The Role of Community Interactions in Shaping the Evolution of Antibiotic Resistance in Oligo-Mouse-Microbiota

A Thesis

submitted to

Indian Institute of Science Education and Research Pune in partial fulfilment of the requirements for the BS-MS Dual Degree Programme

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Date: 15 March, 2025

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From July 2024 to March 2025

INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled "**The Role of Community Interactions in Shaping the Evolution of Antibiotic Resistance in Oligo-Mouse-Microbiota**" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by **Sakshi Anil Kharche** at University of Lausanne under the supervision of Pascale Vonaesch, Professor, Department of Fundamental Microbiology, during the academic year 2024-2025.

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This thesis is dedicated to my parents and my brother.

Declaration

I hereby declare that the matter embodied in the report entitled "**The Role of Community Interactions in Shaping the Evolution of Antibiotic Resistance in Oligo-Mouse-Microbiota**" are the results of the work carried out by me at the Department of Fundamental Microbiology, University of Lausanne, Switzerland, and in affiliation with Indian Institute of Science Education and Research, Pune under the supervision of **Prof. Pascale Vonaesch**, and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

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List of Abbreviations

OMM12	Oligo-Mouse-Microbiota 12
MIC	Minimum Inhibitory Concentration
PMA	Propidium mono-azide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
BHI	Brain Heart Infusion
TAE	Triss-acetate-EDTA
DNA	Deoxyribonucleic acid
OD	Optical Density
qPCR	Quantitative Polymerase Chain Reaction
PI	Propidium Iodide
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight

Abstract

Microbial communities are shaped by interspecies interactions that influence bacterial survival, competition, and adaptation, particularly under antibiotic pressure. However, how these interactions impact resistance evolution remains poorly understood. In this study, we investigated the effects of vancomycin exposure on the evolutionary trajectories of *Enterococcus faecalis* KB1, *Bacteroides caecimuris* I48, and *Clostridium innocuum* I46 within the Oligo-Mouse-Microbiota (OMM12) model. Using two experimental evolutions at distinct vancomycin concentrations (0.5 µg/mL and 10 µg/mL), we analyzed species-specific adaptation and the role of interspecies interactions in modulating antibiotic responses. Our findings reveal distinct bacterial responses to vancomycin within monoculture and community settings. *E. faecalis* KB1 exhibited persistence rather than resistance evolution, maintaining stable minimum inhibitory concentration (MIC) values while surviving prolonged vancomycin exposure. However, its presence in the community remained largely unaffected by interspecies interactions, suggesting its dominance was independent of microbial competition. In contrast, *C. innocuum* I46, which completely collapsed in monocultures under high vancomycin pressure, managed to persist in the community, indicating that interactions with other bacterial species provided a protective effect. Meanwhile, *B. caecimuris* I48 not only acquired resistance, as seen in its increased MIC of vancomycin, but also played a regulatory role in modulating *E. faecalis* KB1's expansion within the OMM12 microbial community. This study highlights the crucial role of microbial interactions in shaping antibiotic susceptibility and resistance evolution. Resistance is not solely determined by intrinsic bacterial traits but is influenced by competition and metabolic dependencies within the community. Antibiotic selection can restructure microbial communities, leading to shifts in species dominance. Understanding these dynamics is essential for predicting resistance development, optimizing antibiotic treatment strategies, and ensuring the stability of microbial ecosystems.

Acknowledgements

I would like to express my sincere gratitude to Prof. Pascale Vonaesch for giving me the opportunity to conduct my thesis in her lab and for her unwavering support throughout this journey. I am also deeply thankful to Prof. Nishad Matange, whose guidance and encouragement have been instrumental in my academic growth. I sincerely appreciate the support from NCCR Microbiomes for funding my stay in Switzerland and IISER Pune and University of Lausanne for providing the platform that enabled me to embark on this endeavor.

I extend my heartfelt thanks to Prof. Sara Mitri and Prof. Stefanie Widder for their valuable insights and collaboration. I am also grateful to Massimo Amicone for his constant support, thoughtful suggestions, and encouragement.

A special thanks to my mentor, Margaux Creze, for her patience, guidance, and unwavering support. Her mentorship has been invaluable to my learning and research experience.

Beyond the research, I am truly grateful to my colleagues who made this journey not just productive but also filled with laughter and camaraderie. A huge shoutout to Sandra Ozáez, Xiaobing Wu, and Youzheng Teo for being my lab family—babysitting me through experiments, putting up with my occasional rants, and tolerating my socially awkward moments. I couldn't have asked for better partners in crime. To Julian Garneau, Abdifatah, Aline Altenried, Sarah McHugh, Naika Prince, Simon Yersin, and the entire Vonaesch lab, thank you for making the lab feel like home.

Finally, to my family and my wonderful friends—Daksh Telang, Sanchi Wankhade, Shriyansh Ranjan, and my IISER Pune gang—thank you for being my constants, for cheering me on, and for always believing in me, even when I doubted myself.

Contributions

Contributor name	**Contributor role**
Pascale Vonaesch, Margaux Creze, Sakshi Kharche	Conceptualization Ideas
Pascale Vonaesch, Margaux Creze, Sakshi Kharche	Methodology
-	Software
Pascale Vonaesch, Margaux Creze, Sakshi Kharche	Validation
Sakshi Kharche	Formal analysis
Sakshi Kharche	Investigation
Pascale Vonaesch	Resources
Sakshi Kharche	Data Curation
Sakshi Kharche	Writing - original draft preparation
Pascale Vonaesch, Margaux Creze, Sakshi Kharche	Writing - review and editing
Sakshi Kharche	Visualization
Pascale Vonaesch, Margaux Creze	Supervision
Pascale Vonaesch	Project administration
Pascale Vonaesch	Funding acquisition

Chapter 1 Introduction

1.1 **Bacterial Communities**

The surface of the Earth is home to a vast array of species, with microbial life, particularly bacteria, comprising the majority of the total biomass on the planet (Flemming & Wuertz, 2019; 'Microbiology by Numbers', 2011). While bacteria are abundant as individual species, they rarely exist in isolation in natural ecosystems. Instead, microbes predominantly form intricate communities in which various microbial species coexist and interact. These microbial communities are not only pervasive across the Earth but are also central to the functioning of natural ecosystems, playing a vital role in maintaining ecological balance. Through their participation in numerous biogeochemical processes, such as nutrient cycling, carbon fixation, and nitrogen transformation, microbial communities contribute significantly to the health and stability of ecosystems (Widder et al., 2016).

In addition to their ecological importance, microbes must contend with the challenges posed by their environment in order to survive. This includes adapting to fluctuations in resource availability, environmental stresses, and interspecies interactions. As a result, microbial communities can have profound effects on their surroundings, influencing the health of plants, animals, and even the atmosphere. These interactions, however, are complex and can have both positive and negative consequences. For example, beneficial microbial communities can promote plant growth and protect against pathogens, while harmful microbes may contribute to disease or environmental degradation (Abreu & Taga, 2016). It is essential to note that such effects are predominantly observed in the context of microbial communities and not in isolated species, underscoring the concept of interdependence and division of labour among microbial members (Ibrahim, Raajaraam, & Raman, 2021; Xavier, 2011).

Within microbial communities, there exists a range of dynamic interactions, including facilitation, competition, and antagonism. These interactions are not limited to direct species-to-species interactions but extend to complex networks of cooperation and

competition that can influence the success and stability of microbial community. Despite the critical role of these interactions, the full complexity of how they shape microbial community structure and function is still not fully understood, with many aspects of microbial behaviour and interaction remaining to be elucidated (Stubbendieck, Vargas-Bautista, & Straight, 2016; Grosskopf & Soyer, 2014).

Studying these complex interactions in natural microbial communities presents significant challenges due to their vast diversity, with hundreds of species coexisting in a single environment. This high level of complexity makes it nearly impossible to replicate and analyse such communities under controlled laboratory conditions. To overcome this limitation, researchers have developed synthetic communities, which are simplified versions of natural microbial ecosystems with a drastically reduced number of species (Grosskopf & Soyer, 2014). These synthetic communities are typically examined in well-defined and simplified media, allowing for more controlled experimental conditions. They serve as valuable models for investigating ecological and evolutionary hypotheses within laboratory settings, providing insights into microbial interactions and community dynamics (Xavier et al., 2011).

In this study, we use the Oligo-Mouse-Microbiota (OMM12), a synthetic bacterial community that stably colonizes mice and provides colonization resistance against enteropathogenic infection (Brugiroux et al., 2016). The OMM12 consists of twelve bacterial species: *Enterococcus faecalis* KB1, *Limosilactobacillus reuteri* I49, *Bifidobacterium animalis* YL2, *Clostridium innocuum* I46, *Blautia coccoides* YL58, *Enterocloster clostridioformis* YL32, *Flavonifractor plautii* YL31, *Acutalibacter muris* KB18, *Bacteroides caecimuris* I48, *Muribaculum intestinale* YL27, *Akkermansia muciniphila* YL44, and *Turicimonas muris* YL45. These species represent the five major eubacterial phyla found in the murine gastrointestinal tract of mice (Brugiroux et al., 2016). Interactions among the twelve bacterial members of the OMM12 community have been extensively studied, including *in vitro* analyses conducted by A S Weiss et al. (2021). Their research demonstrated that the OMM12 interaction network is shaped by both exploitative and interference competition when cultured in nutrient-rich media. Furthermore, their findings revealed that shifts in community structure can occur in response to changes in the nutritional environment. Notably, *Enterococcus faecalis* KB1 was identified as a key driver of community composition, significantly influencing the abundance of several other consortium members *in vitro*.

1.2 Antibiotic Resistance

The discovery of antibiotics marked a groundbreaking advancement in medicine, enabling the treatment of infections that were once fatal. However, nearly a century after their introduction, their effectiveness is now in alarming decline (O'Brien, Baumgartner, & Hall, 2021; Radlinski & Conlon, 2018). Antibiotics have been extensively overused across multiple fields, including human and veterinary medicine, as well as agriculture. This widespread misuse has contributed to a significant global health crisis, the rise of antibiotic-resistant pathogens (Bottery, Pitchford, & Friman, 2021). Infections that were once easily treatable have become increasingly difficult to manage, as some bacterial strains no longer respond to conventional antibiotic treatments. This growing challenge has placed immense pressure on the scientific community to better understand and find solutions to antibiotic resistance (Radlinski & Conlon, 2018).

For years, researchers have carefully examined antibiotic resistance in various bacterial strains by isolating them and analyzing their responses to antibiotics (Bottery, Pitchford, & Friman, 2021). One widely used method is the measurement of the minimum inhibitory concentration (MIC), which determines the lowest concentration of an antibiotic required to prevent bacterial growth (Andrews et al., 2001). These findings serve as the foundation for medical treatments. However, recent advances in microbial ecology have highlighted the importance of studying infections within the broader context of microbial communities, where bacteria interact with one another (de Vos et al., 2017). Investigating antibiotic resistance in infections involving multiple bacterial species, alongside a growing understanding of microbial community dynamics, could provide new insights into addressing this pressing issue (Bottery et al., 2021; O'Brien, Figueroa, & Welch et al., 2021).

1.3 Bacterial Communities and Antibiotic Resistance

Historically, the environmental context from which bacteria are isolated has often been overlooked in studies of antibiotic resistance (Bottery et al., 2021). However, recent research into bacterial community interactions has provided new insights into microbial ecology and the evolution of antibiotic resistance. Baumgartner et al. (2020) found that microbial interactions within a community can either promote antibiotic resistance or, conversely, increase antibiotic susceptibility. Their study demonstrated that a focal

Escherichia coli strain was only able to develop antibiotic resistance in absence of its native microbial community, despite the presence of resistance genes in surrounding bacterial species. In contrast, Bottery et al. (2021) observed the opposite effect, where a sensitive *Pseudomonas aeruginosa* strain could only survive in the presence of multidrug-resistant *Stenotrophomonas maltophilia*. These contrasting findings highlight the complexity of bacterial interactions and suggest that the mechanisms underlying antibiotic resistance within microbial communities are not yet fully understood.

One well-established factor influencing antibiotic resistance within bacterial communities is horizontal gene transfer, which enables the exchange of resistance genes between previously susceptible bacteria (Baumgartner et al., 2020). Additionally, antibiotic susceptibility is not solely determined by genetic factors; ecological interactions such as cross-protection can also play a significant role (Bottery et al., 2021). Competitive interactions for space and resources within a microbial community can restrict bacterial population growth, thereby reducing the likelihood of resistant mutants emerging and lowering the overall probability of antibiotic resistance development (Baumgartner et al., 2020). Another key phenomenon is the inoculum effect, in which high bacterial population density decreases antibiotic efficacy (Karslake et al., 2016). For example, in the case of β-lactam antibiotics, a larger bacterial population leads to increased production of β-lactamase enzymes, which detoxify the environment and reduce the antibiotic's effectiveness (Brook et al., 1989).

