Antibiotic perturbations in experimental microbial community

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Tiivistelmä/Referat – Abstract

The purpose of this thesis was to study the antibiotic perturbations in bacterial populations. Perturbations are common in ecosystems and they can change the composition and functionality of a community substantially. The response of a community is governed by ecological and evolutionary factors: perturbations change the competitive ability of species in the community, but rapid evolution can further affect species fate. This thesis focuses more on the ecological effects. Understanding the community response to a disturbance would be interesting both from a general point of view and from the more practical approach of understanding natural communities under perturbations caused by antibiotics but also, for example, by climate change or chemicals. Thus far, most studies have been performed in one- or two-species systems, not taking into account the effects communities have on the fate of a single species.

To study antibiotic perturbations, a multi-species bacterial community was exposed to a streptomycin pulse of three different levels concentrations. Changes in community composition were studied in the end of the pulse (ecological resistance) and after a recovery period (resilience) from the antibiotic perturbation comparing to the pre-perturbed communities. Further, the presence of species flow was manipulated to examine if it could enhance community resistance and resilience. Based on the analysis, even low antibiotic concentrations can have a long-lasting effect on community composition, but the magnitude of the effect is dependent on the concentration. Community diversity was recovered better than the composition, especially after the weaker perturbations. Species flow aids in community recovery but does not affect resistance. The results were relatively reproducible between replicate communities, and species traits steered the species fate in, pointing to deterministic ecological processes driving the community response. However, repeatability decreased in communities perturbed with the highest antibiotic concentration, which could point to evolution.

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Tiivistelmä/Referat – Abstract
Tässä tutkimuksessa tarkasteltiin antibiootin aiheuttamia häiriöitä bakteeripopulaatioissa. Häiriöt ovat yleisiä ekosysteemeissä ja ne voivat aiheuttaa huomattavia muutoksia yhteisökoostumukseen ja yhteisön toimintaan. Yhteisön vaste määräytyy ekologisten ja evolutiivisten tekijöiden perusteella: häiriöt muuttavat lajien kilpailukykyä yhteisössä, mutta lajien nopea evoluutio voi vaikuttaa lajien kohtaloon. Tässä tutkielmassa keskityttiin pääasiassa häiriöiden ekologisiin vaikutuksiin. Häiriön yhteisövasteen parempi ymmärtäminen on tärkeää yleisesti sekä käytännön kysymysten kannalta niin antibioottihäiriöiden osalta kuin esimerkiksi yhteisöissä, jotka ovat häiriintyneet ilmastonmuutoksen tai erilaisten kemikaalien takia. Tähän asti suurin osa tutkimuksista on käyttänyt yksi- tai kaksilajisia yhteisöjä, jolloin mahdolliset yhteisövaikutukset yhden lajin kohtaloon jäävät huomioimatta.

Antibiootti häiriöitä tutkittiin altistamalla monilajinen bakteeriyhteisö kolmelle eritasoiselle streptomysiinipulssille. Yhteisökoostumuksen muutoksia tutkittiin pulssin lopussa (ekologinen vastustuskyky) ja antibioottipulssin päättymisen ja yhteisön toipumisen jälkeen (palautumiskyky) verraten häiriötä edeltäneeseen yhteisökoostumukseen. Yhteisöön saapuvaa lajivirtaa manipuloimalla tutkittiin parantaisiko se yhteisön vastustus- ja palautumiskykyä. Analyysien perusteella jopa matalilla antibioottikonsentraatioilla voi olla pitkäaikaisia vaikutuksia yhteisökoostumukseen, joskin vaikutuksen suuruusluokka riippui antibioottipitoisuudesta. Yhteisöjen monimuotoisuus palautui koostumusta paremmin etenkin heikompien häiriöiden jälkeen. Lajivirta edesauttoi yhteisöjen palautumista, mutta ei vaikuttanut niiden ekologiseen vastustuskykyyn. Tulokset olivat suhteellisen toistettavia rinnakkaisyhteisöjen välillä, ja lajien ominaisuudet ohjasivat lajien kohtaloa, mikä viittaa deterministisen ekologisten prosessien ohjaavan yhteisövastetta. Kuitenkin toistettavuus väheni yhteisöissä, jotka oli altistettu korkeimman konsentraation antibioottihäiriölle, mikä puolestaan voi viitata evoluutioon.

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2 Abbreviations

ATP	Adenosine triphosphate
ANOVA	Analysis of variance
EMM	Estimated marginal means
GLM	Generalized liner model
GLS	Generalized least squares models
HMSC	Hierarchical modelling of species communities
MIC	Minimum inhibitory concentration
NMDS	Non-metric multidimensional scaling
OD	Optical density
OTU	Operational taxonomic unit
PERMANOVA	Permutational multivariate analysis of variance
PPY	Proteose peptone yeast
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
Sm	Streptomycin

3 Introduction

Antibiotics and antimicrobials are essential tools in modern medicine as suppressors and killers of pathogens otherwise possibly lethal. Despite their benefits, they are a strong disruptive force on the whole microbial community, not only for the targeted pathogens. Antibiotics have been argued to have caused the fastest change in the human gut microbiome since the invention of fire both on the species level and genetic level (Gillings et al., 2015). Antibiotic use has caused a new set of problems in the clinical setting ranging from immediate effects, such as antibiotic diarrhoea, to longer lasting problems, such as the dysbiosis of the gut (Keeney et al., 2014).

Studying antimicrobial perturbations not only could broaden the understanding of clinical consequences, but they can also be used as a model to study larger ecological questions regarding different perturbations. Disturbances are common in natural ecosystems as even a tree falling on the forest floor causes a local disturbance to the animals and vegetation beneath it. Human activities also cause disturbances such as oil spills (Lee et al., 2017) and global warming (Scheffer et al., 2001b). It would be advantageous to be able to predict the response of communities to disturbances, including how well a community can withstand the disturbance and how well it can recover.

It has been reasoned that to better understand natural microbial communities, it is useful to build simpler ecosystems with less confounders (Tecon et al., 2019). After understanding a phenomenon on a smaller scale, it can, to some extent, be used to understand the bigger picture. Disturbances have previously been studied mostly in natural environments such as the gut (Panda et al., 2014) or soil ecosystems (Griffiths et al., 2001) with associative studies but there is a further need to broaden the understanding into the causal factors and mechanisms underlying ecological processes (Kolter & Chimileski, 2018).

The aim of this study was to examine the ecological effects of different concentrations of an antibiotic, here streptomycin, on an experimental microbial community. The experimental design consisted of subjecting the community to a pulse of different concentrations of streptomycin. This allowed examining both the resistance of the community to the antibiotic treatments and its ability to recover afterwards. An

immigration treatment was added to test whether immigration would help with community recovery. Previously collected phenotypic trait data from the community members was used to determine how well the traits of single species could predict community composition during the experiment. Based on the results presented in this master's thesis, even relatively low antibiotic concentrations can cause long-lasting perturbations in bacterial communities. The perturbation severity increases with the antibiotic concentration, but immigration aids the recovery substantially.

3.1 Experimental microbial community ecology

Microbial ecosystems are diverse and exist in all types of environments, including soils, aquatic systems, and outside and inside the human body. Microbes that cohabit the same environment often compete for the same resources leading to the thriving of species possessing the traits most beneficial under the environmental conditions. A change in the conditions might change the proportions of different bacterial species and even cause extinctions of certain taxa, potentially changing the community permanently. Bacteria with high growth rates usually survive well in laboratory conditions where the growth cycle is relatively short and growth medium rich in nutrients (Olson et al., 2002). They can also outcompete slower-growing bacteria by exhausting readily available nutrients. However, bacterial species able to use more complex carbon sources or metabolites from other bacteria can survive by occupying their own ecological niche. Rivett and Bell (2018) show that species abundance could be associated with its ecological function. Higher abundance species were shown to be associated with broader community functions, such as respiration and cell yield, whereas more narrow functionalities, such as the degradation of specific substances, were linked to rare strains. Notably, even the functional properties provided by rare species can be important to a community.

Ecology is a field that studies the interaction of species with their biotic and abiotic surroundings, with community ecology focusing on changes in community composition over time and space. Ecological questions can be studied both in the field and in the laboratory, and each approach has its own advantages. Field studies represent natural states better (Schindler, 1998) whereas laboratory studies are easier to control, monitor and replicate (Fraser & Keddy, 1997). Interactions between different species in their natural environments are intricate and hard to control or monitor. Furthermore, for certain

species with longer generation times, it would take years to gather sufficient data. With simplified microbial models we can determine simple interaction networks that can be used to understand more complex interactions (Fraser & Keddy, 1997). Experimental microbial community models have been used to understand community dynamics and trophic interactions (Benton et al., 2007). Interactions between microbes affect community dynamics as microbes compete for the same resources and might produce metabolites harmful or beneficial to other species (Embree et al., 2015). Microbial community dynamics is also interesting for its own sake. As microbes can be found in all main niches of energy and nutritional flow, as primary producers, consumers, and decomposers, they are crucial for global nutrient cycles and play pivotal roles in all ecosystems.

Ecological networks consist of various types of interaction between species or genotypes. Different species might compete for the same food sources or habitats, have a mutualistic, parasitic or symbiotic relationship, or be seemingly unaffected by each other. One microbial taxon can produce and secrete metabolites that are beneficial, neutral or harmful to other taxa in the same environment. A metabolite can be passed on to other microbial species to be degraded into smaller compounds. Inter-specific relationships can also vary according to the succession stages of the ecosystem (Rivett et al., 2016). Bacterial communities appear to have more negative interactions in the early stages of colonization, but the interactions turn more neutral in later phases. This has been linked to resource dynamics, with more the easily available carbon sources shifting to more energy costly sources as the succession proceeds and the former becomes depleted. Further, community assembly can be directed by priority effects whereby earlier colonizers impact community development and the fate of species arriving later (Fukami, 2015).

Organisms exploiting resources at the expense of the community are known as cheaters (MacLean & Gudelj, 2006), and they might outcompete other taxa under certain conditions. For example, organisms are able to produce energy, or adenosine triphosphate (ATP) molecules, both aerobically by respiration and anaerobically by fermentation, and can enhance their energy production by using both mechanisms (Pfeiffer et al., 2001). However, this depletes the resources from the rest of the community as fermentation exploits energy more inefficiently with an inferior ATP yield compared to respiration, and the efficient carbon source users survive best. Features of the abiotic environment also affect species interactions. For instance, coexistence of bacterial species can be

promoted in spatially structured habitats such as biofilms (Nowak et al., 1994; Kreft, 2004) compared to more homogeneous habitats where species sorting is primarily driven by growth rate (Kreft, 2004). The reason for this is that biofilms offer a diverse set of ecological niches for different bacteria to occupy, including concentration gradients of oxygen, nutrients and metabolites (Nadell et al., 2016).

Understanding community ecology, especially with rapidly reproducing microbes, might require also accounting for evolution which can influence ecological processes even over relatively short timescales. Ecology and evolution were for long mainly viewed as separate fields of science, as evolution was considered to occur too slowly to influence ecological processes, but current evidence suggests that they go hand in hand (Hairston et al., 2005). For instance, the evolution of anti-predatory defense alters classic predator-prey cycles (Yoshida et al., 2003). For similar reasons as those listed above, microbial systems are also highly suited to the study of eco-evolutionary dynamics. To model and understand these systems, it is important to know species traits, as traits determine the fate of a species in an ecosystem (Hart et al., 2019), but the evolution of these traits might also be important (Edwards et al., 2018).

Model communities have certain benefits over natural communities. One of the largest advantages is the reproducibility of an artificial model community. The community can either be constructed from known laboratory strains or strains isolated from the environment. Isolated strains can also be studied alone to obtain phenotypic trait or genome sequence data. This data can help to understand the behaviour of species in a larger community. Additionally, phenotypic and genotypic information opens up new possibilities for studying species evolution.

3.2 Multi-species experimental communities

Until now, the majority of ecological studies on artificial communities have been performed on single- or two-species communities. However, it has been discussed that multi-species communities provide a wider understanding of natural microbial communities as they can broaden the understanding of key functionalities, assembly, and maintenance of a microbial communities with a more controllable and simple system (Kolter & Chimileski, 2018; Tecon et al., 2019). Existing multi-species community studies have already provided important insights. For example, it has been reported that

the community dynamics in multi-species communities are interestingly reproductible and depend on the evolutionary history of the species (Celiker & Gore, 2014), as well as the nutrient source (Goldford et al., 2018). Mounier et al. (2008) show the importance of the interplay between organism groups with their study of bacteria and fungi inhabiting cheese: the bacteria are unable grow in the absence of yeasts.

The main problems related to using model microbial communities have been summarized by Wolfe (2018): When modeling a natural ecosystem, it is unclear how well synthetic communities can represent reality. Further, all species cannot be isolated or cultivated in laboratory conditions, and it is difficult to know which species play an important functional role in the community. Besides, natural environments often contain other organism groups, such as viruses, archaea, fungi, and protists, that also affect community dynamics and functioning, while experimental communities often contain bacteria alone. There is also often low within-species variation, as the communities are started from isolated strains. Moreover, *in situ* conditions can be hard to mimic and laboratory practices such as overnight cultures are far from natural. Finally, it is still uncertain how to study community dynamics accounting for also low-abundance species, as they do not show up with all analysis techniques. These problems are mainly related to the accurate modelling of a specific *in situ* environment. However, this is not always the goal in studies assessing general eco-evolutionary questions, and thus building a community can be simpler. Further, even if a model community does not represent a natural community perfectly, experimental multi-species community studies can provide important information about big ecological questions.

