterwards, as the strains are not able to grow, they are diluted out over the following transfers.

Conclusion

The experiment does not allow to get insights into our hypothesis due to a dysfunctional detoxification mechanism in the conditions. If we need this confirmation, the first step would be to repeat the experiment to rule out technical errors during the experiment. However, if the results remain identical, this indicates that there is a biological cause of the dysfunctional detoxification mechanism. To understand this biological cause, further testing would be required as the most obvious explanations were already explored and were unable to explain the observations.