Beyond competition, cooperative mechanisms among bacteria can also influence antibiotic resistance. *Stenotrophomonas maltophilia*, for instance, has been shown to protect neighboring bacterial species by secreting metallo-β-lactamases, which neutralize antibiotics in the surrounding environment (Bottery et al., 2021). Additionally, biofilm formation is known to enhance bacterial tolerance to antibiotics, further complicating treatment strategies (Høiby et al., 2010).

Given the complexity of bacterial interactions and their influence on antibiotic resistance, it is essential to investigate these dynamics in controlled experimental settings. Thus, in this project, we aim to characterize the evolution of antibiotic resistance in monocultures and coculture of the strains from the OMM12 community. This study builds on the foundational work of Philipp Münch, who investigated the evolution of OMM12 in mice under transient antibiotic exposure (Münch et al., 2023). In addition, we will integrate data from Anna Weiss on microbial interaction networks

within the OMM12 community to inform our analysis (A S Weiss et al., 2023). Our primary objective is to directly compare *in vitro* data generated from our experiments with the available *in vivo* data, with a particular focus on how bacteria respond to antibiotic pressure differently when grown as a community versus when they are grown separately. Conducting these experiments *in vitro* allows us to test a wider array of conditions and provides greater control over variables compared to *in vivo* studies.

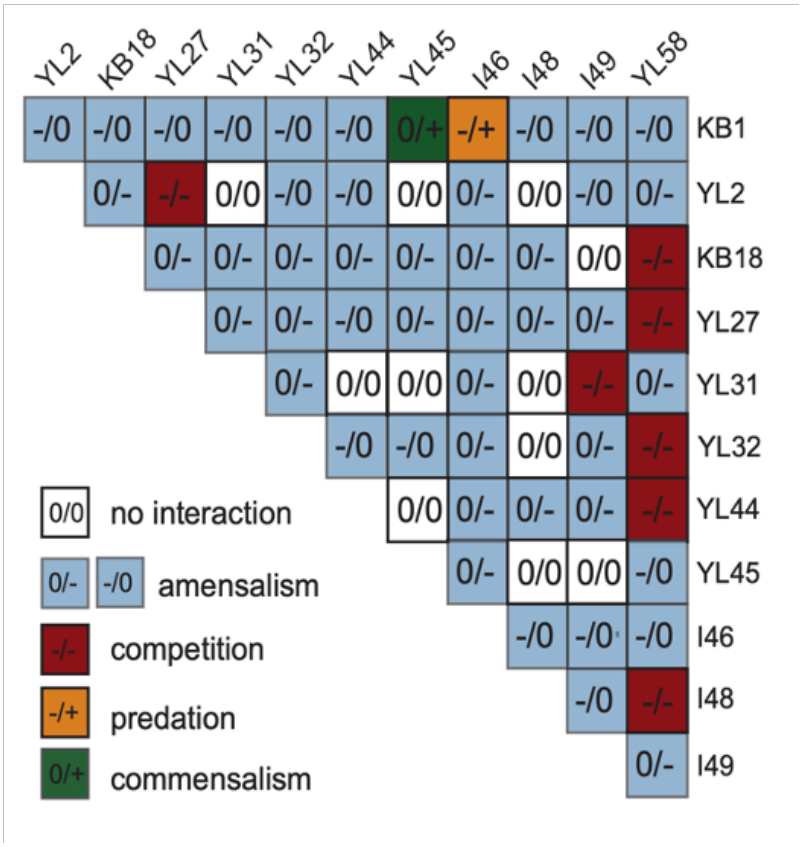

***Figure 1**: Pairwise interactions were determined by comparing the absolute abundance of each strain in co-culture versus monoculture. Interactions were classified as positive (+) if abundance significantly increased, negative (–) if it decreased, and neutral (0) if no significant change was observed (t-test, $p < 0.05$). (A S Weiss et al, 2021).*

1.4 Central Questions and Aims

This project aims to investigate the evolution of antibiotic resistance in the three bacterial strains out of twelve that make up the Oligo-Mouse-Microbiota (OMM12) community. Specifically, we will compare the development of antibiotic resistance when these strains are grown individually in monoculture versus in a complex community setting. A key focus will be to identify and characterize stable genetic changes that arise under different environmental conditions, particularly those leading to changes in antibiotic resistance. These changes will be quantitatively assessed

using minimum inhibitory concentration (MIC) measurements, a standard approach for evaluating bacterial susceptibility to antibiotics. The three focal strains selected for this study are *Enterococcus faecalis* KB1, *Clostridium innocuum* I46, and *Bacteroides caecimuris* I48. The first two were chosen because Philipp Münch isolated them after *in vivo* evolution and conducted detailed analyses of their antibiotic resistance, allowing for a direct comparison with our results. *Bacteroides* species are known for their rapid evolution (Dapa & Xavier et al., 2022), making *Bacteroides caecimuris* I48 a valuable third focal strain. Additionally, all three strains exhibit faster growth compared to other community members, minimizing the likelihood of their loss during the evolutionary process.

One of the central hypotheses of this study is that bacterial species influence each other's population dynamics when cultured together, which in turn may affect their likelihood of evolving resistance mechanisms. For instance, I hypothesize that a species that experiences enhanced growth within a community will have a larger effective population size in co-culture compared to monoculture, increasing the turnover rate resulting in higher mutation rate which increases probability of gaining resistance. Conversely, species that grow more poorly in the community than in monoculture may experience reduced selective pressure, potentially lowering the likelihood of resistance acquisition.

A second hypothesis focuses on the role of microbial interactions, such as competition and facilitation, in shaping antibiotic resistance. I predict that species experiencing strong competition within the community will face higher selective pressure and thus evolve higher antibiotic resistance, as measured by MIC values. In contrast, species that benefit from facilitation, where other community members help detoxify the environment, may develop lower resistance in co-culture and exhibit higher MIC values only in monoculture, where they lack these protective interactions.

1.5 **Experimental design**

To test these hypotheses, we will conduct an evolutionary experiment where the OMM12 strains are grown under selective antibiotic pressure in two distinct conditions: monoculture and co-culture. The bacterial strains will be cultured in permissive media to allow for optimal growth, and evolution will proceed over multiple generations through serial passaging. Each strain and community will be exposed to a panel of

clinically relevant antibiotics. Initially, we will focus on vancomycin, widely used in medical settings. However, the broader experimental framework includes additional antibiotics such as ciprofloxacin, tetracycline, and ampicillin and metronidazole, each of which is frequently employed in human medicine and has been selected to mirror the experimental design of Philipp Münch's research.

During the evolutionary process, bacterial samples will be periodically frozen in glycerol stocks, ensuring that ancestral and intermediate strains are preserved for further analysis. This will enable us to retrospectively examine evolutionary trajectories and verify resistance mutations that arise over time. To assess bacterial viability and population structure, we will perform Propidium mono-azide (PMA) staining followed by quantitative PCR (qPCR), allowing us to distinguish live and dead cells and estimate population sizes across different experimental conditions. This step is critical for determining whether changes in antibiotic resistance correlate with shifts in bacterial abundance and survival dynamics.

At the end of the experiment, MICs will be measured for all evolved focal strains to quantify changes in antibiotic susceptibility. Additionally, whole-genome sequencing will be performed to identify specific genetic adaptations that have emerged in response to antibiotic selection. This genomic analysis will allow us to pinpoint key mutations or horizontal gene transfer events that contribute to resistance development. By comparing genetic changes across monoculture and community settings, we can assess the extent to which microbial interactions influence the pathways through which resistance evolves.

1.6 **Expected outcomes**

This research will provide valuable insights into the role of microbial communities in shaping antibiotic resistance dynamics. By systematically comparing resistance evolution in isolated strains versus complex microbial consortia, we aim to uncover novel mechanisms by which interspecies interactions modulate bacterial adaptation to antibiotics. Understanding these processes is critical, as bacterial infections rarely occur in isolation rather, they develop within polymicrobial communities where species interactions can significantly alter disease progression and treatment efficacy.

Ultimately, the findings from this study could contribute to the development of more effective antimicrobial strategies that account for microbial ecology. If bacterial interactions are found to significantly impact resistance evolution, this knowledge could inform clinical interventions, such as combination therapies designed to exploit competitive dynamics within bacterial communities. By bridging the gap between microbial ecology and antibiotic resistance research, this project has the potential to advance our understanding of how bacterial populations adapt to selective pressures and offer new perspectives on combatting antibiotic resistance in clinical and environmental settings.

As part of my Master's thesis, I will focus on conducting one full evolutionary experiment using vancomycin as the primary antibiotic. This work is embedded within a larger PhD research project conducted by Margaux Crézé, contributing to a broader investigation into bacterial evolution and resistance mechanisms.

Chapter 2 Materials and Methods

2.1 Strains and bacterial cultures

Strains:

1.	*Bifidobacterium animalis* YL2	DSM26074
	Muribaculum intestinale YL27	DSM28989
3.	*Flavonifractor plautii* YL31	DSM26117
4.	*Enterocloster clostridioformis* YL32	DSM26114
5.	*Akkermansia muciniphila* YL44	DSM26127
6.	*Turicimonas muris* YL45	DSM26109
7.	*Blautia coccoides* YL58	DSM26115
8.	*Clostridium innocuum* I46	DSM26113
9.	*Bacteroides caecimuris* I48	DSM26085
10.	*Limosilactobacillus reuteri* I49	DSM32035
11.	*Enterococcus faecalis* KB1	DSM32036
12.	*Acutalibacter muris* KB18	DSM26090

Storage Conditions: All the strains were stored in sealed glass anaerobic vials and PCR strips with 40% glycerol in PBS and Palladium beads (Reducing catylyser) at -80°C.

Media used: AF media was used to carry out all the experiments, taken from [14]. Refer to supplementary data (Table 4) for composition.

Strain rehydration and cultures conditions: For the rehydration of the strains received from DSMZ, the procedure carried out was as follows: the tip of the ampoule was broken inside the anaerobic cabinet. Before this, the ampoule had been heated in the burner flame outside the anaerobic chamber and then quickly placed in ice to induce cracks. Once inside the anaerobic cabinet, the outer ampoule was carefully broken using tweezers, and the wool plug was removed. The inner glass tube was taken out, and the outer ampoule was discarded. The cotton plug was removed, 600 µL of 1x reduced PBS was added to resuspend the pellet, and the plug was replaced. A volume of 200 µL of the cell suspension was transferred into 10 mL of AF media, and another 200 µL was transferred into 10 mL of BHI media. The remaining 200 µL of cells was added to 300 µL of glycerol with palladium (Pd) black beads in an

anaerobic glass vial. The media tubes were placed in a 37°C incubator until the cultures became cloudy, and the anaerobic vials were stored immediately in a -80°C freezer. After 48h of incubation, 750 µL of the liquid cultures was added to anaerobic vials containing 40% glycerol and Palladium beads. The vials were then frozen at -80°C for long-term storage.

Strains were inoculated from glycerol stocks (loop full) into 10 ml of AF media and allowed to grow for 48 hours in an anaerobic chamber (7% H_2, 10% CO_2, 83% N_2) at 37°C. Following this incubation period, a 1% transfer was made to fresh 10ml AF media (100ul in 10ml), and the cultures were again allowed to grow for an additional 48 hours which then were used for further experiments.

2.2 Bacterial growth assay

For the growth curve assay, cultures were diluted in AF media to an initial OD_{600nm} of 0.01 and added to 2ml of total AF media in a 24 well plate separately. After inoculation, the plates were covered with a non-sticky adhesive transparent film (Thermo-fisher scientific 4311971) and 3 holes were punched for gas exchange using a syringe needle (0.31mm thick). Growth curves were then recorded using the Epoch2 plate reader in an anaerobic chamber. Before each OD measurement, a double orbital shake was performed for 10 seconds, and OD_{600nm} was measured with a total experiment time of 48 hours and 1-hour intervals between measurements and the temperature was maintained at 37°C throughout the experiment using an established program on the Gene5 3.11 software. Finally, the results were exported and saved in an Excel file for plotting the growth curves.

2.3 Minimum inhibitory concentration (MIC) determination

2.3.1 MIC determination in liquid culture

To measure minimal inhibitory concentrations for the given antibiotics and each strain, we normalized each strain to an OD_{600nm} to 0.05. For media preparation, the volume of antibiotic for the highest concentration (5mg/ml) wells was calculated using the equation $C1 \times V1 = C2 \times V2$ (C1 and V1 refers to the initial concentration and volume of antibiotic whereas C2 and V2 refers to final concentration and volume respectively), where V1 was determined as $V1 = C2 \times V2 / C1$. The highest concentration wells received

200 - V1µL of fresh media, followed by V1µL of antibiotic solution, ensuring a total volume of 200 µL per well. In the other wells, 180 µL of fresh media was added, and a serial dilution (1:10) was performed by transferring 20 µL from the most concentrated wells to the least concentrated wells. In the control well, 200 µL of fresh media without antibiotics was added. Controls included sterile medium, media with antibiotic of same concentrations as wells with bacteria, and media with bacteria only with OD_{600nm} of 0.05. The appropriate volume of bacteria was inoculated into the wells and growth curves were recorded using the BioStak 4 and Epoch2 in an anaerobic chamber. The same program and settings were used which were used to record the growth curves above.