Sequencing technologies provide new tools for studying the composition and properties of microbial communities as well as single species. 16S ribosomal RNA (rRNA) amplicon sequencing, targeting a fragment of the highly conserved 16S rRNA gene region, has been used to study bacterial communities in a variety of communities ranging from humans (Costello et al., 2009) to water ecosystems (Kent et al., 2004). This makes it possible to acquire information regarding the proportional abundance of bacteria present in an environment unbiased by cultivability. With 16S rRNA amplicon sequencing you can identify bacteria that are hard or impossible to cultivate in laboratory conditions, but as a downside, also unviable bacteria show up in the analysis. Additionally, the analysis reports relative, not absolute values; hence, when comparing a community before and after a treatment, for example, a species might seem to increase in

abundance while in fact the abundance of other species in the community is simply lowered. Furthermore, the analysis method greatly affects the results, as databases are biased towards cultivable and clinically relevant bacteria and have error rates as high as 17% (Edgar, 2018). Although 16S rRNA amplicon analysis provides an extensive overview of the microbiota studied, including changes in communities over time and space, it is important to acknowledge these problems. An upside of using defined experimental communities is that 16S rRNA amplicon data can be aligned with a reference database consisting only of chosen bacteria, making species recognition more straightforward.

3.3 Perturbations and regime shift

A disturbance or perturbation in the environment can change the community drastically either permanently and or temporarily, as illustrated in Figure 1. A disturbance in an ecosystem is caused by an abrupt change in the environment such as a forest fire, flood, or even a falling tree (Shade et al., 2012). Perturbations are classified into two categories depending on the length of the disturbance (Bender, 1984). A momentary disruption in the community that is quickly over, allowing the community to recover, is called a pulse disturbance, whereas a sustained disruption, during which the community reaches a new state of equilibrium, is called a press disturbance. The transition of a community from one stable state (a state of equilibrium in a community where the composition is stable) to another after a change in the ecosystem is called a regime shift (Shade et al., 2012). After reaching a new stable state, the community can become permanently altered, and it may fail to return to its original state even once the perturbation is over. Studying these events is interesting from the ecological point of view as they provide information about the response of communities to environmental change or chemical stress. In experimental microbial ecology, perturbations have been caused, among others, with a heat shock (pulse perturbation) or with a chemical supplement such as heavy metal (Griffiths et al., 2000) or antibiotic in the growth medium (Eckert et al., 2019) (usually press perturbation).

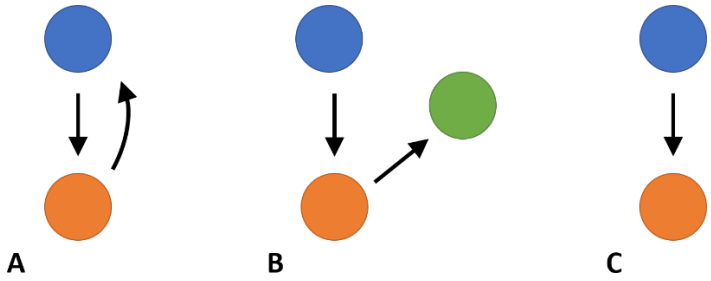

Figure 1. The original community (blue) changes in composition due to a disturbance causing a regime shift (orange). The community can either recover to its original state (A), recover partially (B) and form a new type of community (green), or the change can be permanent (C).

Gonze et al. (2017) discuss three different mechanisms that can explain alternative community types: response to environmental change or perturbation, heterogeneous interaction strengths between community members derived from a metacommunity, and lastly community composition tipping in a multi-stable community. In the last mechanism, the change in the community can be caused by adding or removing taxa, or by changing the growth conditions; however, the community does not return to its original state when the conditions are restored.

Similar to other ecological concepts, the concept of disturbance can be expanded to different fields, including medicine, to help understand community behavior in changing conditions. For example, bacterial vaginosis, a vaginal disease caused by an imbalance in the bacterial community, is often thought to be explained by a disturbance in the vaginal microbiome, that is, the group of microbes cohabiting the same environment (Larsson & Forsum, 2005). The disturbance can be caused, among others, by hormonal contraceptives, the menstrual cycle, or antibiotic treatment (Foster et al., 2008).

3.4 Resistance and resilience

Resistance in ecology is defined as the capability of a community to withstand change in case of a disturbance (Pimm, 1984; Allison & Martiny, 2008). In the literature, resilience is defined as the rate at which a community returns to its original state (Allison & Martiny, 2008) or reaches its equilibrium (Pimm, 1984) after a disturbance. Together resistance and resilience form the concept of community stability: the ability of a community to

withstand change and return to a stable state after disturbance (Pimm, 1984). In Figure 1, community A has the highest resilience as it recovers to its original state, while community C has no resilience. Butler and O'Dwyer (2018) argue that cross-feeding communities are more unstable than competitive communities when resources are depleted. In other words, if microbes not only consume resources but also produce them for others to consume, the community is less stable if the mutualistic relationship is not balanced. However, cross-feeding can also promote community diversity by creating new niches in the community (San Roman & Wagner, 2018).

Resistance and resilience can be measured functionally based on whether the community can perform the same functions as before the disturbance, or at a species level based on whether the species and their proportions stay the same (Holling, 1996). Community diversity before, during and after the disturbance is an easy way to measure the resistance and resilience of a community at the species level. Community diversity consists of how many species are present in a community (species richness) as well as the number of individuals belonging to each species (species evenness). Therefore, communities with a few dominating species are considered to have lower diversity than communities with more equal abundances between species (higher evenness) even with the same amount of species (equal richness). In ecology, diversity is divided into three types: alpha (α), beta (β), and gamma (γ) diversity, corresponding, respectively, to the diversity within a community, the degree of difference between different communities and the combination of within- and between-community diversity (Whittaker, 1960).

Different mathematical equations and indices have been developed to describe diversity and facilitate comparisons. Named after its creator, Simpson's index (Simpson, 1949) is widely used in microbiology to denote alpha diversity. It calculates a diversity value for a community based on the number of species present and their abundance. To describe beta diversity, Brey-Curtis dissimilarity is frequently used. It is based on the presence or absence of species in two communities and the species abundances (Bray & Curtis, 1957). Two identical communities at species occurrence level receive the dissimilarity value of 0 and two communities not sharing any species receive a value of 1. Both alpha and beta diversities can be used to describe change in a community or differences between communities. Alpha diversity indices are simpler metrics for describing the overall diversity in a community and, for example, the decline in diversity after a distribution. Beta diversity can be used to compare compositional changes between different

communities as it takes into account not only species richness and evenness but also differences in the species present in different communities.

Community diversity has been shown to increase the resilience of communities of larger organisms, but the effect in microbial communities is less clear (Shade et al., 2012). Both the properties of communities, such as diversity, and of single species can affect the resistance and resilience of a community. A high growth rate indicates that a species can grow rapidly and utilize the resources effectively after a pulse disturbance and under favorable conditions. Even if this might be beneficial for the species, from a community perspective it lowers resistance as one species will dominate and the resources will be used unevenly (Shade et al., 2012). On the other hand, species with higher growth rates can be beneficial for community resilience as they can recover more rapidly after a disturbance. Cira et al. (2018) studied the effect of transfer volume on neutral, i.e. non-selective (all community members had similar fitness), microbial community diversity. The model used predicted higher diversity and strain count in communities with large amounts of cells transferred compared to low cell count transfer communities, which was consistent with experimental data. However, the intermediate cell count communities, deviating from the model, lost the most diversity with one species dominating. Therefore, the bottleneck caused by inoculation, a common practice in microbial and ecological laboratory procedures, can strongly influence community composition and diversity.

Both resilience and resistance are important and widely studied ecological topics. It has been hypothesized that gradual changes in ecosystems, such as changes in the climate affecting nutrient levels, can lead to loss of resilience in a community, which can, in turn, lead to an abrupt change in community composition at a state denoted as the tipping point (Scheffer et al., 2001a; Lenton et al., 2008). It is essential to understand factors affecting community resilience to understand the response of ecosystems to either natural or human impacted environmental changes, such as global warming and environmental disasters. For example, a study on the crude oil spill affecting a tidal flat in South Korea (Lee et al., 2017) showed that despite a major shift in sediment bacterial community composition at 4 and 9 months after the accident, the community was restored close to its original state by 13 months. The community possessed species harboring important functional properties for degrading oil, increasing the resilience of the community.

3.5 Immigration

Even stable natural environments are prone to experience microbial dispersal from nearby communities as the different environments are not isolated similar to laboratory conditions, where all new bacteria are the product of cell division in the community. For example, natural waters receiving effluents from waste water treatment plants with potential antibiotic residues also receive bacteria from nearby soils and other streams leading to the same pond. Immigration has been hypothesized to lower the extinction rate of an ecosystem (Brown & Kodric-Brown, 1977) and it also provides gene flow to existing species (Lacy, 1987). A continuous immigration of a similar community has been shown to augment community resilience in experimental freshwaters under pulse salinity disturbance (Baho et al., 2012).

Immigration might help to maintain community diversity as it prevents the extinction of the immigrating species in so-called sink populations (Holt & Gomulkiewicz, 1997). Sinks are harsh environments that would not support a population by its own (i.e. the death rate of a species surpasses its growth rate). Immigration can affect community composition especially when the conditions change, and the community has not yet reached a stable state. Additionally, immigration can increase population size and genetic variation in the population, potentially providing genetic variants adaptive to selective conditions. For instance, immigration from non-antibiotic conditions to communities under antibiotic stress has been shown to increase the rate of resistance evolution with lower fitness cost of resistance (Perron et al., 2007). Perron et al. (2008) further showed that sufficient immigration can promote resistance evolution even in rapidly increasing antibiotic concentrations.

Bacterial immigration might be crucial for the survival of certain species in a community that would otherwise be outcompeted. For example, the strains in probiotic supplements eaten by healthy humans have been shown to remain in the gut for a limited time, ranging from hours (Robins-Browne & Levine, 1981) to days (Goldin et al., 1992), after the probiotic treatment has ceased. If the gut microbiome is less stable, the effect of probiotics is much more important, such as in the case of the gut of patients treated with antibiotics (Friedman, 2012), that is, gut communities under dysbiosis.

Fukami et al. (2007) studied the effect of evolutionary history on the diversification of immigrating species. A wild-type strain of *Pseudomonas fluorescens*, a typical model species in ecology, was introduced to the community in early stages, and it diversified quickly specializing to different niches in the spatially structured habitat. If a specialized *P. fluorescens* strain was introduced before or shortly after the wild type, diversification was hindered. Moreover, the specialist caused much less suppression when introduced after the wild type. The study proposes that not only the presence and amount of immigration but also the traits of the immigrated species, that can be shaped by their evolutionary history, can be important for community dynamics.

3.6 Antibiotics

3.6.1 Antibiotic resistance

Antibiotics are substances produced by microbes that inhibit the growth of bacteria. Antibiotics were first discovered in the beginning of the 20th century, with the most famous discovery being penicillin by Alexander Fleming in 1928 (Fleming, 1929). The midcentury was the golden age of antibiotic discovery, prompting widespread use of antibiotics later in the 20th and 21st centuries. Different antibiotics have different modes of action, used to classify them, and are effective towards different bacterial species. To be effective, antibiotics need to reach a sufficiently high concentration to inhibit the growth of bacteria. This concentration is species specific, and the value might also vary between different media and measurement methods (Luber et al., 2003). In this thesis, the term minimum inhibitory concentration (MIC) is used to describe the lowest antibiotic concentration that inhibits bacterial growth. Super-MICs of antibiotics pose a strong selection pressure on bacteria, killing susceptible cells and promoting resistance evolution by enriching resistant cell lines. Lower antibiotic concentrations can also cause strain on a bacterial cell and affect its growth (Andersson & Hughes, 2014) as well as affecting bacterial communities. Sub-MICs of antibiotics can promote the evolution of antibiotic resistant bacteria (Kohanski et al., 2010; Westhoff et al., 2017). Sub-MICs have also been speculated to promote horizontal gene transfer (Ubeda et al., 2005), which would accelerate the spread of resistance genes in a bacterial community, although evidence regarding this is unclear (Lopatkin et al., 2016).

Besides explicit resistance factors, bacteria also have other ways to protect themselves against antibiotics. The same bacterial strains can tolerate much higher antibiotic concentrations in biofilm compared to planktonic lifestyle (Stewart & Costerton, 2001). This could be caused by impermeability of the biofilm, a protective role played by certain bacteria within the biofilm, or a change in pH decreasing antibiotic activity within biofilm microenvironments. Bacteria might also assume a dormant state with lowered metabolic activity and no cell division. These so-called persister cells can be immune to multiple antibiotics and allow the species to survive past antibiotic treatment (Lewis, 2007). Notably, in the batch culture method, where e.g. 1–10% of the old bacterial culture is transferred to fresh medium, the majority of persister cells are lost as they do not reproduce and become diluted out over transfers.

3.6.2 Impact of antibiotics on microbial communities

Antibiotics are one of the important disturbing forces in different environments such as the waters of waste water treatment plants (Baquero et al., 2008) or the human gut (Panda et al., 2014). The effects of antibiotics are usually studied on a genus level for a single bacterium. However, their effect on communities is also important as the decline of one species liberates resources for other bacteria, allowing certain species to thrive in communities under antibiotic pressure compared with the same community in an environment without antibiotics. The effect of an antibiotic perturbation on a microbial community can be studied through an eco-evolutionary framework (Hiltunen et al., 2017) (Figure 2). Antibiotics change the competitive interactions between species (Hall & Corno, 2014), but evolution can further affect species traits (e.g. resistance evolution) and their competitive ability.