2.3.2 MIC determination with E-test

To assess the MIC of vancomycin on the ancestral strains and on the evolved populations, we performed E-test. It consists of a strip showing a gradient of increasing antibiotic concentration, and it is placed on an agar plate. To do so for the ancestral strains, they have been overnight cultured from the glycerol stock at -80°C. Then, by dipping a sterile swab in the overnight culture, the bacteria are streaked and distributed on a AF agar plate; right after, an E-test strip is placed at the center of the agar surface. The plate is then incubated at 37°C for 48 hours in anaerobic conditions. For the evolved population, we either took samples from the -80°C evolved stocks or we picked a colony from the plates streaked with evolved population. We then regrew the bacteria by a re-streak on AF agar, and an overnight culture in 10mL of AF media was afterwards performed. The following morning, we dipped a swab in the overnight culture and spread it on AF agar plates in three directions to be sure the whole plate is covered. Then, the antibiotic strip is carefully disposed in the middle of the AF agar surface. Finally, the plates are incubated at 37°C for 48 hours and the results can be read thanks to the killing zone.

2.4 Maxwell gDNA Extraction

We used the Maxwell® RSC PureFood GMO and Authentication Kit from Promega (AS1600) and the RSC 48 Maxwell Extractor (robot) to extract genomic DNA from the 12 bacterial strains (OMM12 members) . Initially, 1.5 ml of each bacterial culture was transferred to a 2 ml Eppendorf tube and centrifuged at 14,000 g for 2 minutes, after

which the supernatant was removed without disturbing the pellet. 1 mL of CTAB buffer (Promega, MC1411) was added to resuspend the pellets in the Eppendorf tubes. For cell lysis, 1 ml of the samples (with CTAB buffer) was placed in a 2 ml PowerBead Pro tube (Qiagen, 19301), incubated at 95°C for 5 minutes, and subjected to bead beating on a Vortex-Genie 2 mixer at maximum speed for 10 minutes. To precipitate cell debris, the samples were centrifuged at 14,000 x g for 10 minutes, and approximately 600 µL of the supernatant was transferred into new Eppendorf tubes. For protein and RNA degradation, 40 µL of Proteinase K (Promega, MC500C) and 20 µL of RNase A(Promega, A797C) were added to the samples, which were vortexed to mix. The samples were incubated at 70°C for 10 minutes. During this incubation, cartridges were prepared by placing them in the RSC cartridge rack and removing the foil seals. One hundred microliters of elution buffer (Promega, MC501C) were added to the supplied elution tubes, which were then placed in the cartridge rack, and plungers were inserted into the 8th well of the cartridge. Three hundred microliters of lysis buffer (Promega, A828D) were added to well 1 of the cartridges. Finally, the samples were centrifuged for 5 minutes at maximum speed and three hundred microliters of the supernatant were then added to well 1 of the cartridges. The Maxwell RSC was run using the PureFood Protocol, with DNA being eluted in 100 µL, quantified with NanoDrop and stored at 20°C for future use.

2.5 Full-length 16S rRNA gene sequencing

To identify bacterial isolate, we used 16S rRNA full length gene sequencing. To this purpose**,** genomic DNA (gDNA) was extracted from the bacteria using the Maxwell RSC PureFood GMO and Authentication Kit as described above. The PCR master mix was prepared as shown in the table below. Master Mix and 0.5 ul gDNA or water was added in samples and for the negative control respectively, followed by a brief spin to mix.

Firepole PCR Master mix 5x	4uL
Forward primer 27f 100uM (VL041)	0.2uL
Reverse primer 1429r 100uM (VL042)	0.2uL

Nuclease free water	15uL
Total	19.4ul
Total per reaction	19.4uL

The PCR was then run in the Thermocycler using the program below:

cycles	**step**	**temp**	**time**
1x	Initial Denaturation	95°C	3 Min.
35x	Denaturation	95°C	30 Sec.
	Annealing	**50°C**	30 Sec.
	Polymerization	72°C	90 Sec.
1x	Final Polymerization	72°C	7 Min.
1x	Hold	10°C	∞

For gel visualization of the PCR product, a 1% agarose gel was prepared by mixing 100 mL of Tris-acetate-EDTA (TAE)1X with 1 g of agarose and heating until dissolved. This mixture was poured into the gel station and allowed to cool for a few seconds before adding 10 µL of SYBR Safe (Invitrogen, S33102) and mixing with the comb. The comb was placed into the gel and allowed to set for 20-30 minutes. The gel running station was assembled, and TAE buffer was poured in to cover the gel. A mixture of 3 µL of PCR product and 1 µL of loading dye was prepared and the 4 µL, were loaded into each well. Additionally, 4ul of GeneRuler 1Kb DNA ladder (Thermo Fisher, SM0311) was added to a neighboring well. The gel was run at 135 V for 20 minutes, and visualized with GelDoc to check for a band at the desired size (approximately 1400 bp for the 16S gene). For PCR clean-up (Wizard SV Gel and PCR Clean-Up System, A9282) an equal amount of membrane binding solution (20 µL) was added to the PCR tubes containing the product and mixed by pipetting. The mixture was transferred onto a column mounted on a collecting tube and incubated at room temperature for 1 minute before centrifuging at 16,000 g for 1 minute. The flow-through was discarded, and 700 µL of membrane wash buffer was added before spinning the sample at 16,000 g for another minute. The flow-through was again discarded, and the sample was washed a second time with 500 µL of membrane wash buffer, followed by another spin and discarding of the flow-through. The sample was spun again for 1 minute to ensure the membrane was completely dry. Then, 50 µL of

provided nuclease-free water was added directly onto the membrane and incubated for 2 minutes at room temperature to elute the DNA. The column was placed in an Eppendorf tube, and the sample was spun down at 16,000 g for 1 minute to elute the DNA. The tube was labeled, and the DNA sample was stored at 4°C or -20°C for long-term storage. The DNA concentration was measured using a Nanodrop, with the provided nuclease-free water used as a blank, and 12 µL of samples at a concentration of 30-50 ng/µL were sent for 16S rRNA sequencing to Microsynth (commercial provider).

2.6 Live/dead bacterial staining

2.6.1 Propidium Monoazide (PMA) Staining and qPCR

To stain for the proportion of viable and dead bacteria in a sample, we used Propidium Monoazide, PMAxx™ (40069) and a protocol that was adapted from [15]. Four Eppendorf tubes labeled A, B, C, and D were prepared for each strain to be tested. Aliquots of 400 µL of bacterial culture were pipetted into each Eppendorf tube. Half of the population (Eppendorf C and D) was subjected to a heat shock at 90°C for 15 minutes in a hot bath to induce cell death, while Eppendorf A and C remained untreated. The following protocol was performed entirely within an anaerobic chamber. Two Eppendorf tubes were prepared per strain (one with PMA and one without), and 400 µL aliquots of bacterial culture were pipetted into the tubes, resulting in a total of 12 monocultures. Working in low light conditions, the appropriate volume of PMA stock was added to achieve a final concentration of 50 µM (e.g., 1 µL of a 20 mM stock in 400 µL). The tubes were incubated in the dark for 10 minutes at room temperature, with tubes covered in foil to avoid the tubes being exposed to light. After the incubation, the samples were exposed to light for 15 minutes in Biotium's PMA-Lite™ LED Photolysis Device (E90006) to cross-link PMA to DNA. The cells were then pelleted by centrifugation at 5,000 x g for 10 minutes, and the supernatant was removed carefully to avoid disturbing the pellet. DNA extraction was conducted using the Maxwell protocol, after which the extracted DNA could either be frozen or used for qPCR with specific 16S rRNA primers for each strain. For qPCR, universal 16S primers 1492r and 27f were utilized for controls. A mix of specific forward and reverse primers from stock at 100 µM was prepared at 4 µM each, consisting of 920 µL of H_2O, 40 µL of forward primer, and 40 µL of reverse primer. A stock of 5 ng/µL DNA was made for each condition in a 1 mL Eppendorf tube by using the equation C1V1=C2V2

with DNA and nuclease-free water. In the 384-well plate, the following quantities were added to each well (15 µL total): 7.6 µL of SYBR Green (Thermofisher, 4367659, light-sensitive), 1.4 µL of the primer mix (forward + reverse) at 4 µM each, and 6 µL of gDNA at 5 ng/µL or directly from the DNA extraction tube. The plate was sealed with a sticky film (Thermo-fisher scientific 4311971), and the qPCR settings for OMM12 were configured as follows: an initial step at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, with the ramp speed set to standard. We also carried out the same protocol using the Promega Viability PCR Crosslinker Kit (CS333003) according to manufacturer instructions.

2.6.2 Flow cytometry

To initiate the experiment, 0.5 mL of evolved bacterial cultures were prepared per each sample. Propidium Iodide (PI) was used at a final concentration of 5 µg/mL, and SYBR Green was diluted from a 200X stock solution to a 1X final concentration to stain dead and total load of bacteria respectively. A volume of 2.5 µL of PI was transferred into each 0.5 mL sample, followed by the addition of 2.5 µL of SYBR Green (200X stock solution). The samples were incubated in the dark at room temperature for 15 minutes, followed by three washing steps with 1 mL of filtered PBS, with centrifugation at 10,000 × g for 2 minutes after each wash. After the final PBS wash, 200 µL of 4% PFA was added to each sample, followed by incubation on cold dry ice for exactly 30 minutes to fix the bacteria. The samples were then washed three times with PBS (centrifugation, removal of PBS, and resuspension in fresh PBS) and finally resuspended in 1 mL of 0.22 µm-filtered PBS, stored at 4°C in the dark until further analysis. Flow cytometry was performed using FITC (wavelength, 525 nm) and PI (wavelength, 620 nm) lasers to detect SYBR Green and PI staining.

For flow cytometry gating, control samples consisting of a live bacterial population, a heat-killed bacterial population, and samples stained with HADA, RADA, and non-stained controls were analyzed. Live and heat-killed bacterial populations were mixed in various proportions to optimize the gating strategy for distinguishing live and dead bacteria within the sample. CytoFLEX (Beckman Coulter) flow cytometer was used to carry out flow cytomtery.

2.7 Adaptive laboratory evolution

To initiate the experiment, bacterial strains were revived from glycerol stocks (100 µL of bacteria in 20% glycerol) and inoculated into 10 mL AF media in three replicates. AF media was degased overnight inside the anaerobic chamber prior to the experiment. Cultures were incubated anaerobically at 37°C. To ensure all strains were in the exponential phase before the experiment, subculturing was performed at different time points depending on their growth rates.

Strain	Time to reach exponential phase (hrs)
Bifidobacterium animalis YL2	6-8
Muribaculum intestinale YL27	3-4
Flavonifractor plautii YL31	8-10
Enterocloster clostridioformis YL32	8-10
Akkermansia muciniphila YL44	24-26
Turicimonas muris YL45	24-26
Blautia coccoides YL58	8-10
Clostridium innocuum I46	10-12
Bacteroides caecimuris I48	15-16
Limosilactobacillus reuteri I49	24-26
Enterococcus faecalis KB1	3-4
Acutalibacter muris KB18	24-26

The experiment was conducted in two conditions: one condition with antibiotics (vancomycin) and other condition without antibiotics. The 24-well plate contained three monocultures (*Enterococcus faecalis* KB1, *Clostridium innocuum* I46, and *Bacteroides caecimuris* I48) with three replicates each (9 wells), and a coculture well with three replicates (3 wells) with vancomycin and without vancomycin. To standardize the initial bacterial concentrations, OD_{600}nm measurements were taken, and the bacterial volume ($V_{bacteria}$) required for an OD_{600}nm of 0.05 in 2 mL of media was calculated.

For monocultures, (2000 - $V_{bacteria}$) µL of AF media was added to each well, while for cocultures, (2000 - 12 × $V_{bacteria}$) µL of AF media was used. The calculated bacterial volume was then added to each well. To prevent evaporation, water was added to the chamber of each plate, and the plates were incubated at 37°C, with OD_{600} measurements taken every hour.

After two days, the first 1:10 transfer was performed by preparing fresh 24-well plate containing 1980 µL of AF media with or without vancomycin per well based on the condition (Figure 1). A 20 µL transfer of culture from initial passage was added to the plate with fresh AF media, maintaining the same design throught the experiment. Vancomycin was introduced at the concentration of 0.5 µg/µL (Evolution 1) and 10 µg/µL (Evolution 2) , ensuring that all strains were subjected to selective pressure for an additional two days. To facilitate recovery in the absence of antibiotic pressure, cells underwent another 1:10 transfer into fresh media, allowing for nutrient replenishment and antibiotic dilution. These steps were repeated, with adjustments in the number of transfers and duration per step based on observations made during the evolution process. Over the course of the experiment, seven transfers were conducted, leading to a total of eight passages.

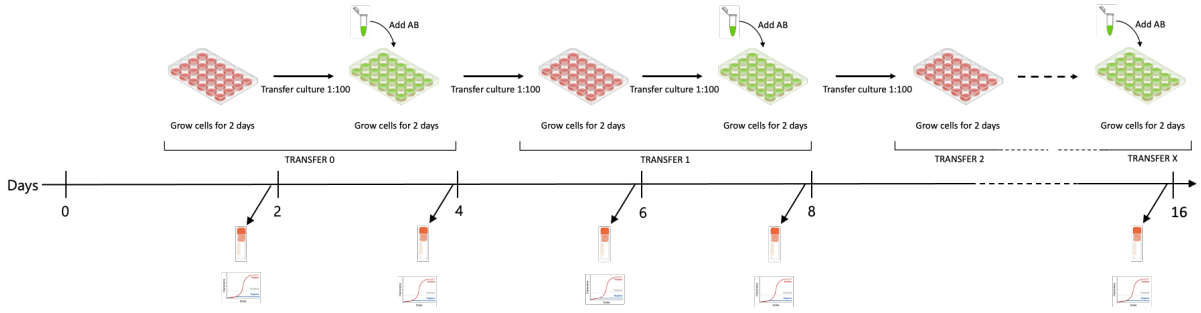

***Figure 2**: Design of the Adaptive Laboratory Evolution.*

At each transfer step, glycerol stocks were prepared by adding 100 µL of 40% glycerol (prepared with PBS and Pd black) to labeled cryotubes, followed by 100 µL of bacterial culture, which was mixed thoroughly and stored at -80°C for future analyses. Additionally, after each transfer, PMA staining followed by qPCR was conducted to assess live and dead populations in cocultures, while flow cytometry using SYBR Green and Propidium Iodide staining was performed to distinguish live and dead bacteria in monocultures. At the end of the experiment, the MIC was determined using an E-test for monocultures and for the strains isolated from cocultures to monitor the development of antibiotic resistance throughout the evolution process.

2.8 Isolation and Identification of evolved bacteria

Bacteria were isolated from both monocultures and cocultures by streaking samples from the -80°C glycerol stocks, preserved during the evolution process, onto AF agar plates under anaerobic conditions. After 48 hours of incubation at 37°C, single

colonies were picked and re-streaked onto fresh AF agar plates. For monocultures, three colonies per sample were selected and incubated for another 48 hours under the same conditions. After this incubation, one colony per plate was picked and inoculated into 10 mL of fresh AF media, where it was allowed to grow for 48 hours under anaerobic conditions. Following this, glycerol stocks were prepared, and an E-test was performed to determine the MIC of the isolated strains.

For cocultures, the same procedure was followed; however, ten distinct colonies were selected based on morphological differences and re-streaked on AF agar plates. After growth, these colonies were inoculated into liquid AF media, and glycerol stocks were prepared. To identify the strains, Matrix-Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) was performed.

For MALDI-TOF grid plate preparation, a single bacterial colony was picked using a sterile toothpick. If a bacterial suspension was used, the cells were pelleted by centrifugation, the supernatant was removed, and the bacterial pellet was used in place of a colony. The sample was first spread onto a target spot on the MALDI grid plate, and using the same toothpick, a portion of the bacterial pellet was transferred to an adjacent target spot to create a thinner layer. This process was repeated for all designated target spots. To facilitate protein extraction, 1 µL of 70% formic acid was added to each spot and allowed to dry for approximately one minute. The matrix solution was prepared by dissolving one tube of α-cyano-4-hydroxycinnamic acid (HCCA) in 250 µL of Bruker standard solvent, ensuring thorough resuspension. Subsequently, 1 µL of the freshly prepared matrix was applied to each dried target spot and left to air-dry, upon which the spots turned yellow. Once dried, the plate was taken for MALDI-TOF mass spectrometry analysis and strains were identified. Following strain identification, an E-test was conducted to determine the MIC of each strain.

2.9 Co-culture Preparation and vancomycin treatment assay

OMM12 strains cultures were inoculated and sub-cultured as described above, followed by normalization to an optical density (OD_{600}) of 0.5. The normalized cultures were then mixed to assemble the background community in separate tubes. A volume of 800 µL of the background community was added to each well of a 24-well plate, followed by the addition of 200 µL of *Enterococcus faecalis* KB1, resulting in a total of

1000ul bacterial suspension. Subsequently, 1,000 µL of AF medium was added to bring the final volume to 2 mL. Control conditions included wells containing only the background community and wells with *E. faecalis* KB1 alone. Cultures were incubated anaerobically at 37°C for 48 hours. After the incubation period, 100 µg/mL of vancomycin was added to each well. Optical density (OD_{600}) measurements and viability assessments using live/dead staining with propidium mono-azide dye were performed at 0 hours (immediately before antibiotic addition) and at 1, 3, 8, and 24 hours post-treatment to evaluate bacterial survival and the impact of vancomycin exposure.

2.10 Data analysis

2.10.1 Relative and absolute abundance

The relative abundances of bacterial strains were determined using quantitative PCR (qPCR) data. qPCR was performed using strain-specific primers targeting OMM12 bacteria and universal 16S rRNA gene primers to quantify the total bacterial load. The relative abundance of each strain in a sample was calculated using the ΔCt method, where ΔCt represents the difference between the Ct value obtained with strain-specific primers and the Ct value obtained with universal 16S rRNA primers. The relative abundance was then computed using the formula 2^(-ΔCt), providing a normalized estimate of each strain's proportion within the total bacterial community.

Absolute abundances were calculated by multiplying the relative abundances by the total DNA concentration (ng/µL), which was used as a proxy for total biomass. This approach allowed for the estimation of the absolute abundance of each strain within the community based on its relative representation and the overall microbial load in the sample.

2.10.2 Flow cytometry

Flow cytometry data were analysed using the CytoFLEX standard program. Samples were stained with SYBR Green and propidium iodide (PI) to distinguish total and dead cells, respectively. Unstained control samples were used to define the background signal by selecting the region containing cells in the unstained samples; any events

outside this region were considered stained cells (Figure 3). This gating strategy was applied to both FITC and PI channels, where FITC fluorescence indicated SYBR Green-stained total cells, and PI fluorescence indicated propidium iodide-stained dead cells. The total and dead cell counts were then determined and used for further data analysis and visualization.

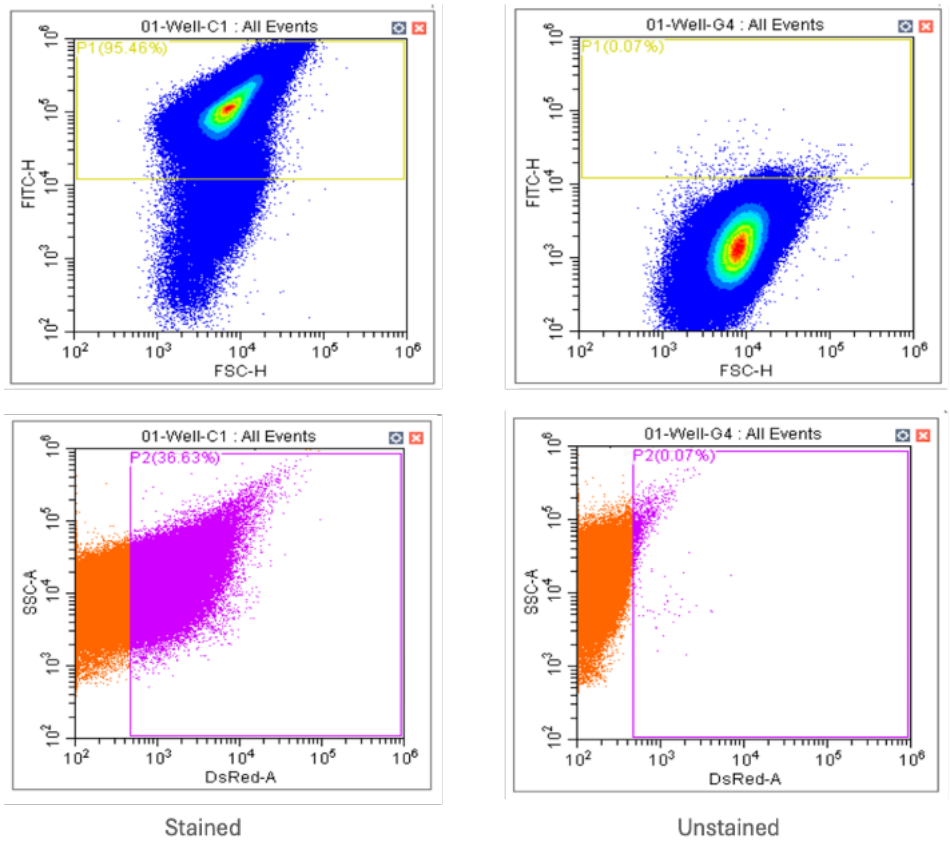

***Figure 3**: Representative image from the CytoFLEX program showing a stained sample (left) and an unstained sample (right), stained with SYBR Green and PI. Detection was performed using the FITC channel (525 nm) for SYBR Green and the PI or Ds-Red channel (620 nm).*

Chapter 3 Results

3.1 *Enterococcus faecalis* exhibits growth autonomy within different microbial consortia

Anna Weiss's study on the OMM12 community highlighted the intricate interactions among its members, identifying *Enterococcus faecalis* KB1 (referred to from now on as *E. faecalis*) as a keystone species that exerts a significant influence on the growth and behavior of other bacteria. However, her experiments were conducted in pairwise co-cultures, leaving open the question of how *E. faecalis* behaves in more complex microbial assemblies. Building on her findings, we sought to investigate *E. faecalis*'s dynamics within a more diverse background community, particularly in the presence of the antibiotic vancomycin, which selectively targets Gram-positive bacteria and has significant clinical relevance due to the rise of vancomycin-resistant enterococci (VRE) (O Ayobami & N Willrich 2011). By incorporating multiple community members, we aimed to determine whether their presence provides *E. faecalis* with any protective advantage such as cross-protection or alters its resilience under antibiotic stress.

Community	Bacterial Strains
C1	*Clostridium innocuum (I46), Akkermansia muciniphila (YL44), Bifidobacterium longum subsp. animalis (YL2), Enterocloster clostridioformis (YL32)*
C2	*Blautia pseudococcoides (YL58), Bacteroides caecimuris (I48), Flavonifractor plautii (YL31), Muribaculum intestinale (YL27)*
C3	*Clostridium innocuum (I46), Bifidobacterium longum subsp. animalis (YL2), Blautia pseudococcoides (YL58), Flavonifractor plautii (YL31)*
C4	*Akkermansia muciniphila (YL44), Enterocloster clostridioformis (YL32), Bacteroides caecimuris (I48), Muribaculum intestinale (YL27)*
C5	*Clostridium innocuum (I46), Akkermansia muciniphila (YL44), Blautia pseudococcoides (YL58), Bacteroides caecimuris (I48)*
C6	*Bifidobacterium longum subsp. animalis (YL2), Enterocloster clostridioformis (YL32), Flavonifractor plautii (YL31), Muribaculum intestinale (YL27)*
C7	*Clostridium innocuum (I46), Enterocloster clostridioformis (YL32), Blautia pseudococcoides (YL58), Muribaculum intestinale (YL27)*
C8	*Akkermansia muciniphila (YL44), Bifidobacterium longum subsp. animalis (YL2), Bacteroides caecimuris (I48), Flavonifractor plautii (YL31)*

Table 1: The table shows the bacterial strain compositions of eight background communities (C1–C8), each consisting of four strains. Strain names are listed with their respective identifiers in parentheses

To investigate the impact of vancomycin on *E. faecalis* in the presence of a microbial community, we designed an experiment incorporating *E. faecalis* alongside a background community. Certain strains were excluded due to experimental constraints; *Acutalibacter muris* (KB18) and *Limosilactobacillus reuteri* (I49) were omitted due to their slow growth. Additionally, *Turicimonas muris* (YL45) exhibited a translucent appearance, making it difficult to quantify bacterial cells using OD-based measurements (Figure 1F). We finally opted to include *E. faecalis* in background communities with four other bacterial strains from the OMM12 (Oligo-Mouse Microbiota) community. Eight different community configurations were established, each consisting of *E. faecalis* and four background strains (Table 1). The concentration of vancomycin was set at 100 µg/mL, approximately ten times the minimum inhibitory concentration (MIC) of *E. faecalis* KB1, which ranges between 5–10 µg/mL to see an immidiate response.

Optical density (OD) measurements of *E. faecalis* within different microbial communities (C1–C8) showed comparable overall growth patterns across configurations (Figure 4A). The OD of the background communities alone followed a distinct trajectory compared to those co-cultured with *E. faecalis.* Additionally, the initial OD before addition of vancomycin of each background community was higher when grown alone compared to when *E. faecalis* was present (Figure 4A-B).

At one-hour post-vancomycin treatment, a decrease in OD was observed for microbial communities co-cultured with *E. faecalis* (Figure 4C). However, OD remained stable or showed signs of recovery over time. A similar trend was observed in the background communities grown alone (Figure 4D). In contrast, *E. faecalis* grown in monoculture did not exhibit any initial decrease in OD following vancomycin treatment. Instead, OD increased slightly until three hours post-treatment, followed by a slight decrease at the eight-hour time point, before recovering again by 24 hours (Figure 4E).