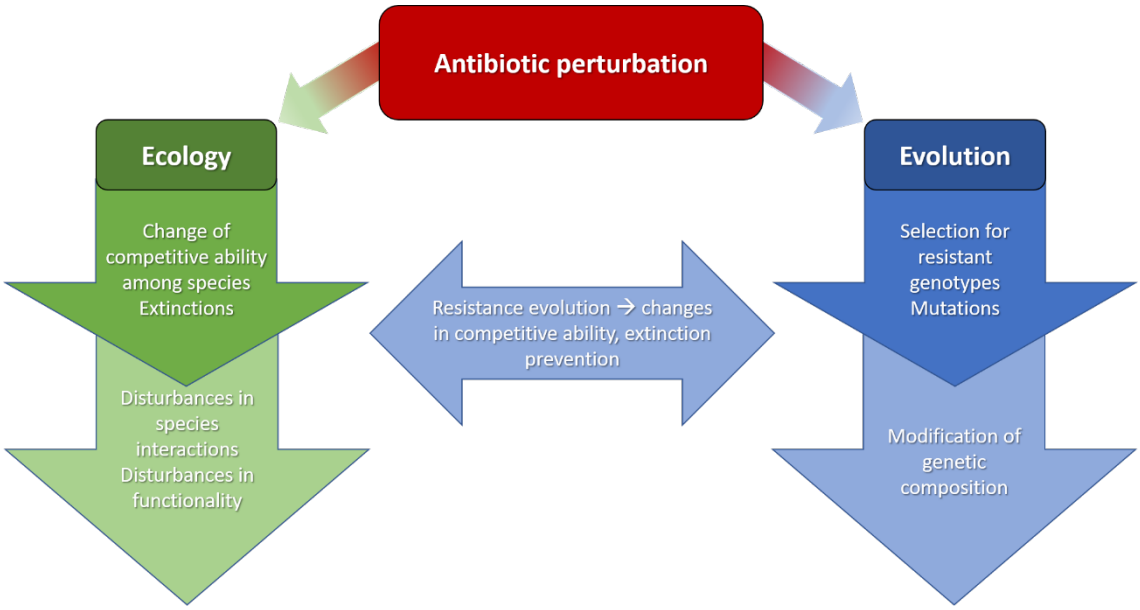

Figure 2. Ecological and evolutionary impact of antibiotic perturbation on microbial community: Antibiotics change the competitive ability of the species in the community and the susceptible species can go extinct. This further disturbs the species interactions, such as cross-feeding, and can cause changes in the functions of the community. This can, in turn, cause the community to become more sensitive to, for example, invasive species. Moreover, antibiotics select for resistant genotypes within species, which changes the genetic composition of the community even if the species composition does not change or recovers after the perturbation. Rapid resistance evolution can cause changes in species growth ability in the presence of antibiotics, feeding back to ecological dynamics, including prevention of extinctions.

Antibiotics are the corner stone of modern medicine and, while indeed effective towards susceptible pathogens, they also affect the microbiota inside the body, with most of the antibiotic perturbance studies focusing on human and animal gut microbiota. After antibiotic treatment, the patient becomes much more susceptible to infections from different pathogens, as proven in animal models (Douce & Goulding, 2010; Kamada et al., 2012). Gut dysbiosis, an altered state of the gut's microbial composition, has been linked further to other diseases, for example asthma (van Nimwegen et al., 2011) and rheumatoid arthritis (Scher et al., 2013). Antibiotic perturbations of the normal microbiota cause new health problems as different strains colonize the gut, which might cause diarrhea (McFarland, 2008), and can cause long term changes in the composition of the gut microbiota (Jernberg et al., 2007; Jakobsson et al., 2010), in turn, creating further medical problems (Keeney et al., 2014). Understanding the resilience and recovery of the normal gut microbiota after antibiotic treatment is important from a clinical perspective.

Palleja et al. (2018) reported that the gut microbiota of young and healthy research subjects had high resilience, displaying a relatively high rate of recovery within six months after treatment with multiple antibiotics. Nevertheless, certain bacterial strains went extinct or their frequency was reduced below the detection limit without recovery during the follow-up period.

Beardmore et al. (2018) suggest that there is a tipping point in a community containing antibiotic susceptible and resistant strains, where the resistant strains might dominate the community even after the antibiotic is removed. The authors propose that the simultaneous variation of antibiotic treatment and glucose availability guides the community into a state of multi-stability, where a community can have more than one stable state in the same conditions, and this could lead to tipping. The study was performed using only two yeast strains and the hypothesis cannot be applied directly to more complex communities, but it provides insights on the potential shifts occurring in communities after drug treatment. Even if the reversal of resistance is possible in a community, as resistance often leads to otherwise reduced fitness (Andersson & Levin, 1999), the reversal process is slow (Levin, 2001), and even if only a low frequency of resistant bacteria remains in the community, they can re-emerge during the next drug treatment.

Stein et al. (2013) used extended Lotka-Volterra equations, i.e. predator-prey equations, to model clindamycin perturbation and the following *Clostridium difficile* colonization in mouse gut and found signs of multi-stability in the gut community. The shift between these stable states could be introduced by clindamycin treatment or *C. difficile* introduction. Further, communities perturbed by clindamycin were susceptible to *C. difficile* colonization, whereas unperturbed communities suppressed *C. difficile* growth. The authors proposed that the antibiotic treatment suppressed the gut community stabilizing genera *Coprobacillus, Akkermansia*, and *Blautia*, allowing *Enterococcus* to increase in abundance, which in turn may facilitate *C. difficile* colonization.

To model gut communities, Bucci et al. (2012) divide the microbiota into two groups, the bacteria resistant to the antibiotic in question and the bacteria susceptible to it. According to their model, antibiotic exposure causes multi-stability, assuming that the sensitive group can inhibit the growth of the tolerant group, and that the effect of the treatment can last for long after the exposure. The addition of noise, represented by an influx (or efflux)

of bacteria, increases the chance of the sensitive bacteria to dominate, as the probability of extinction decreases. Additionally, the recovery time is highly dependent on the amplitude of noise, and the recovery of totally isolated communities was deemed highly unlikely according to this model.

Substance concentrations may vary greatly in environments as they usually have point origins such as antibiotic producing microbes in soil or contamination sources. Especially in the human body, antibiotic concentrations might vary significantly during antibiotic treatment and even between different body fluids (Elliott et al., 1995). Besides high concentrations, also sublethal concentrations of antibiotics could alter microbial communities by increasing fitness variance (Trindade et al., 2012), selecting for resistant strains (Gullberg et al., 2011) and altering competitive interactions between bacterial species (Hall & Corno, 2014). However, there is still very little experimental evidence regarding the effects of sub-MICs on multi-species communities. While it has been reported that even low antibiotic concentrations can reduce the diversity and density of bacterial communities, as well as affect community composition, the effects might be lost in more complex ecological settings including, for example, trophic interactions or biofilms (Cairns et al., 2018c). As previously described, biofilms can protect even susceptible strains from the adverse effects of antibiotics (Stewart & Costerton, 2001). Moreover, some community members might be able to inactivate or degrade the antibiotics, which protects the whole community (Cairns et al., 2018b; Murray et al., 2018).

3.6.3 Streptomycin

Streptomycin (Sm) (Figure 3) was first isolated from *Streptomyces griseus* by Albert Schatz in 1943, although Selman Walksman was credited long for Schatz's work (Schatz et al., 1944; Schatz, 1993). It used to play a major role in the clinic as it is effective against *Mycobacterium tuberculosis* infections, but it is less widely used today owing to its side-effects, including harming the inner ear and balance organs (Sköld, 2011). It belongs to the aminoglycoside group, an antibiotic group of actinomycetes derived protein synthesis inhibitors, and inhibits the growth of a broad range of gram-negative bacteria (Krause et al., 2016). Sm functions by binding to the ribosome with the help of the S12 protein in the small 30S ribosomal unit and disturbing protein synthesis through inhibiting the

formation of the initiation complex between the ribosome and mRNA (Sköld, 2011). Sm also causes misreading of the mRNA template resulting in premature termination of protein synthesis. Sm resistance can be caused, among others, by mutations in the S12 protein (encoded by the gene *rpsL*) that prevent target binding of the antibiotic.

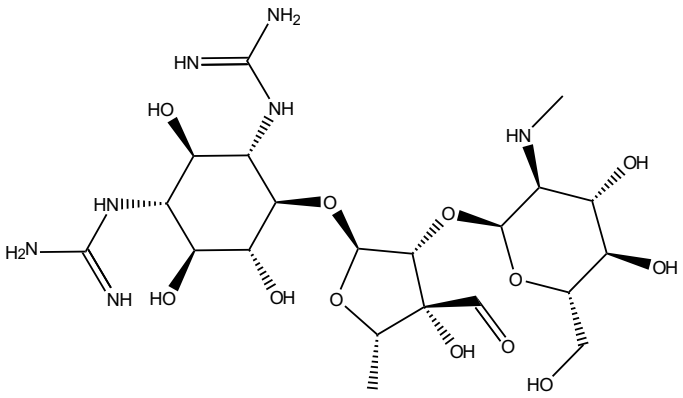

Figure 3. Chemical structure of streptomycin. Drawn with ChemDraw JS 17.1.1.5 (PerkinElmer).

4 Research objectives

The main research objective in this study was to increase understanding concerning the effect of different antibiotic concentrations on community composition and the ability of communities to recover from perturbation. The research interest was geared toward providing general mechanistic insights regarding the community effects of perturbations, rather than understanding the effects of antibiotics in a particular environment (e.g. human gut). For this reason, a controlled experimental setup with a defined artificial community was employed.

The following predictions were made:

i. Higher Sm concentrations should have stronger effects on community composition compared to lower concentrations, including extinction of the most susceptible species. The role of low concentrations is less clear and therefore of special interest.

ii. Deterministic community dynamics indicates species sorting by pre-existing phenotypic traits influencing fitness in a given environment (e.g. growth ability without and intrinsic resistance level with antibiotic).

iii. In contrast to deterministic community dynamics, apparent stochasticity in population trajectories could occur either due to ecological processes (e.g. population bottlenecks) or evolutionary processes (e.g. *de novo* resistance mutations), warranting further study.

iv. Immigration should increase diversity after the Sm treatment especially with high concentrations as extinct species are reintroduced to more favorable conditions. Immigration could also maintain diversity in all communities preventing extinctions that would otherwise occur in antibiotic sinks. This buffering effect of immigration should cause decreased variation in community composition although this might be countered by an increased supply of adaptive genetic variation such as random resistance mutation events.

5 Materials and methods

5.1 Strains and culture conditions

The liquid medium used in the experiment was specifically developed for complex communities and a long culture cycle. An artificial bacterial community consisting of 34 species (Table 1, Figure 4) was almost entirely chosen from the HAMBI Culture Collection, University of Helsinki, except for *Escherichia coli* K-12 strain JE2571 (Datta et al., 1971). The bacteria are gram-negative and represent three classes (Alpha-, Beta- and Gammaproteobacteria) in the phylum Proteobacteria and three classes (Chitinophagia, Flavobacteriia and Sphingobacteriia) in the phylum Bacteroidetes. The strains are not representative of a particular natural system but were rather selected based on growth in simple, uniform laboratory conditions. Different versions of the artificial community have been used in two previous studies (Cairns et al., 2018a; Cairns et al., 2018c), where details are reported regarding its construction and the phenotypic and genomic characteristics of the strains.

Table 1. Species used in the experiments.

Species	HAMBI strain ID
Acinetobacter lwoffii	HAMBI 97
Aeromonas caviae	HAMBI 1972
Agrobacterium tumefaciens	HAMBI 105
Azorhizobium caulinodans	HAMBI 216
Azospirillum brasilense	HAMBI 3172
Bordetella avium	HAMBI 2160
Brevundimonas bullata	HAMBI 262
Chitinophaga filiformis	HAMBI 1966
Chitinophaga sancti	HAMBI 1988
Citrobacter koseri	HAMBI 1287
Comamonas testosteroni	HAMBI 403
Cupriavidus necator	HAMBI 2164
Elizabethkingia meningoseptica	HAMBI 1875
Enterobacter intermedius	HAMBI 1299
Escherichia coli K-12 substrain JE2571 (RP4)	Not applicable
Hafnia alvei	HAMBI 1279
Microvirga lotoninidis	HAMBI 3237
Moraxella canis	HAMBI 2792
Morganella morganii	HAMBI 1292
Myroides odoratus	HAMBI 1923
Niabella yanshanensis	HAMBI 3031
Paraburkholderia caryophylli	HAMBI 2159
Paracoccus denitrificans	HAMBI 2443
Paraburkholderia kururiensis	HAMBI 2494
Phyllobacterium myrsinacearum	HAMBI 1992
Pseudomonas chlororaphis	HAMBI 1977
Pseudomonas putida	HAMBI 6
Psychrobacter proteolyticus	HAMBI 2948
Roseomonas gilardii	HAMBI 2470
Sphingobacterium multivorum	HAMBI 1874
Sphingobacterium spiritivorum	HAMBI 1896
Sphingomonas yanoikuyae	HAMBI 1842
Stenotrophomonas maltophilia	HAMBI 2659
Thermomonas haemolytica	HAMBI 2467

A medium was specifically refined for the selected community and long culture cycles. The co-culture medium contains 1 g l^{-1} R2A broth (Labema, Helsinki, Finland) and

0.5 g l^{-1} of cereal grass medium (Ward's Science, St Catharines, ON, Canada) in M9 salt solution. The cereal grass medium stock was prepared by autoclaving it in deionized H_2O and filtering through 5 µl to remove particulate matter.