Relative and absolute abundances of *E. faecalis* KB1 within the background communities were measured at multiple time points following vancomycin treatment. The temporal trends in both relative and absolute abundance closely paralleled the OD trajectory of *E. faecalis* grown in monoculture, with an initial stability or slight increase, followed by a minor decline at 8 hours, and subsequent recovery by the 24-hour time point (Figure 4E, G-H).

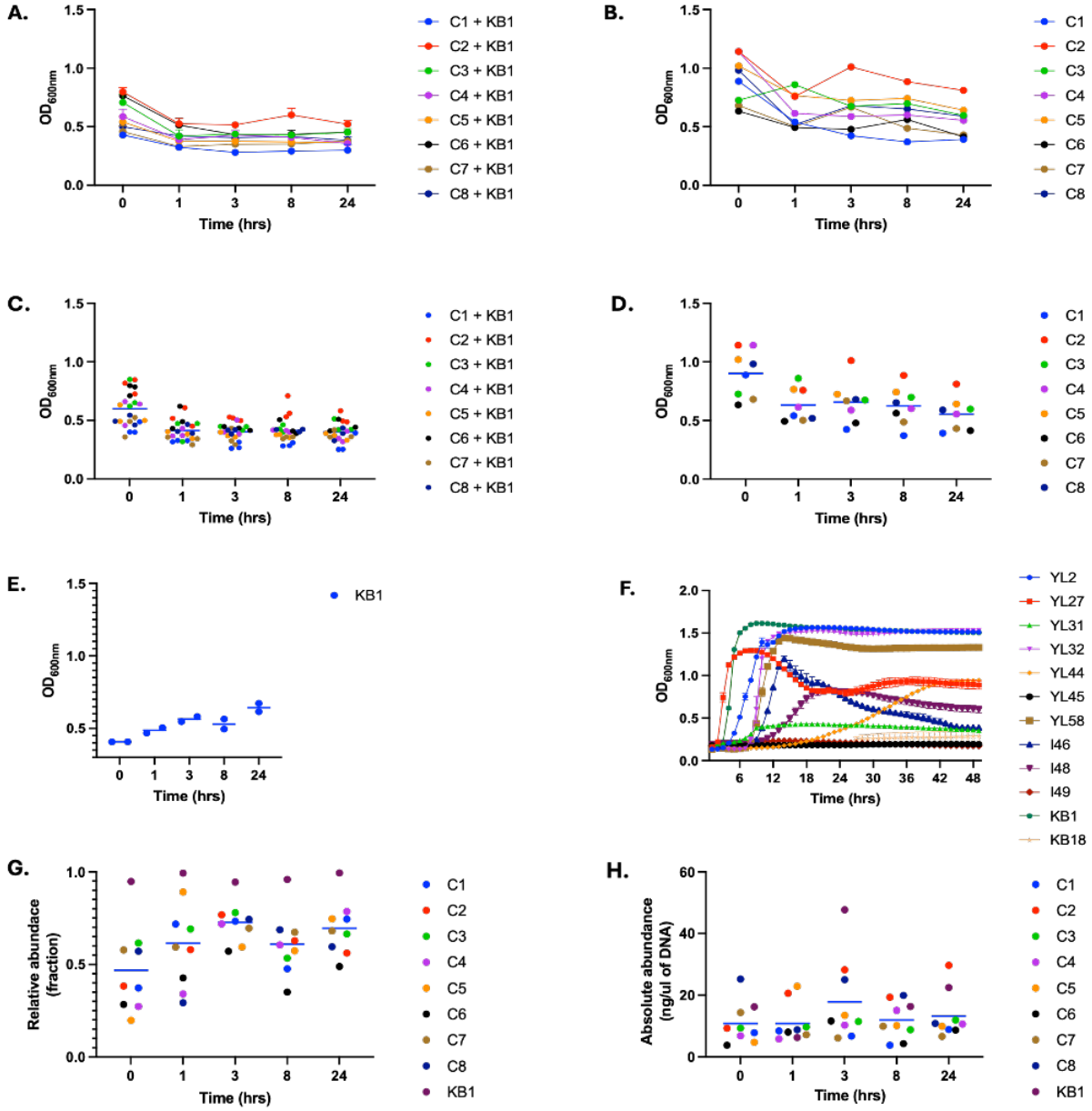

Figure 4: *Optical density (OD) measurements, growth curves, and* E. faecalis *KB1 abundance in microbial communities post-vancomycin exposure. (A) OD measurements of background microbial communities co-cultured with* E. faecalis *KB1 at different time points post-vancomycin exposure, represented as line graphs. (B) OD measurements of background communities grown without E. faecalis KB1 at different time points post-vancomycin exposure, represented as line graphs. (C) OD measurements of background communities co-cultured with KB1 at different time points post-vancomycin exposure, represented as a scattered point plot with grand mean. (D) OD measurements of background communities grown without E. faecalis KB1, represented as a scattered point plot with grand mean. (E) OD measurements of E. faecalis KB1 grown in monoculture at different time points post-vancomycin exposure. (F) Growth curves calculated for individual OMM12 community strains. (G) Relative abundance of E. faecalis KB1, represented as a fraction (0-1), assessed via qPCR using KB1-specific primers in both the background community and KB1 monoculture, shown as a scattered point plot with grand mean. (H) Estimated absolute abundance of E. faecalis KB1, represented as ng/µL of DNA (calculated as relative abundance × total biomass), assessed via qPCR in the background community and KB1 monoculture, shown as a scattered point plot with grand mean. All measurements were performed with three biological replicates (N=3).* (*Enterococcus faecalis*–KB1, *Bacteroides caecimuris*–I48, *Clostridium innocuum*–I46, *Flavonifractor plautii*–YL31, *Blautia coccoides*–YL58, *Lactobacillus reuteri*–I49, *Bifidobacterium longum subsp. animalis*–YL2, *Muribaculum intestinale*–YL27, *Acutalibacter muris*–KB18, *Akkermansia muciniphila*–YL44, *Turicimonas muris*–YL45, and *Clostridium clostridioforme*–YL32)

3.2 Identifying optimal vancomycin concentrations for microbial adaptations

To assess how vancomycin affects microbial community structure, we cultured the OMM12 community across a gradient of vancomycin concentrations ranging from 5 mg/mL to 0.05 µg/mL in ten-fold serial dilutions, with a no-antibiotic control. After 48 hours, we analyzed relative and absolute abundance data alongside live/dead staining.

At high concentrations (500 µg/mL and 50 µg/mL), bacterial viability was significantly reduced, with *Enterococcus faecalis* (KB1) and *Bacteroides caecimuris* (I48) showing reduced presence, while *Clostridium innocuum* (I46) was completely eliminated. At intermediate concentrations (5 µg/mL and 0.5 µg/mL), selective pressure altered species abundance without causing total community collapse, making these conditions ideal for studying microbial adaptation and interactions (Figure 5A).

MIC determination assays in liquid media showed that *E. faecalis*, *C. innocuum*, and *B. caecimuris* exhibited a similar MIC range (4.8–9.7 µg/mL) (Figure 5B). The MIC of vancomycin was largely consistent between monocultures, assessed via optical density, and co-cultures, assessed via qPCR, for most strains, including *E. faecalis* KB1, *B. caecimuris* I48, *C. innocuum* I46, *Flavonifractor plautii* YL31, and *Blautia coccoides* YL58 (Figure 5C-D). However, for the remaining strains, MIC determination within microbial communities proved challenging, as these strains were frequently observed to be either inhibited or eliminated within the consortia, making it difficult to establish a definitive MIC threshold in the community (Figure 5C).

Based on these findings, we selected 0.5 µg/mL and 10 µg/mL as the optimal concentrations for evolutionary studies. The lower concentration (0.5 µg/mL) allowed all community members to survive under selective pressure, while the higher concentration (10 µg/mL) imposed stronger selection pressure.

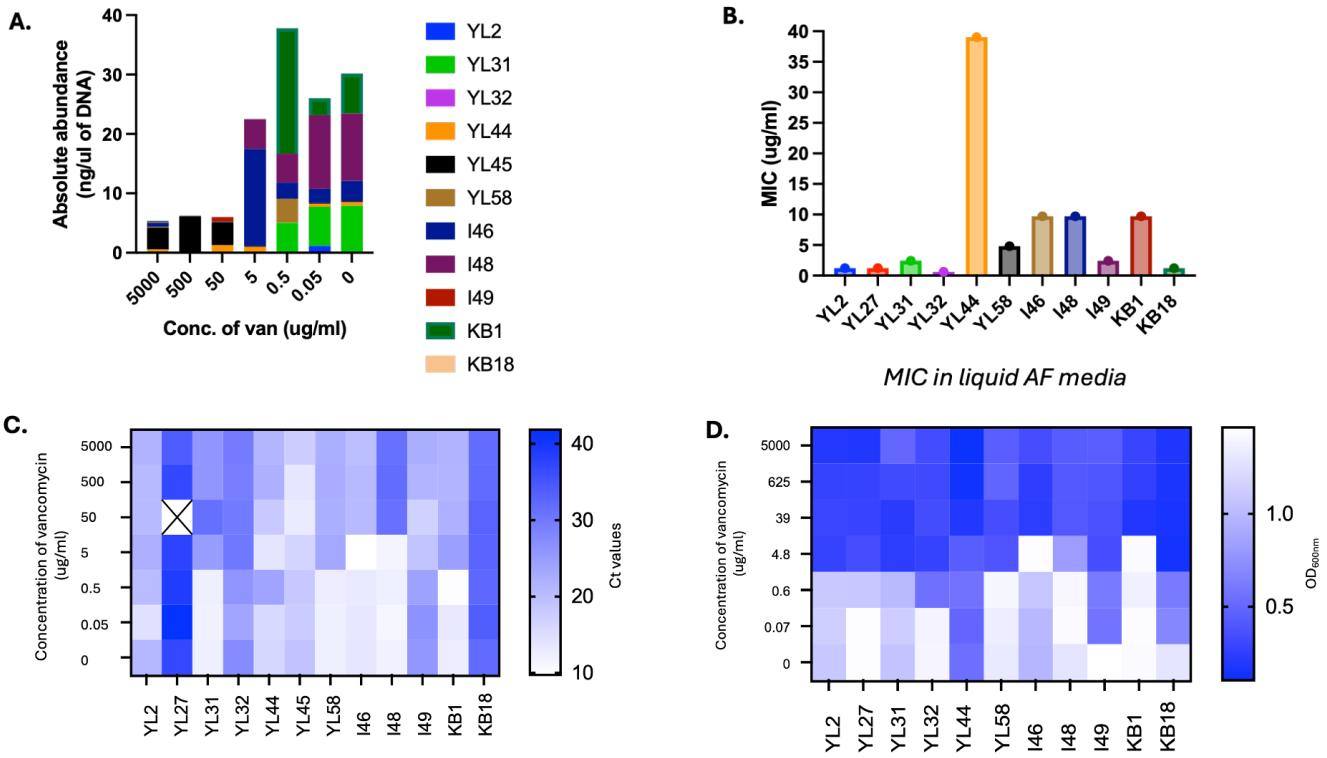

*Figure 5: Estimated absolute abundance, minimum inhibitory concentration (MIC), and response of OMM12 species to vancomycin exposure. (A) Estimated absolute abundance of OMM12 species after 48 hours of vancomycin exposure, represented as ng/µL of DNA (calculated as relative abundance × total biomass), assessed via qPCR in co-cultures. (B) Minimum inhibitory concentration (MIC) of OMM12 species in liquid AF media in response to vancomycinnin mono-cultures. (C) Heatmap of Ct values for strain-specific primers, assessed via qPCR, plotted against vancomycin concentration for each OMM12 strain after 48 hours of exposure in co-cultures. (D) Heatmap of optical density (OD600 nm) plotted against different vancomycin concentrations for OMM12 strains in liquid AF media in mono-cultures. All measurements were performed with three biological replicates (N=3). (*Enterococcus faecalis*–KB1, *Bacteroides caecimuris*–I48, *Clostridium innocuum*–I46, *Flavonifractor plautii*–YL31, *Blautia coccoides*–YL58, *Lactobacillus reuteri*–I49, *Bifidobacterium longum subsp. animalis*–YL2, *Muribaculum intestinale*–YL27, *Acutalibacter muris*–KB18, *Akkermansia muciniphila*–YL44, *Turicimonas muris*–YL45, and *Clostridium clostridioforme*–YL32)*

3.3 Microbial adaptation and competitive exclusion under low vancomycin pressure

In the first evolution experiment, bacterial communities were exposed to a low concentration of vancomycin (0.5 µg/mL) over eight passages, with four antibiotic exposures throughout the experiment. This concentration was set to ensure that all community members initially survived, allowing interspecies interactions to shape adaptive responses.

MIC determination post-evolution revealed that *Clostridium innocuum* (I46) exhibited a decrease in MIC when grown in co-culture without antibiotics compared to its parent strain, while in all other conditions, the MIC remained unchanged (Figure 6A). *Enterococcus faecalis* (KB1) did not show any change in MIC compared to its parent strain across all conditions (Figure 6B). *Bacteroides caecimuris* (I48) could not be

successfully isolated from co-cultures due to its slow growth, which allowed faster-growing species to outcompete it on agar plates. However, relative and absolute abundance data confirmed that *B. caecimuris* remained present within the community (Figure 7A–E). Additionally, MIC assays of *B. caecimuris* isolates from monocultures exposed to vancomycin showed a slight increase in MIC (Figure 6C).

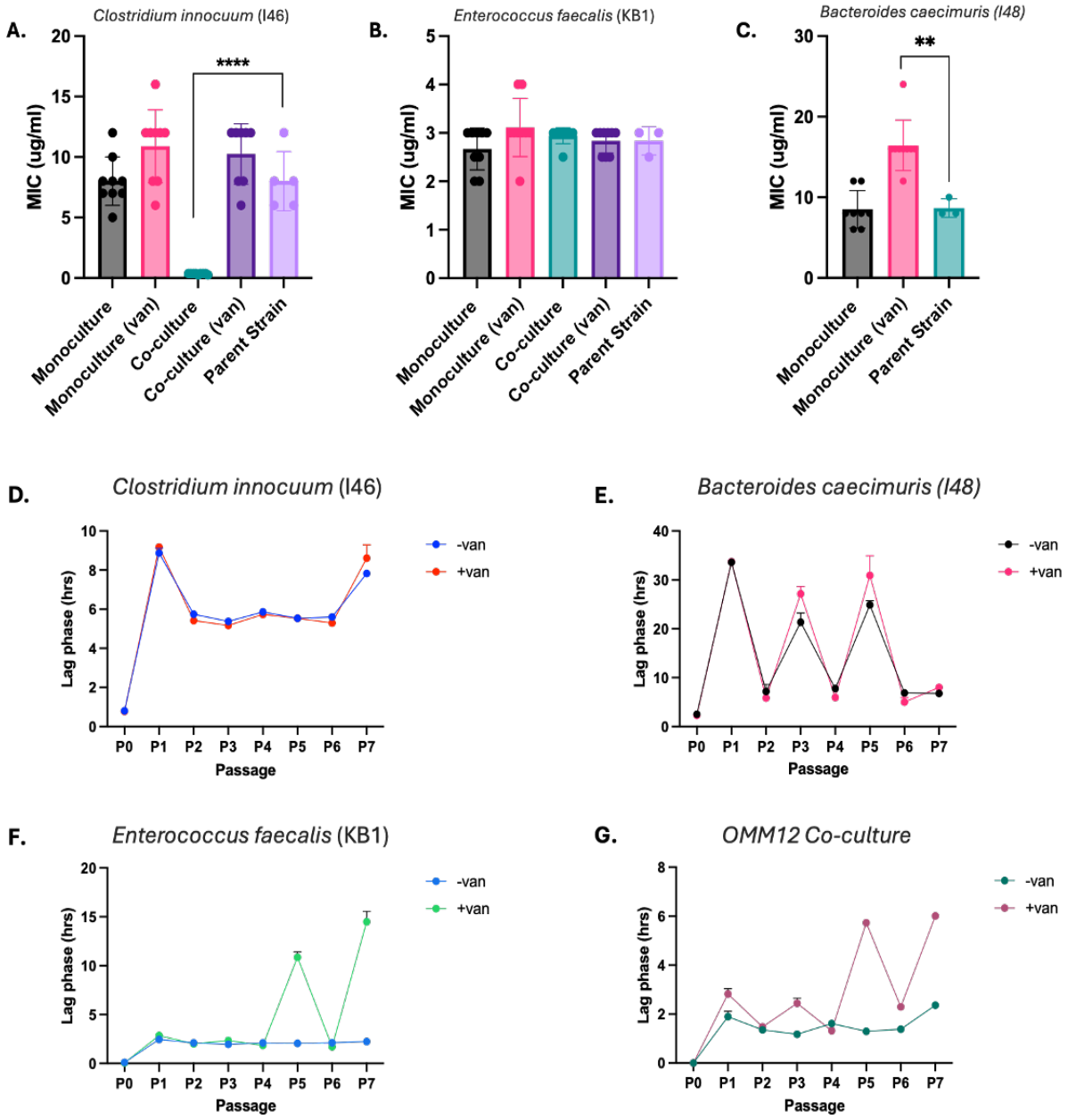

*Figure 6: Minimum inhibitory concentration (MIC) and lag phase dynamics post-evolution under vancomycin exposure. (A) MIC of Clostridium innocuum (I46) determined using E-test after Evolution 1 (0.5 µg/mL), for isolates from monocultures and co-cultures in the presence and absence of vancomycin. (B) MIC of Enterococcus faecalis (KB1) determined using E-test after Evolution 1 (0.5 µg/mL), for isolates from monocultures and co-cultures in the presence and absence of vancomycin. (C) MIC of Bacteroides caecimuris (I48) determined using E-test after Evolution 1 (0.5 µg/mL), for isolates from monocultures in the presence and absence of vancomycin. (D) Lag phase duration over successive passages during Evolution 1 (0.5 µg/mL) for C. innocuum (I46) monocultures grown in the presence and absence of vancomycin. (E) Lag phase duration over successive passages during Evolution 1 (0.5 µg/mL) for B. caecimuris (I48) monocultures grown in the presence and absence of vancomycin. (F) Lag phase duration over successive passages during Evolution 1 (0.5 µg/mL) for E. faecalis (KB1) monocultures grown in the presence and absence of vancomycin. (G) Lag phase duration over successive passages during Evolution 1 (0.5 µg/mL) for OMM12 co-cultures grown in the presence and absence of vancomycin. All measurements were performed with three biological replicates (N=3). p value****<0.0001,***<0.001, **<0.01,*<0.1*

The lag phase is the initial period of bacterial growth during which cells acclimate to their environment, synthesize essential enzymes, and prepare for active division without a significant increase in cell number. Lag phase adaptation serves as a key indicator of bacterial stress response and persistence. *E. faecalis* exhibited a notable increase in lag phase at passage 6 (P5) under vancomycin exposure, while it remained stable in antibiotic-free conditions (Figure 6F). A similar trend was observed in co-cultures (Figure 6G), whereas in *Bacteroides caecimuris* (I48) and *Clostridium innocuum* (I46), the lag phase remained unchanged between vancomycin-treated and untreated bacteria (3D-E). At the same passage, the relative and absolute abundance of *E. faecalis* declined in co-cultures (Figure 7A-E). However, *E. faecalis* population recovered in subsequent passages when vancomycin was removed, only to decline again upon re-exposure to vancomycin in passage 8 (P7).

The relative and absolute abundance data indicate that in the community grown without vancomycin exposure, the overall community composition remained relatively stable throughout the evolutionary experiment. However, minor shifts in dynamics were observed over time, such as an increase in the abundance of *Turicimonas muris* (YL45) over passages and a decrease in the abundance of *Akkermansia muciniphila* (YL44), which partially recovered by the final passage. Some strains, such as *Muribaculum intestinale* (YL27) and *Acutalibacter muris* (KB18), were either knocked down from the first passage or detected at very low abundances. *Bifidobacterium longum* (YL2) was initially present but was progressively lost over passages, becoming nearly undetectable by the end of the experiment, similar to *Lactobacillus reuteri* (I49) (Figure 7 A-C, Appendix Figure 3).

In contrast, the vancomycin-exposed community exhibited distinct dynamics. When the community was exposed to 0.05 µg/mL of vancomycin, *E. faecalis* KB1's abundance increased sharply, leading to a concurrent decrease in the abundance of *Bacteroides caecimuris* (I48), *Clostridium innocuum* (I46), and *Blautia coccoides* (YL58) compared to the antibiotic-free control. This composition remained relatively stable until passage 6 (P5), when the abundance of KB1 suddenly dropped, allowing these strains to regain abundance. When the antibiotic was removed, the community composition recovered toward its pre-treatment state, but re-exposure to vancomycin restored the same selective shift observed earlier (Figure 7 A, D-E).

Flavonifractor plautii (YL31) exhibited minimal changes in overall composition throughout the experiment, remaining unaffected by vancomycin exposure or the sudden community shifts at P5 (Figure 7A, Appendix Figure 3). Additionally, *Turicimonas muris* (YL45), which had gradually increased in abundance in the antibiotic-free community, exhibited a more pronounced increase over passages in the presence of vancomycin compared to the untreated condition (Figure 7A, Appendix Figure 3).

Live/dead staining of bacterial populations revealed that co-cultures exposed to vancomycin at P5 had a higher proportion of dead cells. While *E. faecalis* remained viable throughout the experiment, its transient drop in abundance at P5 influenced overall community stability (Figure 7F).

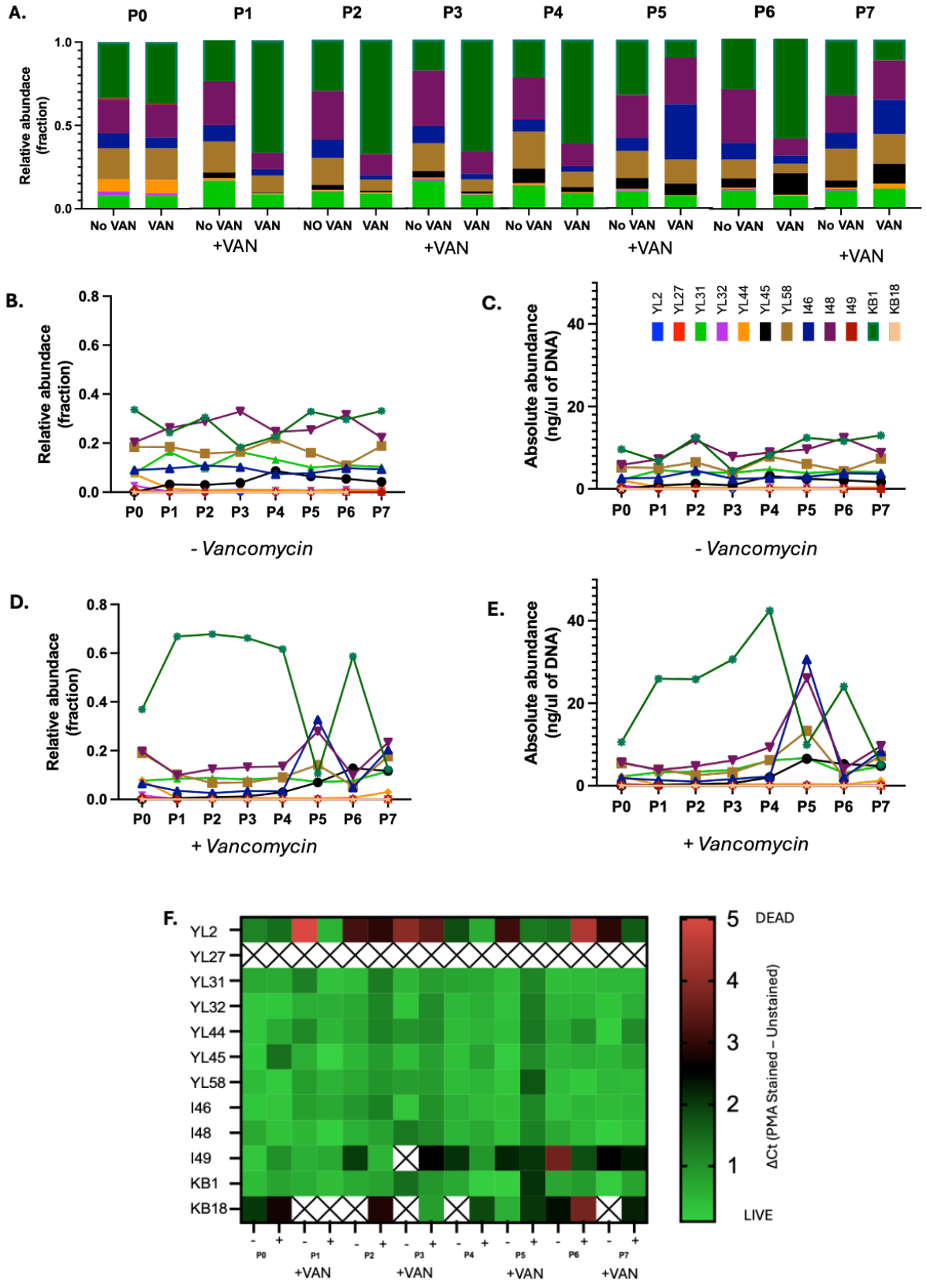

Figure 7: Relative and estimated abundance of OMM12 strains in co-cultures over passages during evolution, and live-dead staining analysis. (A) Relative abundance of OMM12 strains in co-cultures over passages during evolution, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers, shown as stacked bar plots for co-cultures grown in the presence and absence of vancomycin. (B) Relative abundance of OMM12 strains in co-cultures over passages during evolution, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the absence of vancomycin, represented as line graphs. (C) Estimated absolute abundance of OMM12 strains in co-cultures over passages during evolution, represented as ng/μL of DNA (calculated as relative abundance × total biomass), assessed via qPCR using OMM12-specific

primers for co-cultures grown in the absence of vancomycin, represented as line graphs. (D) Relative abundance of OMM12 strains in co-cultures over passages during evolution, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the presence of vancomycin, represented as line graphs. (E) Estimated absolute abundance of OMM12 strains in co-cultures over passages during evolution, represented as ng/µL of DNA (calculated as relative abundance × total biomass), assessed via qPCR using OMM12-specific primers for co-cultures grown in the presence of vancomycin, represented as line graphs. (F) Live-dead staining of co-cultures over passages during evolution using Propidium Monoazide (PMA) dye, assessed via qPCR. The heatmap plots ΔCt values (PMA-stained – unstained), where ΔCt values close to 4 indicate a predominantly dead population, while values close to 1 indicate a mostly live population. All measurements were performed with three biological replicates (N=3). (*Enterococcus faecalis*–KB1, *Bacteroides caecimuris*–I48, *Clostridium innocuum*–I46, *Flavonifractor plautii*–YL31, *Blautia coccoides*–YL58, *Lactobacillus reuteri*–I49, *Bifidobacterium longum subsp. animalis*–YL2, *Muribaculum intestinale*–YL27, *Acutalibacter muris*–KB18, *Akkermansia muciniphila*–YL44, *Turicimonas muris*–YL45, and *Clostridium clostridioforme*–YL32)

3.4 High vancomycin pressure drives competitive elimination, adaptation- and persistence-based survival

The second evolution experiment tested a higher vancomycin concentration of 10 µg/mL mirroring the design of the first one: eight passages, with four antibiotic exposures exchanging with two days of relaxation of the antibiotic pressure. This condition applied stronger selective pressure to determine how community members adapted or were eliminated due to antibiotic stress.