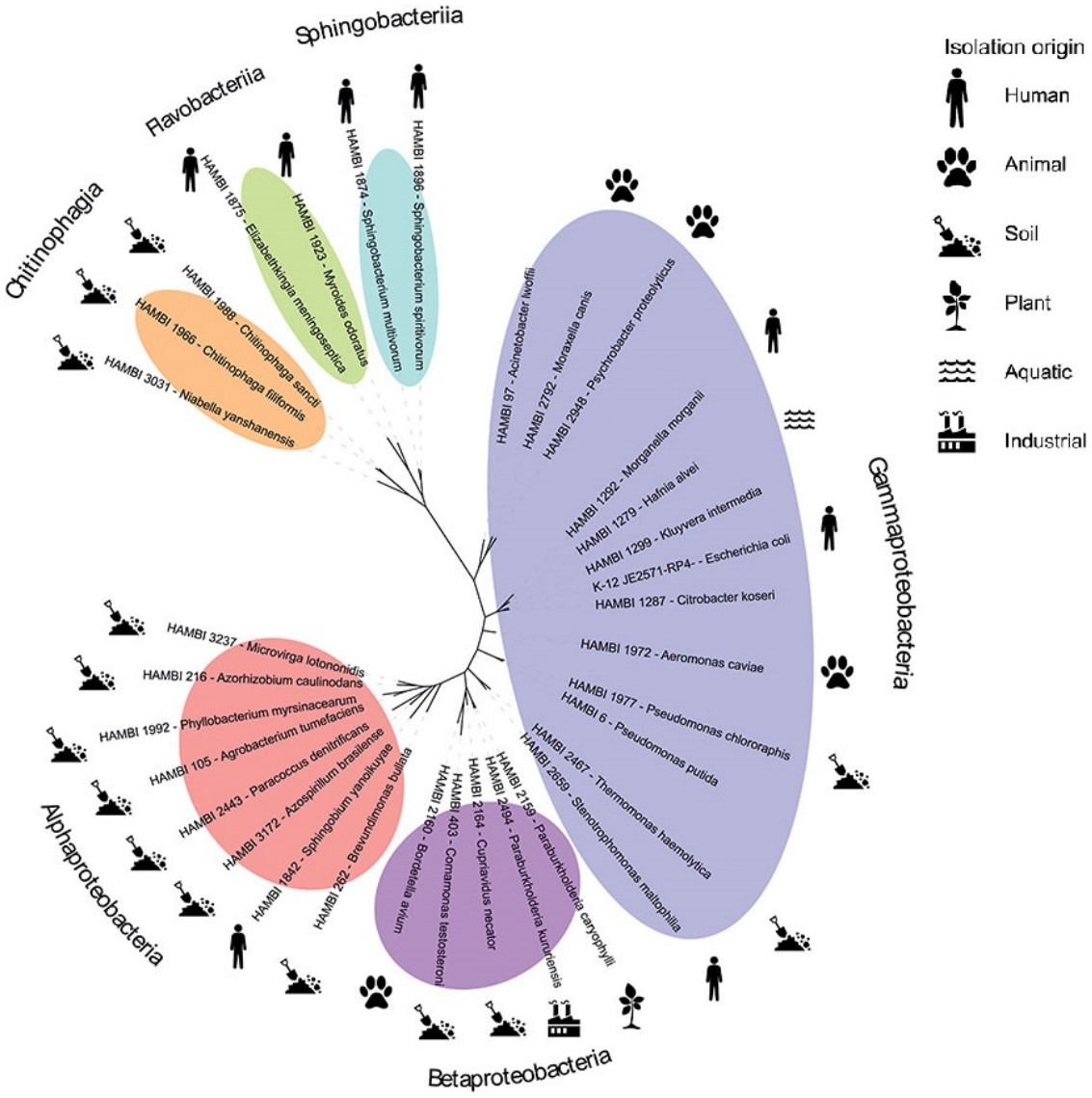

Figure 4. Phylogenetic tree depicting the community members. Modified and reprinted with permission (Cairns et al., 2018a).

5.2 Serial passage antibiotic pulse experiment

A 48-day serial passage antibiotic pulse experiment was performed consisting of three epochs: 16 days without Sm to allow the community composition to acclimatize to experimental conditions, 16 days with Sm at concentrations 4, 16, and 128 µg ml^{-1}, and 16 days without antibiotics to allow the community to recover (Figure 5). The experiment included an antibiotic-free control treatment. The experiment was performed in a full-factorial design without and with immigration consisting of adding an inoculum of the original community at each transfer. Each treatment combination was replicated eight times.

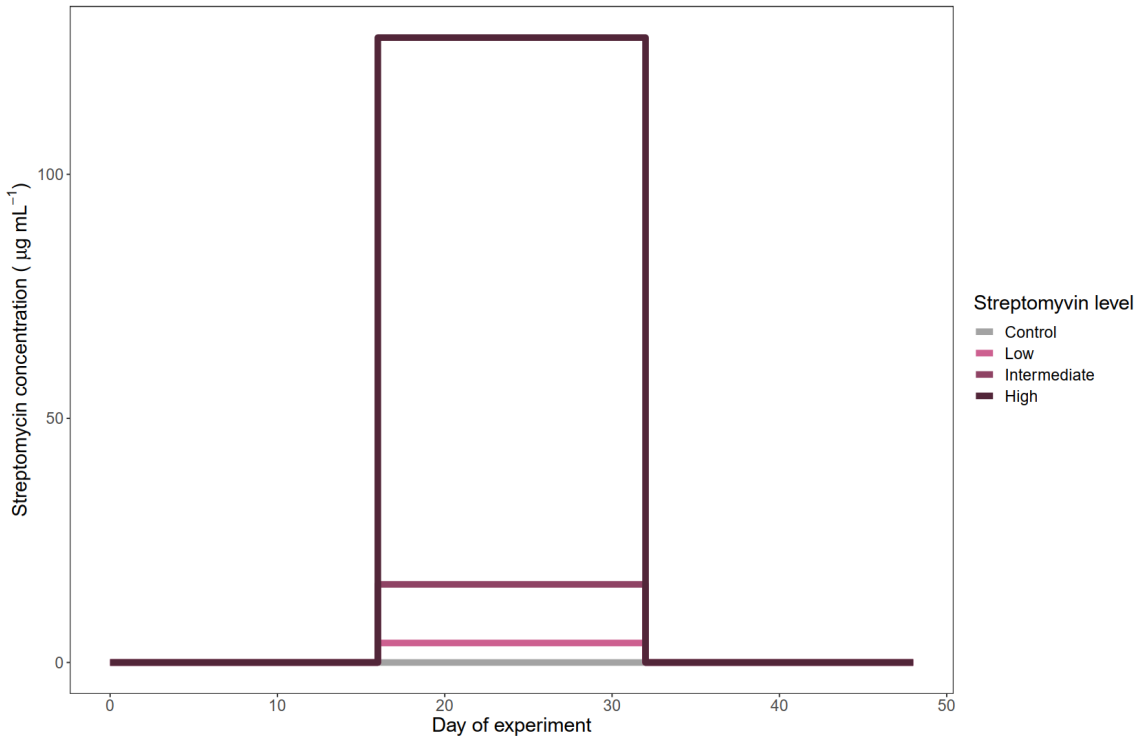

Figure 5. Experimental flow depicted as streptomycin concentration as a function of time.

The experiment was conducted in ABgene™ 96 Well 2.2 ml Polypropylene Deepwell Storage Plates (Thermo Fisher Scientific, Waltham, MA, USA) in the co-culture medium.

Prior to starting the experiment, all the strains were transferred to the co-culture medium and cultured for 96 hours at 28 °C / 50 rpm. Following this, they were pooled together in equal volumes and freeze-stored with 30% glycerol at –80 °C. To start the experiment, 10 µl of 100-fold diluted freezer-stock community was added to each well containing 500 µl of medium and 50 µl of sterile dH_2O to compensate the dilution caused by Sm additions. The experiment was maintained every 96 hours by transferring 50 µl, about 10%, to fresh medium prepared as in the beginning of the experiment. For the immigration treatment, 10 µl of 100-fold diluted freeze-stored community was also added. For cultures containing Sm, the dH_2O was replaced with an equal volume of appropriate Sm stock.

To monitor bacterial density, optical density values at 600 nm wavelength (OD_{600nm}) were obtained from old cultures at each transfer using a well plate reader (Tecan Infinite M200 well-plate reader, Tecan Trading AG, Switzerland). Samples from time points 16 days (before Sm addition), 32 days (last time point with Sm) and 48 (final time point) days were also frozen in glycerol at –80 °C for further analysis.

5.3 DNA extraction and sequencing for 16S rRNA amplicon analysis

DNA was extracted from three technical replicates of the original freezer-stock community, and three out of eight experimental replicate communities from days 16, 32 and 48 in the serial passage experiment. DNA extraction was performed with the DNeasy 96 Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions using 400–600 µl of sample. DNA concentrations were measured with the Qubit™ 2.0 (Life Technologies Corporation, Carlsbad, CA, USA) fluorometer using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Paired-end 16S rRNA amplicon sequencing was performed by a third party, the Institute for Molecular Medicine Finland (FIMM), using the Illumina MiSeq platform by amplifying the V3 and V4 regions as previously described (Cairns et al., 2018a).

Adapters were first removed from the unpaired raw reads using Cutadapt 1.12 (Martin, 2011) with options --minimum-length 100 to discard all processed reads under the length of 100. Pairing was done with the paired-end read merger Pear 0.9.11 (Zhang et al., 2014). Read quality was controlled with FASTQC (www.bioinformatics.babraham.ac.uk/projects/fastqc) before and after running Cutadapt and Pear, and the reads were further

trimmed 5 nucleotides from the left and 40 from the right of the reads with PRINSEQ (Schmieder & Edwards, 2011) to obtain better quality reads and minimize loss of data during quality filtering. USEARCH 10 (Edgar, 2010) was used to quality filter the reads using the --fastq-filter command with the parameter --fastq-maxee 1.0 to discard all reads with > 1.0 total expected errors. Unique sequences were obtained by dereplicating with VSEARCH 2.3.4 (Rognes et al., 2016). The reads were mapped to a reference database containing the 16S rRNA gene sequences of the 34 experimental species with USEARCH -closed_ref command with > 97% identity requirement. Problems associated with closed reference operational taxonomic unit (OTU) clustering for environmental bacterial communities (Edgar, 2017), such as false positive genus names, should not apply to this case as the community is defined and has its own reference database.

For downstream analyses, the species abundance data was further normalized with the USEARCH -otutab_norm command, converted into relative abundance with the -otutab_counts2freqs command, and used to compute alpha diversity with the -alpha_div command. Furthermore, a phylogenetic tree was created using QIIME 2 2018.8 (qiime2.org) aligning the 16S rRNA sequences from the 34 species detected with PyNAST alignment method using the QIIME align_seqs.py command.

5.4 Pheno- and genotypic data collected for community members

Certain phenotypic trait data for the community members (Sm MIC values and carbon source utilization data), collected in this master's thesis project, have been published previously (Cairns et al., 2018a). Sm MIC values for each species were obtained with an E-test (Liofilchem, Roseto degli Abruzzi, Italy). For this, a bacterial suspension of 0.5 McFarland units was spread-plated evenly on 50% PPY agar plates. The plates were incubated at 28 °C for 1–2 days, and the results were interpreted according to manufacturer's instructions. Furthermore, to determine the ability of the species to utilize different carbon sources, a growth experiment was performed on EcoPlates™ (Biolog Inc., Hayward, CA, USA) containing 31 different carbon sources, each with three technical replicates and a negative control. The species were grown and starved similar to the filtrate experiment, a 100-fold dilution was prepared of every species, and 150 µl of the dilution were pipetted on each well in EcoPlates™ (separately for each species). The plates were cultured at 28 °C for 7 days, and OD values at 590 nm were measured

using the Tecan Infinite M200 well-plate reader. The procedure was modified from MacLean et al. (2004). The OD values for each carbon source were compared to OD values of the negative control. A significantly higher OD value, based on a one-tailed *t*-test, was interpreted as the ability to utilize a given carbon source. For each species, the total number of different carbon sources utilized was computed for later analysis.

A growth experiment was performed for all the 34 strains in the co-culture medium in the Bioscreen C MBR (Oy Growth Curves Ab Ltd, Helsinki, Finland) well-plate reader at 28 °C with shaking and using a wideband (420–580 nm) filter to perform OD measurements every 5 minutes. The data was analyzed with the Growthcurver package (Sprouffske, 2018) in R version 3.4.0 (R Core Team, 2011) to obtain estimates for carrying capacity and intrinsic growth rate for each species.

Information about genetic aminoglycoside resistance was previously collected from whole genome data (Cairns et al., 2018a). All aminoglycoside resistance genes were pooled into species specific binary information (presence or absence). The metadata used in this study is summarized in Table S1 in Appendix 1.

5.5 Statistical analysis

Statistical analyses for community composition in the serial passage experiment were performed using R and the vegan package version 2.4-4 (Oksanen et al., 2017). Most of the figures were created using the ggplot2 package (Wickham, 2009). Permutational multivariate analysis of variance (PERMANOVA) was performed, using 999 permutations, on a Bray-Curtis dissimilarity matrix of square root transformed species abundance data for the time points zero and 16 days with antibiotic concentration, immigration, and time point as explanatory variables to test whether experimental manipulations change the community significantly prior to the antibiotic pulse. Group dispersion levels were tested with betadisper function, a multivariate analogue to Levine's test (Oksanen et al., 2017), combined with variance analysis (ANOVA) to test whether group dispersions differ between explanatory variable groupings and affect the liability of *p*-values in PERMANOVA. This showed that the experimental treatments (note that only immigration applied at this stage) did not affect community composition prior to antibiotic administration (Sm: $p = 0.17$, immigration: $p = 0.10$, Sm × immigration: $p =$

0.59; for full results see Appendix 2, Table S1), although some group dispersion occurred (ANOVA: $p = 0.0085$; for full results, see Appendix 2, Table S2). .

Community resistance was analyzed using PERMANOVA as previously for the final time point of the antibiotic pulse epoch (day 32). Furthermore, pairwise PERMANOVA was performed to test for differences between Sm concentrations using the RVAideMemoire package (Hervé, 2018) with Pillai's trace test and the Benjamini–Hochberg procedure (FDR in R) to correct *p*-values. Community resilience was analyzed similarly by comparing community compositions from days 16 and 48 (before antibiotic pulse versus after recovery period). Since there was a strong interaction between immigration and Sm treatment, to understand this better, the analysis was performed separately for communities with and without immigration. To visualize similarities between communities, non-metric multidimensional scaling (NMDS) was used as a dimensionality reduction technique, and the first two axes were plotted using the graphics package by Lasse Ruokolainen (https://github.com/laruokol/Graphics, accessed December 4th, 2017).

To determine the relative contribution of experimental conditions and species traits on species occurrence (also accounting for phylogenetic correlation), hierarchical modelling of species communities (HMSC) was performed on the community composition in the last time point using the HMSC package (Blanchet et al., 2017) with the probit model with 20,000 iterations and 10,000 algorithm burning phase iterations. Extremely rare species at time point 48, accounting for under 20 reads in all samples combined, and one species, *Thermomonas haemolytica*, lacking some of the trait data, were removed before analysis. *T. haemolytica* was not highly abundant in any stage in any community in the experiment. Altogether 19 species remained for the analysis. The phenotypic traits used in the analysis included carrying capacity, intrinsic growth rate, log transformed Sm MIC data, the number of carbon sources each species is capable of utilizing, and the presence or absence of aminoglycoside resistance genes in the genome of the species. Because HMSC was used for species occurrence data, not abundance, to cross-validate trait results, a separate 4th corner analysis (traitglm) was performed with the mvabund package (Wang et al., 2018). Abundance data on day 32 was further analyzed with 4th corner analysis (traitglm), to see the effect of traits on species abundance during the antibiotic pulse.

Diversity was analyzed with the vegan package (Oksanen et al., 2017) using the inverse Simpson's index comparing day 16 communities separately to the two later time points representing community resistance and resilience, respectively. Generalized least squares models (gls), accounting for treatment-specific variance structures, were used to test for differences in diversity over time and across treatments, using the nlme package (Pinheiro, 2017). For post hoc analyses, estimated marginal means (EMM) or least-square means were computed using the emmeans package (Lenth, 2018). Gls was also used in population size analyzes with log-transformed OD_{600} values and AR1 autocorrelation structure.

The community data was used to determine the species, across all treatments, that went extinct between the time points 16 and 32 days or 16 and 48 days. A logistic regression (binomial glm) model was constructed to test for the effect of experimental treatments on the extinction of the 17 species that went extinct in at least one treatment.

6 Results

6.1 General features of communities

From the 34 bacterial species community, only 29 were present at detectable frequency in at least one time point and community in the experiment. Two to four species dominated all the communities (Figure 6). The population size increased over time ($p < 0.0001$; for full results, see Appendix 2, Table S2), and Sm affected population size ($p = 0.025$).

Figure 6. Square root transformed species abundances as a function of time in the serial passage experiment in different experimental treatments. The shaded area represents the middle antibiotic pulse epoch. Sm = streptomycin, numbers following represent concentrations in μg ml^{-1}. The twelve named species represent the most abundant species across all the treatments and time points and the rest of the species were grouped to others.

6.2 Community resistance

Streptomycin explained 49% of variation in community composition at the end of the antibiotic pulse (day 32) (PERMANOVA: $p < 0.001$; for full results, see Appendix 2, Table S4), while immigration did not affect community composition (PERMANOVA: $p = 0.55$). Communities also differed between all the Sm concentrations (pairwise PERMANOVA: all comparisons $p = 0.002$). Sm group dispersals did not differ significantly (ANOVA: $p = 0.88$), supporting this conclusion (Figure 7).

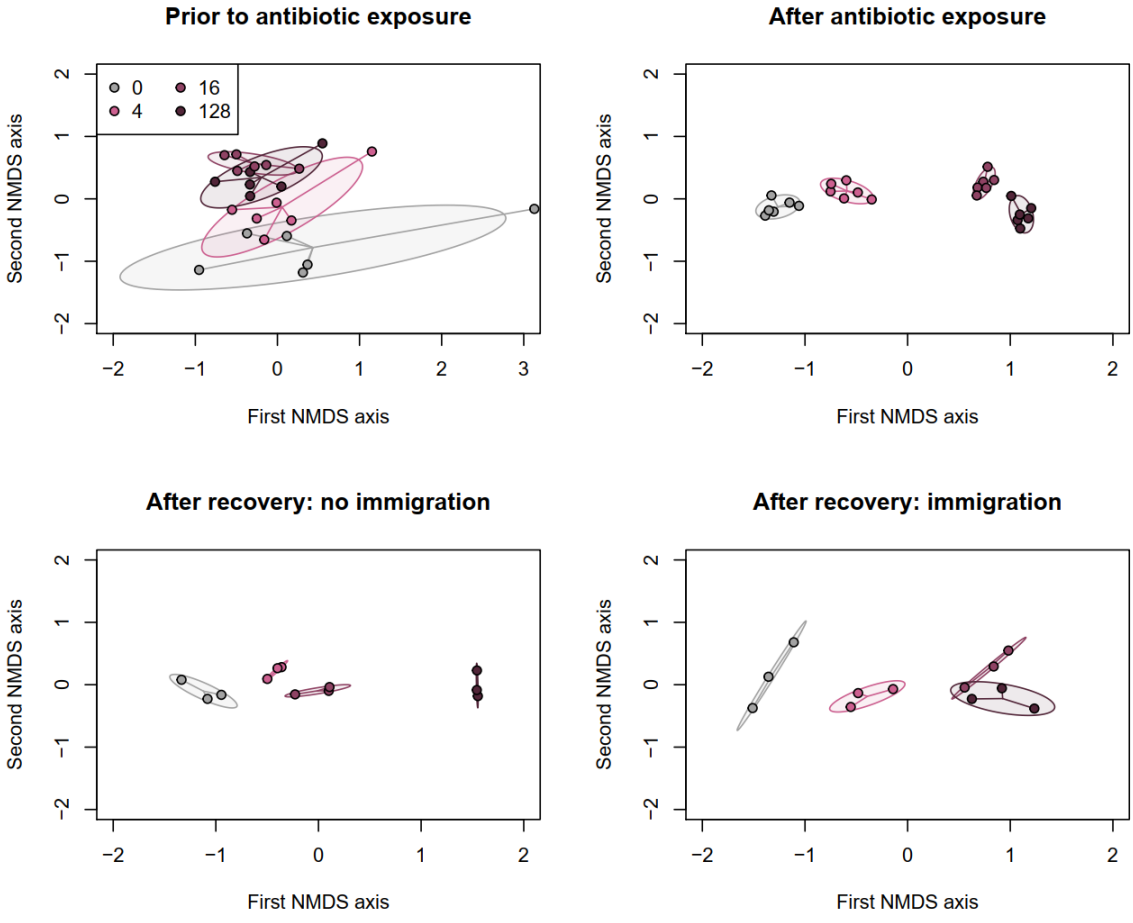

Figure 7. Ordination plots (NMDS) showing differences between community composition following antibiotic pulse (upper panel) and after antibiotic pulse with immigration and immigration-free communities displayed separately (bottom panel).

Diversity was affected by both Sm (ANOVA: $p < 0.001$; for full results, see Appendix 2, Table S5, Figure 8) and immigration (ANOVA: $p < 0.001$), but immigration affected only the control treatment (post hoc test: $p < 0.001$; for full results, see Appendix 2, Table S6) by enhancing the diversity, and the highest Sm treatment ($p = 0.0017$) by slightly reducing the diversity. Immigration did not prevent the decline in diversity in the presence of low or intermediate levels of Sm (post hoc test: Sm 4 µg ml^{-1}: $p = 0.33$, Sm 16 µg ml^{-1}: $p = 0.71$). Diversity declined over time with Sm in all but the control treatment (post hoc test: 0 µg ml^{-1}: $p = 0.77$, all Sm concentrations: $p < 0.001$).

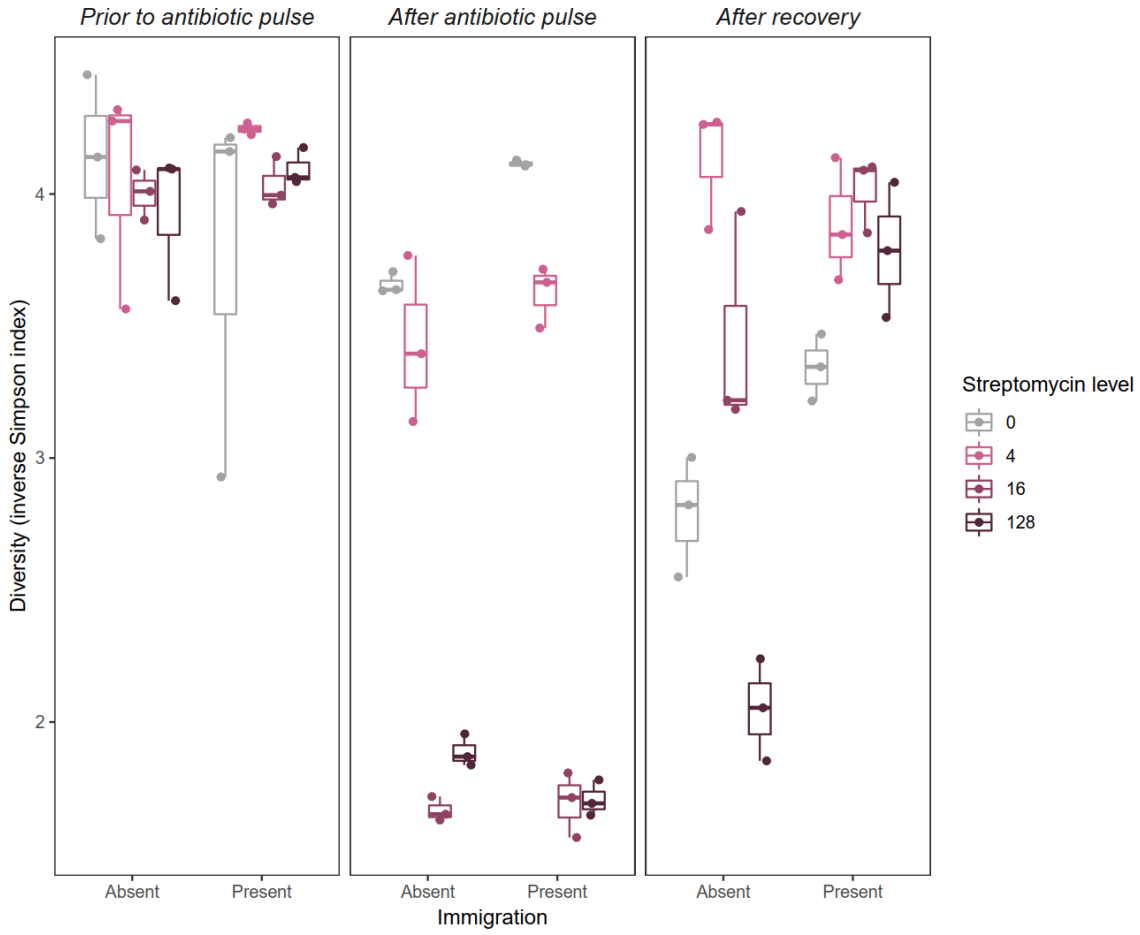

Figure 8. Alpha diversity across different treatments and time points.

The species *Elizabethkingia meningoseptica, Citrobacter koseri, Aeromonas caviae*, and *Pseudomonas chlororaphis* were prominent in communities both with and without immigration (Figure 6). However, the abundance of the latter two species responded strongly to increasing Sm concentrations (including the lowest concentration for *A. caviae*), especially without immigration where extinctions and displacement by other species, most notably, *Sphingobacterium spiritivorum* and *Comamonas testosteroni* (both possessing higher Sm MIC values) was observed. Interestingly, certain species with low measured MIC values seemed to survive in higher Sm concentrations than expected based on the MIC value. For instance, *A. caviae* with an MIC of 0.75 µg ml^{-1} survived without immigration through a 16-day pulse of 4 and even 16 µg ml^{-1} Sm, representing over 20-fold its MIC value. Immigration kept the community more similar between different treatments, preventing changes in the dominant species. Traitglm analysis revealed no significant results on day 32.

6.3 Community resilience

Overall, species occurrence was mostly explained by the experimental treatments (Figure 9; see below for contribution of phenotypic traits). In communities without immigration, Sm explained 83% of the differences in community composition when only the last time point was analyzed (PERMANOVA: $p < 0.001$, for full results, see Appendix 2, Table S7). As the sample size is small, communities were also analyzed with the time point before antibiotic exposure: Sm explained 34% of variation in community composition when the time points before antibiotic exposure (day 16) and after recovery (day 48) were compared (PERMANOVA: $p < 0.001$; for full results, see Appendix 2, table S8), while time point explained 11% of the variation ($p < 0.001$). Antibiotic and time point also had a strong interaction (34%; $p = 0.002$), as the effect of antibiotic was restricted to day 48. Community composition varied with Sm (pairwise PERMANOVA: 0 vs. 4 µg ml^{-1} $p < 0.05$; 0 vs. 16 µg ml^{-1} $p = 0.015$; 0 vs. 128 µg ml^{-1}: $p = 0.012$) but not between different Sm concentrations (all comparisons $p = 0.155$). Group dispersions were inflated on day 48 (ANOVA: $p = 0.0055$; for full results, see Appendix 2, Tables S9 and S10) explained by the inflation in the highest Sm concentration treated communities (ANOVA: $p < 0.001$; for full results, see Appendix 2, Tables S11 and S12), indicating that differences in community composition increased between replicate communities.

When only time point 48 days was analyzed, Sm accounted for 30% of the variation between communities in communities with immigration (PERMANOVA: $p = 0.02$; for full results, see Appendix 2, Table S13). Again, time points 16 and 48 days were grouped to attain a larger sample size, and the effect of Sm was only marginally significant (PERMANOVA: $p = 0.085$; for full results, see Appendix 2, Table 14, Figure 8). In the HMSC analysis, when the effect of experimental treatments was accounted for, residual species associations displayed very low correlations (–0.15 to 0.15), indicating that synergistic or antagonistic species interactions likely played a negligible role in community dynamics at species occurrence level.

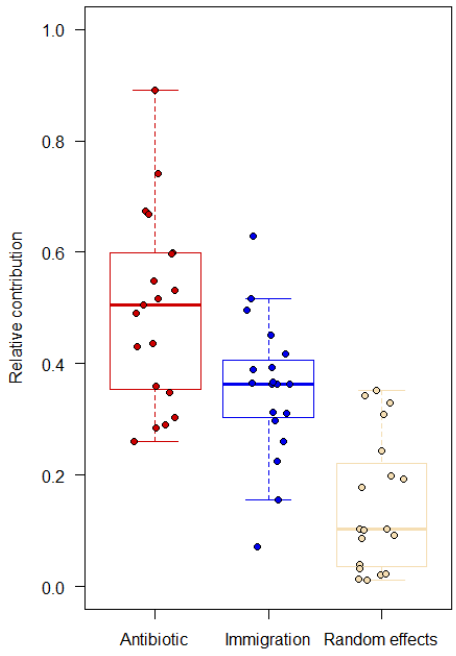

Figure 9. Relative contribution of experimental treatments and random effects on species occurrence after recovery from antibiotic pulse according to HMSC analysis. Each observation represents one species.