At this higher antibiotic concentration, survival patterns changed dramatically. *Clostridium innocuum* (I46) suffered a complete population collapse in mono-cultures upon initial vancomycin exposure and did not recover in later passages, indicating that it lacked the ability to develop either resistance or persistence mechanisms (Figure 8 A-B). However, it was detected in the community exposed to vancomycin until passage 6 before being knocked down (Figure 8 K-L).

Bacteroides caecimuris (I48), on the other hand, initially exhibited poor growth in all replicates when revived from glycerol stocks, likely due to cell death or reduced viability over time in storage. As a result, the pre-inoculum grew very slowly, affecting its initial adaptation. When this slow-growing pre-inoculum was used to continue the evolution experiment, *B. caecimuris* struggled in the first passage, displaying a prolonged lag phase in monoculture but ultimately managing to recover over successive passages. Additionally, MIC assays at the end of the evolution experiment revealed a significant increase in MIC in *B. caecimuris* isolates from monocultures exposed to vancomycin (Figure 8 C-D, H). However, in co-culture settings, *B. caecimuris* was completely outcompeted and eliminated (Figure 8 I-L, Appendix Figure 4).

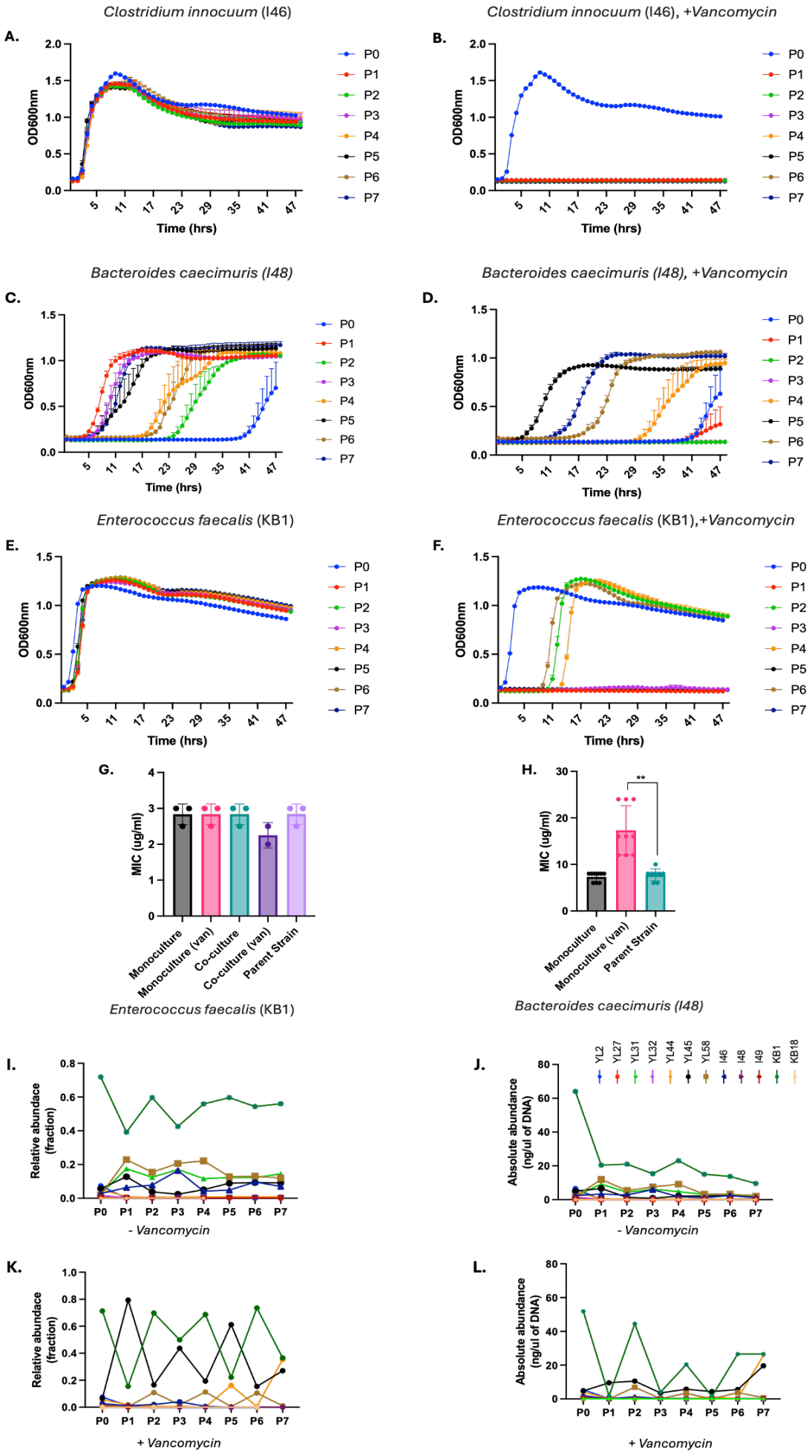
A.
Clostridium innocuum (I46)
OD600nm
Time (hrs)
P0
P1
P2
P3
P4
P5
P6
P7
B.
Clostridium innocuum (I46), +Vancomycin
C.
Bacteroides caecimuris (I48)
D.
Bacteroides caecimuris (I48), +Vancomycin
E.
Enterococcus faecalis (KB1)
F.
Enterococcus faecalis (KB1),+Vancomycin
G.
MIC (ug/ml)
Monoculture
Monoculture (van)
Co-culture
Co-culture (van)
Parent Strain
H.
**
Enterococcus faecalis (KB1)
Bacteroides caecimuris (I48)
I.
Relative abundace (fraction)
- Vancomycin
J.
YL2
YL27
YL31
YL32
YL44
YL45
YL58
I46
I48
I49
KB1
KB18
Absolute abundance (ng/ul of DNA)
K.
+ Vancomycin
L.

Figure 8: *Growth curves, MIC determination, and relative abundance of OMM12 strains during evolution 2 under vancomycin exposure. (A) Growth curve (OD600 nm) over passages during Evolution 2 (10 µg/mL) for* Clostridium innocuum *(I46) in the absence of vancomycin. (B) Growth curve (OD600 nm) over passages during Evolution 2 (10 µg/mL) for* C. innocuum *(I46) in the presence of vancomycin. (C) Growth curve (OD600 nm) over passages during Evolution 2 (10 µg/mL) for* Bacteroides caecimuris *(I48) in the absence of vancomycin. (D) Growth curve (OD600 nm) over passages during Evolution 2 (10 µg/mL) for* B. caecimuris *(I48) in the presence of vancomycin. (E) Growth curve (OD600 nm) over passages during Evolution 2 (10 µg/mL) for* Enterococcus faecalis *(KB1) in the absence of vancomycin. (F) Growth curve (OD600 nm) over passages during Evolution 2 (10 µg/mL) for* E. faecalis *(KB1) in the presence of vancomycin. (G) MIC of* E. faecalis *(KB1) determined using E-test post-Evolution 2 (10 µg/mL) for isolates from monocultures and co-cultures in the presence and absence of vancomycin. (H) MIC of* B. caecimuris *(I48) determined using E-test post-Evolution 2 (10 µg/mL) for isolates from monocultures in the presence and absence of vancomycin. (I) Relative abundance of OMM12 strains in co-cultures over passages during Evolution 2, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the absence of vancomycin, represented as line graphs. (J) Estimated absolute abundance of OMM12 strains in co-cultures over passages during Evolution 2, represented as ng/µL of DNA (calculated as relative abundance × total biomass), assessed via qPCR using OMM12-specific primers for co-cultures grown in the absence of vancomycin, represented as line graphs. (K) Relative abundance of OMM12 strains in co-cultures over passages during Evolution 2, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the presence of vancomycin, represented as line graphs. (L) Estimated absolute abundance of OMM12 strains in co-cultures over passages during Evolution 2, represented as ng/µL of DNA (calculated as relative abundance × total biomass), assessed via qPCR using OMM12-specific primers for co-cultures grown in the presence of vancomycin, represented as line graphs. All measurements were performed with three biological replicates (N=3).* (*Enterococcus faecalis*–KB1, *Bacteroides caecimuris*–I48, *Clostridium innocuum*–I46, *Flavonifractor plautii*–YL31, *Blautia coccoides*–YL58, *Lactobacillus reuteri*–I49, *Bifidobacterium longum subsp. animalis*–YL2, *Muribaculum intestinale*–YL27, *Acutalibacter muris*–KB18, *Akkermansia muciniphila*–YL44, *Turicimonas muris*–YL45, and *Clostridium clostridioforme*–YL32)

Enterococcus faecalis (KB1) exhibited a recurring pattern of population decline upon vancomycin exposure, followed by recovery in antibiotic-free passages. The MIC of *E. faecalis* remained unchanged throughout the evolution experiment, indicating no detectable increase in resistance (Figure 8 E-G).

The relative and absolute abundance data of OMM12 strains in the community indicate that *E. faecalis* (KB1) largely dominated the community, with its abundance being relatively higher compared to evolution 1 passage 0, likely due to the absence of *Bacteroides caecimuris* (I48) (Figure 7 B-E ,8 I-L). Apart from *E. faecalis*, *Blautia coccoides* (YL58) was the second most abundant species (Figure 8 I-L).

In the community not exposed to vancomycin, similar dynamics to evolution 1 were observed, with *Bifidobacterium longum* (YL2), *Muribaculum intestinale* (YL27), *Acutalibacter muris* (KB18), and *Lactobacillus reuteri* (I49) being knocked out over passages. Meanwhile, *Akkermansia muciniphila* (YL44) showed a pattern of decline followed by reappearance in the final passages, while *Turicimonas muris* (YL45) exhibited a gradual increase in abundance over time (Figure 8 I-J).

In the community exposed to 10 µg/mL vancomycin, a drastic shift in community dynamics was observed. *E. faecalis* (KB1) exhibited a cyclical pattern, decreasing in abundance when exposed to vancomycin, recovering in antibiotic-free passages, and

decreasing again upon re-exposure. A similar trend was observed for *Blautia coccoides* (YL58). In contrast, *Turicimonas muris* (YL45) displayed the opposite pattern, increasing in abundance in passages exposed to vancomycin and decreasing in its absence. *Akkermansia muciniphila* (YL44) followed a similar trend but remained at low abundance in the first five passages before recovering and mirroring the dynamics of YL45 (Figure 8 K-L, Appendix Figure 4). *Flavonifractor plautii* (YL31) and *Clostridium clostridioforme* (YL32) were initially present at low abundance but were eventually eliminated as vancomycin exposure continued over passages (Figure 8 K-L, Appendix Figure 4).

Chapter 4 Discussion

Microbial communities are shaped by complex interactions between species, where competition, facilitation, and antagonism play crucial roles in determining community structure and function. Studying these interactions is challenging in natural ecosystems due to their vast diversity, which is why simplified synthetic communities, such as the Oligo-Mouse-Microbiota (OMM12), have been used as models to investigate ecological and evolutionary dynamics in controlled laboratory settings.