Diversity was recovered after low and intermediate Sm levels without immigration and after all Sm levels with immigration (Figure 8), with Sm and immigration explaining the

differences in diversity (ANOVA: both $p < 0.001$; full results in Appendix 2, Table S16). Immigration restored diversity in communities perturbed with high (post hoc test: $p < 0.001$; for full results, see Appendix 2, Table S17) and intermediate ($p = 0.034$) Sm levels and maintained an elevated diversity in the control treatment ($p < 0.001$), whereas communities exposed to low concentrations recovered with and without immigration ($p = 0.20$).

Antibiotic concentration and immigration both significantly affected whether a species went extinct during the experiment (logistic regression: antibiotic $p < 0.001$, immigration $p = 0.035$; for full results see Appendix 2, Table S18, Figure 10). Extinction probability showed an overall increasing tendency with increasing concentrations of Sm and decreased in the presence of immigration.

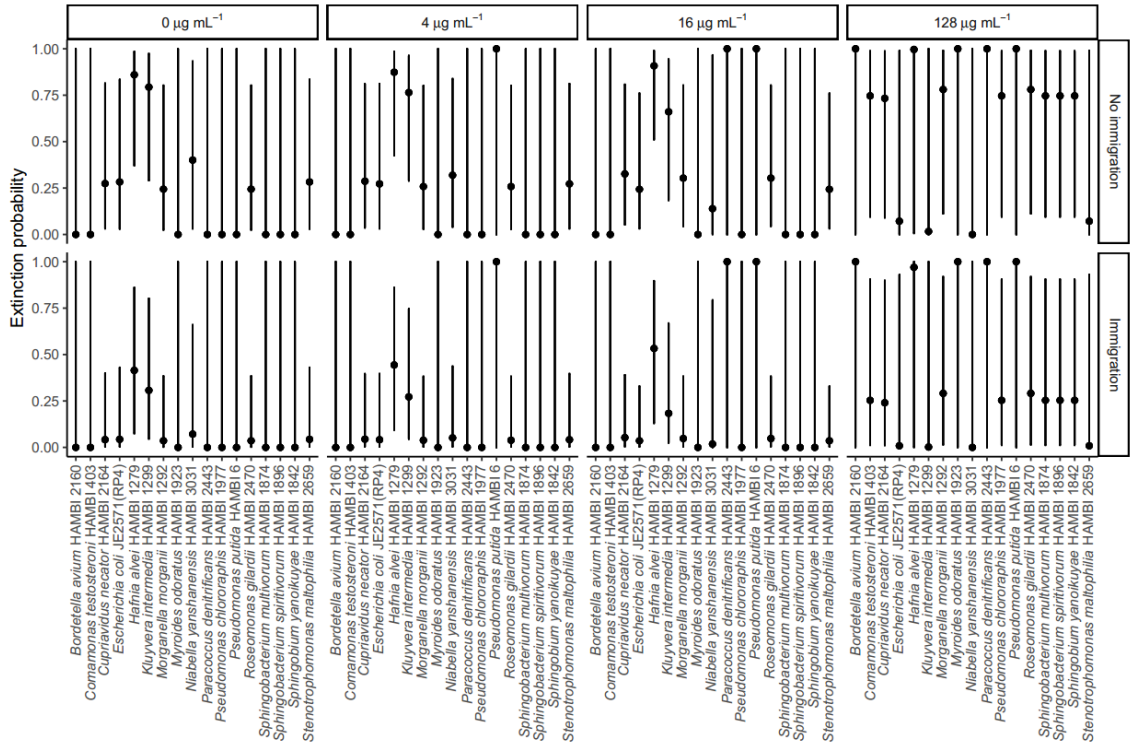

Figure 10. Extinction probability with 95% confidence intervals for each species in different experimental treatments based on logistic regression.

6.4 Role of phenotypic traits

According to HMSC variation partitioning analysis, the phenotypic traits of species explained 15% of species occurrence after recovery from antibiotic disturbance. Sm resistance, both in terms of MIC level and the presence of aminoglycoside resistance genes in the genome (which are not strongly correlated), affected species occurrence and abundance (Figure 11) positively. Carrying capacity affected species occurrence and abundance positively in communities with immigration but negatively with Sm. Intrinsic growth rate seemed to also have a positive effect on species occurrence in communities with immigration, while the number of carbon sources utilized seemed to have a negative effect.

The Sm susceptibility of a species, in terms of both MIC level and presence/absence of aminoglycoside resistance gene in its genome, had a significant effect on its survival probability between day 16 and either of the later timepoints (logistic regression: $p < 0.001$, for full results, see Appendix 2, Table S18).

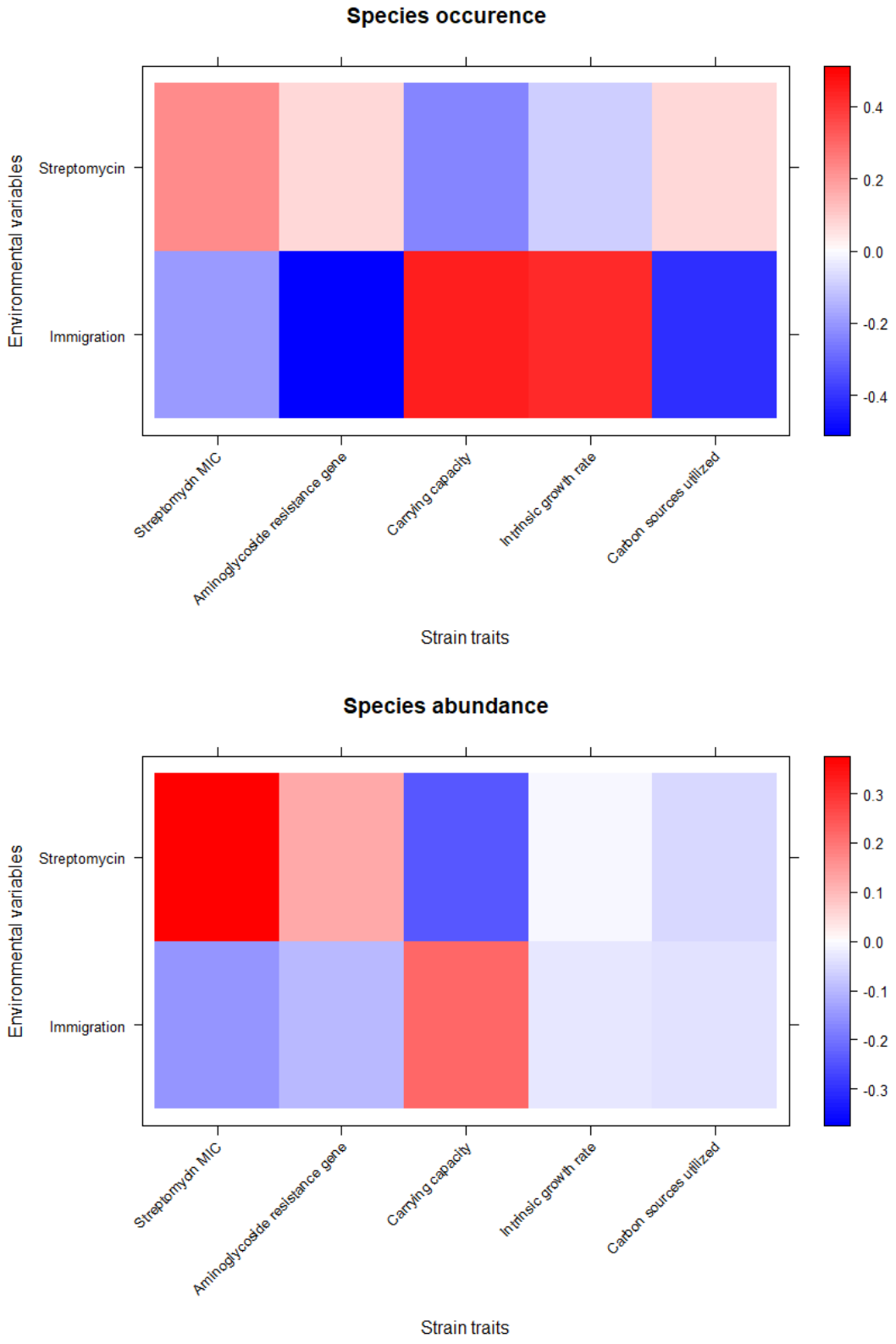

Figure 11. Heat map showing how different traits increased (red) or decreased (blue) species occurrence in different experimental treatments after recovery from antibiotic pulse based on HMSC analysis (species occurrence; top panel) and 4th corner analysis (traitglm) (species abundance; bottom panel). The baseline for comparison is the antibiotic-free treatment without immigration.

7 Discussion

7.1 Streptomycin concentration determines the level of perturbation

All streptomycin concentrations examined affected community composition and lowered diversity during the exposure. Bucci et al. (2012) explain the long lasting effect after antbiotic treatment with the shift of a multi-stable community. As predicted (i.), the effect was somewhat continuous, with the degree of community shift growing with increasing Sm concentrations, and shows also lower antibiotic concentrations have an effect on the community. Further, the results support the notion that even relatively low antibiotic concentrations can cause extinctions, although extinction probability increases with increasing antibiotic concentration. The extinction of even low-abundance species has the potential to cause a substantial change in community functioning, since low-abundance species can have important functions (Rivett & Bell, 2018). For instance, sub-therapeutic antibiotic concentrations have been used to promote growth in livestock (Cromwell, 2002), which might be due to an altered gut microbiota and long-lasting metabolic changes in the host caused by the alteration (Cox et al., 2014).

Species flow (immigration) played a relatively minor role in enhancing community resistance, maintaining increased diversity only in the control treatment. The priority effect might also limit the ability of immigrating species to occupy or modify the niches present (Fukami, 2015). It has been shown previously that communities with specialized species are less affected even by the immigration of specialized species than communities with maladapted species (Fukami et al., 2007). Moreover, immigrating bacteria were not accustomed to the experimental conditions, whereas the residing bacteria had undergone several generations potentially allowing them to adapt to the conditions. One reason for the weak effect could also be the unevenness of species in the immigration stock: dominant species were dominant already in the stock community, and species flow of rare species with low competitive ability was low.

7.2 Even low concentrations cause long-lasting composition shifts

The Sm pulse caused changes in the communities that lasted throughout the last recovery epoch. The highest concentration also caused the community trajectories of the recovered

replicate communities to deviate more compared to lower concentrations, again consistent with the first (i.) prediction. This is in line with studies on human gut microbiota changes after broad-range antibiotic treatment: the treatments have been reported to cause long-term changes in the microbiota composition even after years from the treatment (Jernberg et al., 2007; Jakobsson et al., 2010), and repeated exposures cause even more drastic changes (Dethlefsen & Relman, 2011). The drastic changes caused by the high Sm concentration, such as the extinction of several species, could account for the differentiation in replicate communities in later time points through increased ecological drift in population bottlenecks. Notably, the extinction rate was higher in communities exposed to higher concentrations. Moreover, if cross-feeding interactions exist in the community, the consumer is affected by the extinction of the producer, especially if it can use a limited set of resources.

The communities treated with the lowest Sm concentration were able to recover in diversity even though the community compositions did change, which again is consistent with gut microbiome studies (Jakobsson et al., 2010). The diversity not only recovered after the two lower Sm concentrations but the recovered diversity was higher than in the control community at the same timepoint, which seems to follow the intermediate disturbance hypothesis (Connell, 1978). The mechanisms behind this hypothesis have been disputed, and Fox (2013) proposes that diversity in communities with non-additive species (communities where species growth rate response to competition changes based on the conditions (Chesson, 1994)), the storage effect (different species thrive under favourable and unfavourable conditions (Chesson, 1994)) can benefit from disturbances. As discussed earlier, even rare taxa can be important for community function (Rivett & Bell, 2018). Diversity has been considered an important measure of community function (Cavigelli & Robertson, 2000; Griffiths et al., 2001), but also community composition and species interactions play an important role (Bell et al., 2005). Thus, the extinction of species might cause significant changes in the community function even if the diversity recovers, illustrating why it is important to not only study the alpha diversity of communities but also the beta diversity incorporating community composition.

Contrary to resistance, immigration had a considerable effect on community resilience. Diversity recovered to near-pre-exposure levels in all concentrations, and only communities exposed to the highest concentration exhibited reduced diversity (as did the control treatment), further supporting the intermediate disturbance hypothesis (Connell,

1978). As immigration reduced extinction probability, the higher survival rate could be linked to higher diversity in communities with immigration. The community composition also recovered quite well compared to the absence of immigration, although the separate analysis of the last time point for communities with immigration still showed a strong effect of antibiotics. This is consistent with the extinction probability decreasing in immigration treated communities and with previous study (Brown & Kodric-Brown, 1977; Bucci et al., 2012). Similarly, administration of normal mice gut microbiota to antibiotic treated and resistant *Enterococcus* colonized mice has been reported to decrease the abundance of the resistant pathogen and enhance the recovery of the community composition (Ubeda et al., 2013). These results favor the use of probiotics in the treatment of perturbed human gut microbiota after antibiotics (Friedman, 2012), but no direct conclusions can be drawn. Alternatively, immigration could also have had an adverse effect on community recovery based on increased adaptive mutations due to species flow (Perron et al., 2007) which could shift the stable state of the community. These results provide insight on the fourth (iv.) prediction: immigration does prevent extinctions, maintain diversity, and decrease variation between replicate communities after the Sm pulse. Furthermore, the results are inconsistent with a potentiating effect of immigration on adaptive evolution, which should lead to increased variability between replicate communities.

7.3 General dynamics and future prospects

Even if the viable cell proportions in the starting and immigration bacterial stock could not be determined with absolute certainty as previously discussed (Cairns et al., 2018a), different treatments groups did not differ from each other significantly at day 16 before the antibiotic pulse. Thus, comparing the remaining time points to pretreatment communities should provide reliable results regarding community changes. Nevertheless, having only three replicates for each treatment combination complicates the statistical analyses, as the within-group variation can obstruct the detection of between-group variation. Despite this, clear treatment effects were observed even for the low antibiotic concentration treatment, showing that these effects are strong enough to surpass the noise. The remaining 5/8 replicated populations are in the process of being amplicon sequenced, which will allow cross-validating and strengthening the results.

Community dynamics was mostly highly repeatable between biological replicates, as shown previously for multi-species experimental microbial communities (Celiker & Gore, 2014; Goldford et al., 2018). This points to deterministic dynamics rather than stochastic dynamics, which answers the second (ii.) prediction. In other words, the dynamics depend on deterministic processes selecting species, such as environmental variables and biotic interactions, as modulated by species traits affecting species response to these two factors, rather than random processes, such as environmental stochasticity or ecological drift. However, the replicate communities exposed to the highest streptomycin concentration were an exception and the end dynamics seemed more random, following the third (iii.) prediction. This could be caused, for example, by ecological stochasticity from a stronger bottleneck when the community was transferred to fresh medium, as the high antibiotic concentration drove the population sizes of several species to low levels, or evolutionary changes such as selection for resistant genotypes.

Although species with higher intrinsic antibiotic resistance levels were enriched in communities after recovery from the antibiotic pulse, it is, however, more difficult to determine the traits beneficial for survival during the Sm pulse: even though Sm resistance lowers the extinction rate and resistant species survive and enrich during the recovery epoch, antibiotic resistance did not predict the species abundance during the antibiotic pulse. Highly abundant species are less susceptible to Sm that their MIC values would have predicted, and even less abundant species behave unexpectedly. Besides species Sm resistance, the ability to utilize resources is probably important for species survival. It is probable, that as the more susceptible strains went extinct under the antibiotic exposure, the resistant strains were able to gain abundance after the pulse due to competitive release when the niches were released. Further, the antibiotic pulse and dominant strains restrain their growth during the Sm epoch. Immigration affects which traits are beneficial for species prevalence and abundance in the communities after recovery and is especially associated with species growth rate. This implies that certain species with good competitive ability in the base conditions go extinct during the Sm pulse, but they are able to recover later with immigration.

Of the overall top four species, all had MIC values of 24 μg ml^{-1} or lower, yet two of them, *E. meningoseptica* and *C. koseri* were abundant in all communities, and *A. caviae* and *P. chlororaphis*, with with MIC values of 0.75 and 8 μg ml^{-1} respectively, survive in all but the highest Sm concentration without immigration. Notably, MIC values were

measured on solid media with E tests which can overestimate antibiotic susceptibility (Schulz & Sahm, 1993; Luber et al., 2003). Further, microbial communities have protective properties against antibiotics, such as biofilms (Stewart & Costerton, 2001). The majority of bacteria live in biofilms (Flemming & Wuertz, 2019) and biofilms are associated also with pathogenetic bacteria (Costerton et al., 1999). Extracellular DNA in biofilms have been reported to protect *Pseudomonas aeruginosa* against aminoglycosides (Chiang et al., 2013). Communities can also include species with antibiotic inactivating functions which aid not only the species in question but the whole community (Cairns et al., 2018a). Thus, the protective effect of the community could mitigate the Sm perturbation. Although signals of strong positive species associations during antibiotic exposure were not observed in species occurrence level analyses, further analyses incorporating species abundance data could be used to investigate this more precisely. Additionally, some cell could go to a dormant state during the antibiotic pulse and survive the pulse as persister cells (Lewis, 2007).

It is also possible that resistance evolution has occurred during the experiment. This might not be limited to the higher concentrations as even sub-MICs of Sm have been reported to cause adaptation (Westhoff et al., 2017). Higher antibiotic concentrations could also select for more resistant genotypes. Immigration has, moreover, been reported to enhance resistance evolution by providing more genetic variation and new possible mutations (Perron et al., 2008). However, evolutionary events affecting ecological dynamics should introduce variability between replicate communities, and are unsupported by the high repeatability of community trajectories observed here. Nevertheless, the role of evolution is unclear based on this data alone. Metagenomics analysis of the same samples is underway and will allowing testing these questions.

8 Conclusions

This thesis provides new information about the effects of antibiotic perturbations of different magnitudes on multi-species bacterial communities. This thesis studied a classical system of rapid evolution (bacteria responding to antibiotics), but the results indicate the communities are driven by deterministic ecological dynamics as the response to experimental variables was highly repeatable. This indicates a negligible role for

evolution in the ecological disturbance response of multi-species communities. Further, even though the effect of the perturbation increases with increasing antibiotic concentrations, a clear community response is observed even at low concentrations. Nevertheless, further studies are required, as community level studies of low antibiotic effects are still rare. Immigration could increase the effect of evolution but in this thesis it rather aided the recovery of the communities as predicted by previous studies. To conclude, the results indicate that ecology determines the community response and the role of evolution could be overestimated when a more restricted system is studied. The results also highlight the major role of species influx in the recovery of communities from disturbance.

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Appendix 1

Table S1. Species trait table containing traits used for the statistical analysis. Different carbon sources are coded with 0/1 coding, 1 meaning the species could utilize the carbon source in question in the EcoPlate™ assay (Biolog Inc., Hayward, CA, USA). Sum of carbon sources column summarizes all the assayed substrates to one variable. Aminoglycoside resistance gene column reports whether the species genome contained at least one gene encoding aminoglycoside gene (1 = yes, 0 = no). The table continues for eight pages.

Species	HAMBI code	Carrying capacity
Escherichia coli K-12 substrain JE2571 (RP4)	Not applicable	0.246179391
Agrobacterium tumefaciens	HAMBI 105	0.473820741
Hafnia alvei	HAMBI 1279	0.099442655
Morganella morganii	HAMBI 1292	0.215482466
Citrobacter koseri	HAMBI 1287	0.387744757
Kluyvera intermedia	HAMBI 1299	0.301864607
Sphingomonas yanoikuyae	HAMBI 1842	0.428166099
Sphingobacterium multivorum	HAMBI 1874	0.373996547
Elizabethkingia meningoseptica	HAMBI 1875	0.277622314
Sphingobacterium spiritivorum	HAMBI 1896	0.324579121
Myroides odoratus	HAMBI 1923	0.05669627
Aeromonas caviae	HAMBI 1972	0.312050581
Pseudomonas chlororaphis	HAMBI 1977	0.427565047
Chitinophaga sancti	HAMBI 1988	0.379086658
Paraburkholderia caryophylli	HAMBI 2159	0.406740709
Azorhizobium caulinodans	HAMBI 216	0.199279343
Bordetella avium	HAMBI 2160	0.051625781
Cupriavidus necator	HAMBI 2164	0.097600234
Paracoccus denitrificans	HAMBI 2443	0.498963241
Thermomonas haemolytica	HAMBI 2467	NA
Roseomonas gilardii	HAMBI 2470	0.550307931
Brevundimonas bullata	HAMBI 262	0.187735054
Stenotrophomonas maltophilia	HAMBI 2659	0.081574677
Moraxella canis	HAMBI 2792	0.243003144
Niabella yanshanensis	HAMBI 3031	0.338082085
Azospirillum brasilense	HAMBI 3172	0.314382281
Comamonas testosteroni	HAMBI 403	0.206252382
Pseudomonas putida	HAMBI 6	0.386629841
Acinetobacter lwoffii	HAMBI 97	0.198313455

Species	Instinct growth rate (OD hour^{-1})	MIC Sm (µg ml^{-1})	Pyruvic acid methyl ester	Tween 40
E. coli JE2571 (RP4)	0.888076378	1024	0	0
A. tumefaciens	0.339024646	12	0	0
H. alvei	0.884446275	0.25	0	1
M. morganii	0.849023977	0.19	0	0
C. koseri	0.619079739	24	1	0
K. intermedia	0.722410045	0.75	1	0
S. yanoikuyae	0.270343491	48	0	1
S. multivorum	0.53320551	192	0	0
E. meningoseptica	0.500555121	16	0	1
S. spiritivorum	0.352044528	1024	0	0
M. odoratus	0.234300491	12	0	0
A. caviae	0.820623772	0.75	1	1
P. chlororaphis	0.833646843	8	1	1
C. sancti	0.089235126	0.5	0	0
P. caryophylli	0.398287684	0.38	1	1
A. caulinodans	0.3530889	3	0	1
B. avium	0.227472073	6	1	1
C. necator	0.545281868	2	0	0
P. denitrificans	0.412778243	2	0	0
T. haemolytica	NA	0.125	0	0
R. gilardii	0.108955825	0.75	1	0
B. bullata	0.326003468	0.38	0	1
S. maltophilia	0.80488747	48	1	1
M. canis	0.334865345	0.5	0	0
N. yanshanensis	0.200186876	6	0	0
A. brasilense	0.379717492	1.5	1	0
C. testosteroni	0.758360388	32	1	1
P. putida	0.509971013	6	1	1
A. lwoffii	0.36930207	0.19	0	0

OD = optical density, MIC = minimal inhibitory concentration, Sm = streptomycin

Species	Tween 80	α-cyclodextrin	glycogen	D-cellobiose	α-D-lactose	ß-methyl-D-glucoside
E. coli JE2571 (RP4)	1	0	1	0	0	0
A. tumefaciens	1	0	0	1	1	1
H. alvei	1	0	1	0	0	0
M. morganii	0	0	0	0	0	0
C. koseri	1	0	0	1	0	1
K. intermedia	1	0	0	1	1	1
S. yanoikuyae	0	0	0	1	1	1
S. multivorum	0	1	1	0	1	1
E. meningoseptica	1	1	0	0	1	0
S. spiritivorum	0	1	1	1	1	1
M. odoratus	0	0	1	0	1	1
A. caviae	0	1	1	1	1	1
P. chlororaphis	1	0	0	0	0	0
C. sancti	1	0	0	0	0	0
P. caryophylli	1	0	0	0	0	0
A. caulinodans	1	1	1	0	0	0
B. avium	1	0	1	0	0	0
C. necator	0	0	0	0	0	0
P. denitrificans	1	0	0	0	0	0
T. haemolytica	1	0	1	0	0	0
R. gilardii	0	0	0	0	0	0
B. bullata	1	0	0	0	0	0
S. maltophilia	1	0	0	1	0	0
M. canis	0	0	0	0	0	0
N. yanshanensis	1	1	1	1	1	1
A. brasilense	1	0	0	0	0	0
C. testosteroni	1	0	1	0	1	0
P. putida	1	0	1	0	0	0
A. lwoffii	1	0	0	0	0	0

Species	D-xylose	i-erythritol	D-mannitol	N-acetyl-D-glucosamine	D-glucosaminic acid
E. coli JE2571 (RP4)	1	0	0	0	0
A. tumefaciens	1	0	1	1	0
H. alvei	1	0	0	0	0
M. morganii	0	0	0	0	0
C. koseri	1	0	1	1	1
K. intermedia	1	0	1	1	0
S. yanoikuyae	0	0	0	1	0
S. multivorum	1	0	0	1	0
E. meningoseptica	0	0	1	1	0
S. spiritivorum	1	0	1	1	0
M. odoratus	0	0	1	0	0
A. caviae	0	0	1	1	0
P. chlororaphis	0	0	1	1	1
C. sancti	0	0	0	0	1
P. caryophylli	1	0	1	1	1
A. caulinodans	1	0	0	0	0
B. avium	1	0	0	0	0
C. necator	0	0	0	0	0
P. denitrificans	1	0	0	0	0
T. haemolytica	0	0	0	0	0
R. gilardii	0	0	0	0	0
B. bullata	0	0	0	0	0
S. maltophilia	1	0	0	0	0
M. canis	0	0	0	0	0
N. yanshanensis	0	0	0	1	0
A. brasilense	0	0	0	0	0
C. testosteroni	1	0	0	0	0
P. putida	0	0	0	0	0
A. lwoffii	1	1	0	0	0

Species	Glucose-1-phosphate	D,L-α-glycerol phosphate	D-galactonic acid	D-galacturonic acid	2-hydroxy benzoic acid
E. coli JE2571 (RP4)	0	0	0	0	0
A. tumefaciens	1	0	1	0	0
H. alvei	0	0	0	0	1
M. morganii	0	1	0	0	0
C. koseri	1	1	0	1	0
K. intermedia	1	0	0	1	0
S. yanoikuyae	0	0	0	0	0
S. multivorum	0	0	0	0	0
E. meningoseptica	0	0	0	0	1
S. spiritivorum	0	1	0	1	1
M. odoratus	1	1	0	1	1
A. caviae	0	0	0	0	1
P. chlororaphis	0	1	1	0	0
C. sancti	0	0	0	0	1
P. caryophylli	1	0	1	1	1
A. caulinodans	0	0	1	1	1
B. avium	0	0	0	0	1
C. necator	0	0	1	0	1
P. denitrificans	1	0	0	0	0
T. haemolytica	0	0	0	0	0
R. gilardii	0	0	0	0	1
B. bullata	0	0	0	0	1
S. maltophilia	0	0	0	0	0
M. canis	0	0	0	0	0
N. yanshanensis	1	0	0	1	1
A. brasilense	0	0	1	1	1
C. testosteroni	0	1	0	0	1
P. putida	0	0	0	1	0
A. lwoffii	0	0	0	0	1

Species	4-hydroxy benzoic acid	γ-hydroxybutyric acid	Itaconic acid	α-ketobutyric acid	D-malic acid
E. coli JE2571 (RP4)	0	0	0	0	0
A. tumefaciens	1	0	0	0	1
H. alvei	0	1	0	0	0
M. morganii	0	0	0	1	0
C. koseri	0	0	0	0	1
K. intermedia	1	1	0	0	0
S. yanoikuyae	1	0	0	0	0
S. multivorum	0	0	0	0	0
E. meningoseptica	0	0	0	0	0
S. spiritivorum	0	1	0	0	0
M. odoratus	0	0	1	0	1
A. caviae	0	0	0	0	0
P. chlororaphis	1	0	1	0	1
C. sancti	0	0	0	0	0
P. caryophylli	1	0	0	1	0
A. caulinodans	1	1	1	1	1
B. avium	0	0	0	0	0
C. necator	0	0	0	0	0
P. denitrificans	0	0	0	0	0
T. haemolytica	0	0	0	0	0
R. gilardii	1	0	0	0	1
B. bullata	1	0	0	0	0
S. maltophilia	0	0	0	0	0
M. canis	0	0	0	0	0
N. yanshanensis	0	0	0	0	0
A. brasilense	1	0	1	1	1
C. testosteroni	1	1	1	1	1
P. putida	1	1	0	0	1
A. lwoffii	0	1	0	1	0

Species	L-arginine	L-asparagine	L-phenylalanine	L-serine	L-threonine
E. coli JE2571 (RP4)	0	0	0	0	0
A. tumefaciens	1	1	0	1	1
H. alvei	0	0	0	0	1
M. morganii	0	0	0	0	1
C. koseri	0	1	0	1	1
K. intermedia	0	0	0	1	1
S. yanoikuyae	0	1	0	0	0
S. multivorum	0	0	0	0	0
E. meningoseptica	0	1	0	0	0
S. spiritivorum	1	1	0	1	0
M. odoratus	0	0	0	0	0
A. caviae	1	1	0	1	1
P. chlororaphis	1	1	0	1	0
C. sancti	0	0	0	0	0
P. caryophylli	1	1	0	1	0
A. caulinodans	0	1	0	0	1
B. avium	0	1	1	0	0
C. necator	0	0	0	0	0
P. denitrificans	0	0	0	0	0
T. haemolytica	0	0	0	0	0
R. gilardii	0	0	0	0	1
B. bullata	1	0	1	1	1
S. maltophilia	0	0	0	0	0
M. canis	0	0	0	0	0
N. yanshanensis	0	0	0	0	0
A. brasilense	1	1	0	0	1
C. testosteroni	0	1	1	0	1
P. putida	1	1	0	1	1
A. lwoffii	0	0	0	0	0

Species	Glycyl-L-glutamic acid	Phenylethylamine	Putrescine	Sum of carbon sources	Aminoglycoside resistance genes
E. coli JE2571 (RP4)	0	0	0	3	1
A. tumefaciens	0	0	0	15	0
H. alvei	0	0	0	7	1
M. morganii	0	0	0	3	1
C. koseri	1	0	0	16	1
K. intermedia	1	0	0	15	1
S. yanoikuyae	1	0	0	8	0
S. multivorum	0	0	0	6	0
E. meningoseptica	1	0	0	9	0
S. spiritivorum	1	0	0	16	0
M. odoratus	0	0	0	10	0
A. caviae	1	0	1	16	0
P. chlororaphis	0	1	1	16	1
C. sancti	0	0	0	3	0
P. caryophylli	1	0	1	18	1
A. caulinodans	0	0	0	15	0
B. avium	1	0	0	9	1
C. necator	0	0	0	2	1
P. denitrificans	0	0	0	3	1
T. haemolytica	0	0	0	2	0
R. gilardii	0	0	0	5	0
B. bullata	1	0	0	9	0
S. maltophilia	1	0	0	6	1
M. canis	0	0	0	0	0
N. yanshanensis	0	0	0	10	0
A. brasilense	0	1	0	13	1
C. testosteroni	0	0	0	16	0
P. putida	0	1	1	14	1
A. lwoffii	1	1	0	8	0

Appendix 2

Abbreviations used in this appendix

Df	Degree of freedom
im.	Immigration
Mean Sq	Mean of squares
PCoA	Principal coordinates analysis
R^2	Explanatory power
SE	Standard error
Sum Sq	Sum of squares

Table S1. PERMANOVA performed on day 0 and day 16 communities to examine the effect of experimental conditions before streptomycin exposure with 999 random permutations. Model: distance ~ antibiotic × immigration × time point

Model terms	Df	Sum Sq	Mean Sq	*F*-value	R^2	*p*-value
antibiotic	1	0.017	0.017	1.48	0.038	0.17
immigration	1	0.020	0.020	1.76	0.046	0.10
time point	1	0.14	0.14	12.61	0.33	0.001
antibiotic × immigration	1	0.0082	0.0082	0.71	0.019	0.59
residuals	22	0.25	0.011		0.57	
total	26	0.44			1	

Table S2. ANOVA on the homogeneity of multivariate dispersions of community dissimilarity between days 0 and 16.

	Df	Sum Sq	Mean Sq	F-value	*p*-value
groups	1	0.019	0.019	8.15	0.0085
residuals	25	0.059	0.0024		

Table S3. ANOVA on the generalized least squares fit on the population density in the regime shift experiment. Model: antibiotic × immigration × time point.

Model terms	Df	F-value	*p*-value
intercept	1	75787	0.001
antibiotic	1	5.09	0.025
immigration	1	0.12	0.73
transfer	1	65.04	0.001
antibiotic × immigration	1	0.02	0.90
antibiotic × transfer	1	0.55	0.46
immigration × transfer	1	0.33	0.56
antibiotic × immigration × transfer	1	1.27	0.26
	184		

Table S4. PERMANOVA on communities at time point 32 days with 999 permutations. Model: distance ~ antibiotic × immigration

Model terms	Df	Sum Sq	Mean Sq	*F*-value	R^2	*p*-value
antibiotic	1	0.60	0.60	20.40	0.49	0.001
immigration	1	0.012	0.012	0.41	0.010	0.55
antibiotic × immigration	1	0.015	0.015	0.50	0.012	0.54
residuals	20	0.59	0.030		0.48	
total	23	1.22			1	

Table S5. ANOVA on the generalized least squares fit on diversity between days 16 and 32 i.e. the community resistance. Model: antibiotic × immigration × time point.

Model terms	Df	*F*-value	*p*-value
intercept	1	510953	0.001
antibiotic	3	3609	0.001
Immigration	1	1033	0.001
Time point	1	3612	0.001
antibiotic × Immigration	3	35.70	0.001
antibiotic × time point	3	198.6	0.001
Immigration × time point	1	0.40	0.54
antibiotic × Immigration × time point	3	2.20	0.11
	32		

Table S6. Post hoc test on the generalized least squares fit on diversity between days 16 and 32 with estimated marginal means. Confidence level 0.95, significance level alpha = 0.05

Treatment	Contrast	Estimate	SE	Df	*t*-ratio	*p*-value
antibiotic = 0, time point = 16	0 - 1 (im.)	0.37	0.46	32	0.82	0.42
antibiotic = 0, time point = 32	0 - 1 (im.)	0.46	0.02	32	18.61	0.001
antibiotic = 4, time point = 16	0 - 1 (im.)	0.19	0.25	32	0.79	0.44
antibiotic = 4, time point = 32	0 - 1 (im.)	0.19	0.19	32	0.98	0.33
antibiotic = 16, time point = 16	0 - 1 (im.)	0.03	0.08	32	0.42	0.68
antibiotic = 16, time point = 32	0 - 1 (im.)	0.03	0.08	32	0.38	0.71
antibiotic = 128, time point =16	0 - 1 (im.)	0.17	0.17	32	0.96	0.34
antibiotic = 128, time point =32	0 - 1 (im.)	0.18	0.05	32	3.42	0.0017
Averaged over the levels of immigration						
antibiotic = 0	16 - 32 (day)	0.07	0.23	32	0.29	0.77
antibiotic = 4	16 - 32 (day)	0.62	0.16	32	3.97	0.001
antibiotic = 16	16 - 32 (day)	2.34	0.05	32	42.97	0.001
antibiotic = 128	16 - 32 (day)	2.22	0.09	32	24.67	0.001
Averaged over the levels of streptomycin						
immigration = 0	16 - 32 (day)	1.37	0.10	32	13.75	0.001
immigration = 1	16 - 32 (day)	1.25	0.11	32	11.40	0.001

Table S7. PERMANOVA on the Brey-Curtis dissimilarity between communities without immigration at timepoint 48 (to measure resilience) with 999 permutations. Model: distance ~ antibiotic

Model terms	Df	Sum Sq	Mean Sq	*F*-value	R^2	*p*-value
antibiotic	1.00	0.66	0.66	48.49	0.83	0.001
residuals	10.00	0.14	0.014		0.17	
total	11.00	0.80			1.00	

Table S8. PERMANOVA on the Brey-Curtis dissimilarity between communities without immigration at timepoint 16 and 48 (to measure resilience) with 999 permutations. Model: distance ~ antibiotic × time point

Model terms	Df	Sums Sq	Mean Sq	*F*-value	R^2	*p*-value
antibiotic	1	0.33	0.33	32.53	0.34	0.001
time point	1	0.11	0.11	10.68	0.11	0.001
antibiotic × time point	1	0.34	0.34	32.86	0.34	0.001
residuals	20	0.21	0.010		0.21	
total	23	0.99			1	

Table S9. Homogeneity of multivariate dispersion between time points 16 and 48 days without immigration.

Average distance to median:	
16	48
0.077	0.22

Eigenvalues for PCoA axes:

(Showing 8 of 23 eigenvalues)

PCoA1	PCoA2	PCoA3	PCoA4	PCoA5	PCoA6	PCoA7	PCoA8
0.81	0.092	0.040	0.026	0.022	0.014	0.0097	0.0078

Table S10. ANOVA on the group dispersals of community dissimilarity at timepoints 16 and 48 days without immigration with time point grouping.

	Df	Sum Sq	Mean Sq	*F*-value	*p*-value
groups	1	0.12	0.12	9.47	0.0055
residuals	22	0.27	0.012		

Table S11. Homogeneity of multivariate dispersion between different Sm treatment groups without immigration at timepoints 16 and 48.

Average distance to median:			
0	4	16	128
0.11	0.081	0.098	0.26

Eigenvalues for PCoA axes:
(Showing 8 of 23 eigenvalues)

PCoA1	PCoA2	PCoA3	PCoA4	PCoA5	PCoA6	PCoA7	PCoA8
0.81	0.092	0.040	0.026	0.022	0.014	0.0097	0.0078

Table S12. ANOVA on the group dispersals of community distances at timepoints 16 and 48 days without immigration with antibiotic treatment grouping.

	Df	Sum Sq	Mean Sq	*F*-value	*p*-value
groups	3	0.12	0.039	49.40	0.001
residuals	20	0.016	0.00080		

Table S13. PERMANOVA on the Bray-Curtis dissimilarity of immigration treated communities at timepoint 48 days (resilience) with 999 permutations. Model: distance ~ antibiotic

Model terms	Df	Sum Sq	Mean Sq	*F*-value	R^2	*p*-value
antibiotic	1	0.037	0.037	4.37	0.30	0.02
residuals	10	0.086	0.0086		0.70	
total	11	0.12			1	

Table S14. PERMANOVA on the Bray-Curtis dissimilarity of immigration treated communities at timepoints 16 and 48 days (resilience) with 999 permutation. Model: distance ~ antibiotic × time point

Model terms	Df	Sum Sq	Mean Sq	*F*-value	R^2	*p*-value
antibiotic	1	0.018	0.018	1.96	0.063	0.085
time point	1	0.050	0.050	5.55	0.18	0.001
antibiotic × time point	1	0.034	0.034	3.76	0.12	0.004
residuals	20	0.18	0.0090		0.64	
total	23	0.28			1	

Table S16. ANOVA on the generalized least squares fit on diversity between days 16 and 48 i.e. the community resilience. Model: antibiotic × immigration × time point.

Model terms	Df	*F*-value	*p*-value
intercept	1	140931	0.001
antibiotic	3	109.2	0.001
immigration	1	65.13	0.001
time point	1	99.08	0.001
antibiotic × immigration	3	31.26	0.001
antibiotic × time point	3	14.29	0.001
immigration × time point	1	22.46	0.001
antibiotic × immigration × time point	3	8.68	0.001
	32		

Table S17. Post hoc test on the generalized least squares fit on diversity between days 16 and 48 with estimated marginal means. Confidence level 0.95, significance level alpha = 0.05

Treatment	Contrast	Estimate	SE	Df	*t*-ratio	*p*-value
antibiotic = 0, time point = 16	0 - 1 (im.)	0.37	0.46	32	0.82	0.42
antibiotic = 0, time point = 48	0 - 1 (im.)	0.55	0.15	32	3.67	0.001
antibiotic = 4, time point = 16	0 - 1 (im.)	0.19	0.25	32	0.79	0.44
antibiotic = 4, time point = 48	0 - 1 (im.)	0.25	0.19	32	1.30	0.20
antibiotic = 16, time point = 16	0 - 1 (im.)	0.03	0.08	32	0.42	0.68
antibiotic = 16, time point = 48	0 - 1 (im.)	0.57	0.26	32	2.21	0.034
antibiotic = 128, time point = 16	0 - 1 (im.)	0.17	0.17	32	0.96	0.34
antibiotic = 128, time point = 48	0 - 1 (im.)	1.74	0.19	32	9.39	0.001
Averaged over the levels of immigration						
antibiotic = 0	16 - 48 (day)	0.89	0.24	32	3.69	0.001
antibiotic = 4	16 - 48 (day)	0.14	0.16	32	0.90	0.37
antibiotic = 16	16 - 48 (day)	0.29	0.13	32	2.13	0.041
antibiotic = 128	16 - 48 (day)	1.09	0.13	32	8.67	0.001
Averaged over the levels of streptomycin						
immigration = 0	16 - 48 (day)	0.93	0.12	32	7.72	0.001
immigration = 1	16 - 48 (day)	0.28	0.12	32	2.30	0.028

Table S18. Summary of binominal glm model explaining the species extinction probability: extinction ~ immigration+ antibiotic + aminoglycoside resistance gene. Null deviance: 200.77 on 223 degrees of freedom. Residual deviance: 166.13 on 220 degrees of freedom.

Model terms	Estimate	SE	*z*-value	*p*-value
intercept	-2.85	0.45	-6.40	0.001
immigration	-0.86	0.41	-2.11	0.035
antibiotic	0.013	0.0034	3.80	0.001
aminoglycoside resistance gene	1.68	0.44	3.84	0.001