In this study, we examined how bacterial species within the OMM12 model adapt to vancomycin exposure and whether interspecies interactions influence their evolutionary trajectories. Specifically, we investigated the evolutionary dynamics of microbial communities under vancomycin pressure, focusing on the persistence and adaptation of key strains, including *Enterococcus faecalis* (KB1), *Bacteroides caecimuris* (I48), and *Clostridium innocuum* (I46). By conducting two independent evolution experiments at different antibiotic concentrations (0.5 µg/mL and 10 µg/mL vancomycin), we analysed how antibiotic pressure, community structure, and interspecies interactions affected bacterial survival, MIC changes, and competitive exclusion.

Throughout both evolutionary experiments, *Enterococcus faecalis* (KB1) demonstrated its role as a key driver of the microbial community by maintaining stable MIC values despite prolonged vancomycin exposure. Even after evolving under 10 µg/mL vancomycin, a concentration close to its MIC, *E. faecalis* (KB1) did not exhibit increased resistance, instead relying on persistence mechanisms for survival. Persistence is a phenotypic adaptation wherein a subpopulation of bacteria enters a dormant state, allowing them to tolerate antibiotics without acquiring genetic resistance. Unlike resistant bacteria, persister cells resume normal growth once the antibiotic is removed, distinguishing this process from true resistance evolution.

In all experiments where *E. faecalis* (KB1) was inoculated directly into high vancomycin concentrations, it failed to grow. However, when *E. faecalis* (KB1) was first allowed to grow for two days before vancomycin was added, it remained viable even at 10 times its MIC. This behavior is likely explained by vancomycin's mechanism of action, the antibiotic inhibits cell wall synthesis, which primarily affects actively dividing cells. When *E. faecalis* (KB1) is in the log phase, vancomycin blocks

peptidoglycan crosslinking, leading to cell lysis. However, in the stationary phase, bacterial cells are not actively dividing, rendering vancomycin significantly less effective and the presence of persister cells could explain why *E. faecalis* (KB1) tolerated high antibiotic concentrations when already established in stationary-phase growth. Regardless of the precise mechanism, it is evident that *E. faecalis* (KB1) exhibits persistence to vancomycin rather than resistance evolution.

Our findings also align with interaction network analyses conducted by A S Weiss et al. (2021)**,** which showed that *E. faecalis* (KB1) negatively affects most OMM12 species. This is consistent with our observation that when *E. faecalis* (KB1) was co-cultured with the background community, it inhibited other members, leading to a lower initial OD in communities containing *E. faecalis* (KB1) compared to background-only communities. A S Weiss et al (2021) also indicated that other species do not significantly impact *E. faecalis* (KB1) in co-cultures, which aligns with our results, as *E. faecalis* (KB1) remained unaffected even in a background community with four different species.

Interestingly, our findings also support *in vivo* observations by Münch, Philipp C. et al. (2023)**,** who reported that *E. faecalis* (KB1) was initially present in low abundance following vancomycin treatment in mice. In contrast, in our *in vitro* experiment, *E. faecalis* (KB1) rapidly took over the community. However, at higher vancomycin concentrations, we observed a drastic decrease in *E. faecalis* (KB1) abundance, after which it persisted but did not recover within the experimental timeframe. This aligns with Münch, Philipp C. et al (2023). findings, where *E. faecalis* (KB1) was able to persist under vancomycin stress and eventually adapted and grew, a process that we may not have observed in our study due to a shorter experimental duration. Moreover, both our study and Münch, Philipp C. et al (2023) found no significant MIC changes in *E. faecalis* (KB1) clones isolated after evolution to vancomycin, suggesting that *E. faecalis* (KB1) primarily relies on persistence rather than resistance mechanisms for survival.

A key observation in the 0.5 µg/mL vancomycin evolution experiment was that *Clostridium innocuum* (I46) exhibited a significant decrease in MIC when grown in the community without vancomycin, an effect not observed under antibiotic pressure. This suggests that microbial interactions, such as metabolic dependencies or competition, may play a role in sensitizing *C. innocuum* (I46) to vancomycin in the

absence of selective pressure. Interestingly, *C. innocuum* intrinsically resists vancomycin through the Ddl *C. innocuum* ligase and C. innocuum racemase, which cooperate to synthesize D-Ala-D-Ser peptidoglycan precursors with low vancomycin affinity (David et al. 2004). This mechanism enables *C. innocuum* to survive vancomycin exposure by altering its cell wall composition, reducing antibiotic binding, and maintaining structural integrity. However, the observed MIC reduction in the absence of vancomycin suggests that community interactions might interfere with this resistance mechanism, potentially limiting *C. innocuum* (I46)'s ability to sustain its low-affinity precursors.

In contrast, in the 10 µg/mL vancomycin evolution experiment, *C. innocuum* (I46) exhibited a different survival pattern. In monoculture, its population collapsed entirely after the first exposure to vancomycin, whereas in community settings, it persisted at low abundance until the fourth vancomycin exposure before being completely eliminated. This suggests that while *C. innocuum* (I46)'s intrinsic resistance was insufficient to sustain survival under high antibiotic pressure alone, the presence of other bacterial species temporarily supported its persistence. Community interactions may have facilitated its survival by modulating stress responses or metabolic exchanges, delaying but ultimately not preventing its eradication under prolonged vancomycin selection.

As for *Bacteroides caecimuris* (I48), an important observation was that it exhibited an increase in MIC in monocultures across both evolutionary experiments, suggesting that *B. caecimuris* (I48) gained resistance to vancomycin over time. This aligns with the broader understanding that *Bacteroides* species are highly adaptable and capable of rapid evolution under selective pressures. Dapa & Xavier et al (2022), demonstrated that *Bacteroides thetaiotaomicron* evolves quickly in response to dietary shifts, acquiring mutations that enhance mucin glycan degradation. Similarly, in our study, the observed MIC increase in *B. caecimuris* (I48) highlights its ability to rapidly adapt under antibiotic stress, reinforcing the idea that *Bacteroides* species are dynamic members of the gut microbiome capable of evolving under changing environmental conditions.

In Evolution 2 (10 µg/mL vancomycin)**,** we observed a striking shift in community composition, where *Turicimonas muris* (YL45) and *Akkermansia muciniphila* (YL44) became the dominant species in passages exposed to vancomycin. Their ability to

take over the community can likely be attributed to their high MIC values, which allowed them to withstand vancomycin pressure more effectively than other species. As more susceptible strains were gradually eliminated, *T. muris* (YL45) and *A. muciniphila* (YL44) capitalized on the reduced competition, thriving in an environment where antibiotic selection favored their survival.

Interestingly, in Evolution 1 (0.5 µg/mL vancomycin**)**, we observed that *Bacteroides caecimuris* (I48) appeared to regulate the dominance of *Enterococcus faecalis* (KB1). When *B. caecimuris* (I48) was present, *E. faecalis* (KB1) maintained a balanced presence within the no-antibiotic control communities. However, in its absence in Evolution 2, *E. faecalis* (KB1) completely dominated the microbial landscape, suggesting that *B. caecimuris* (I48) played a critical regulatory role in limiting its expansion. This regulatory effect was likely driven by resource competition or antagonistic interactions, which prevented *E. faecalis* (KB1) from outcompeting other species in the community.

This observation contrasts with the findings of A S Weiss et al. (2021)**,** who demonstrated that *E. faecalis* (KB1) exhibits an amensalistic effect on *B. caecimuris* (I48) in co-culture, negatively impacting its growth. However, in our study, we observed the opposite effect at the community level, where *B. caecimuris* (I48) appeared to restrict *E. faecalis* (KB1). The key difference between the two studies is that A S Weiss et al. (2021) examined this interaction in a pairwise co-culture, whereas we observed it within a complex community setting, where additional species may have influenced these dynamics. This raises the possibility that microbial interaction networks become more complex and context-dependent when multiple species are present, potentially modifying direct pairwise relationships observed in simplified systems. The presence of other community members may introduce indirect interactions, metabolic dependencies, or competitive pressures that reshape the dynamics between *E. faecalis* (KB1) and *B. caecimuris* (I48), leading to regulatory effects that were not apparent in the isolated co-culture experiments.

Community interactions played a role in shaping microbial composition in both evolutionary experiments. In no-antibiotic control communities, *Bifidobacterium longum subsp. animalis* (YL2), *Muribaculum intestinale* (YL27), *Lactobacillus reuteri* (I49), and *Acutalibacter muris* (KB18) were progressively eliminated by the end of

the evolution, likely due to competitive exclusion rather than antibiotic stress. This pattern closely resembles Münch, Philipp C. et al**.** *in vivo* findings, where *A. muris* (KB18) and *B. longum* (YL2) were completely lost, and *M. intestinale* (YL27) reappeared in later passages. The difference in our study is that our evolution experiment was not long enough to detect a possible reappearance of *M. intestinale* (YL27) at later timepoints of the evolution.

Furthermore, Münch, Philipp C. et al. (2023) observed that *A. muciniphila* (YL44) was lost after initial vancomycin treatments but eventually recovered over passages, suggesting that it evolved over time. This aligns with our findings, where *A. muciniphila* (YL44) initially decreased but later recovered, reinforcing the idea that this strain can adapt and re-establish itself within a microbial community under antibiotic pressure. Similarly, Münch, Philipp C. et al. (2023) found that *C. innocuum* (I46) and *B. caecimuris* (I48) increased after vancomycin treatment, while *B. coccoides* (YL58) and *L. reuteri* (I49) declined. While we observed a similar trend with *C. innocuum* (I46), *L. reuteri* (I49) and *B. caecimuris* (I48), we did not detect a major change in the dynamics of *B. coccoides* (YL58) in our study.

The community context plays a crucial role in shaping MIC values and resistance evolution, as interspecies interactions can either enhance or limit a strain's ability to survive antibiotic pressure. In our study, *Clostridium innocuum* I46 exhibited a decrease in MIC when grown in the community without vancomycin, suggesting that the presence of other species influenced its antibiotic susceptibility, possibly through metabolic dependencies or competition. However, under 10 µg/mL vancomycin (Evolution 2), *Clostridium innocuum* I46 persisted longer in the community than in monoculture, where it was immediately eliminated. This indicates that while microbial interactions can temporarily support the survival of certain species, their ability to withstand antibiotic pressure may be influenced by the surrounding microbial environment rather than just intrinsic resistance mechanisms.

Furthermore, in Evolution 2, *Turicimonas muris* YL45 and *Akkermansia muciniphila* YL44 transitioned from being nearly undetectable to dominating the community under vancomycin selection. Their expansion followed the elimination of *Clostridium innocuum* I46 and *Flavonifractor plautii* YL31 and the persistence of *Enterococcus faecalis* KB1 and *Blautia coccoides* YL58, highlighting that the removal of susceptible species can restructure microbial dynamics, allowing previously minor

members to emerge as dominant. Interestingly, while some species tolerated vancomycin exposure, they did not necessarily evolve higher MIC values (*E. faecalis*), reinforcing that resistance and survival in a community setting depend not only on intrinsic antibiotic resistance but also on competitive interactions and ecological shifts.

Moving forward, we are actively working on isolating *Bacteroides caecimuris* (I48) from co-culture experiments to assess its MIC more accurately, as well as obtaining pure clones for further investigations such as whole-genome sequencing. This will provide deeper insights into the genetic basis of its observed resistance development in monocultures and its competitive exclusion in co-culture settings.

Additionally, we plan to send key strains for whole genome sequencing, including *Clostridium innocuum* (I46), where we observed a significant MIC decrease during evolution, and *Enterococcus faecalis* (KB1) at passage 6, where we noted a sudden shift in its population dynamics. Whole-genome sequencing of these strains will help uncover potential mutations and resistance mechanisms.

Beyond vancomycin, we aim to extend our evolution experiments using different antibiotics, including ampicillin, tetracycline, ciprofloxacin, and metronidazole. This will allow us to investigate whether similar persistence, resistance, and community adaptation dynamics occur under different selective pressures or if antibiotic-specific effects drive distinct evolutionary outcomes in this microbial consortium.

Furthermore, we plan to expand our analysis to additional focal strains to assess whether different positions within the microbial interaction network influence resistance evolution. While we have not yet examined how the positioning of *Bacteroides caecimuris* (I48) and *Clostridium innocuum* (I46) within the network correlates with their resistance patterns, this could provide valuable insights into how interspecies interactions shape antibiotic adaptation. By investigating species at different network positions, we aim to determine whether microbial interactions create ecological constraints that promote persistence, resistance, or extinction under antibiotic selection

Ultimately, these findings could help us understand how resistance evolves in pathogens within complex microbial communities and how antibiotic exposure shapes community composition, potentially leading to the emergence of resistant

opportunistic pathogens. Studying how bacteria like *Escherichia coli, Klebsiella pneumoniae,* or *Clostridioides difficile* respond to antibiotics in the presence of other microbes could reveal whether resistance develops more easily in certain community structures or if some species help suppress it.

At the same time, if microbial interactions influence resistance in commensals, this could open new possibilities for slowing down resistance evolution within the microbiome before it becomes a clinical concern. By identifying commensal species that naturally limit the spread of resistance, we could explore how microbial communities can be managed to reduce antibiotic resistance risks. Investigating how bacteria at different positions in microbial interaction networks respond to antibiotics may also help determine whether certain community structures promote or restrict resistance evolution**.**

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Appendix

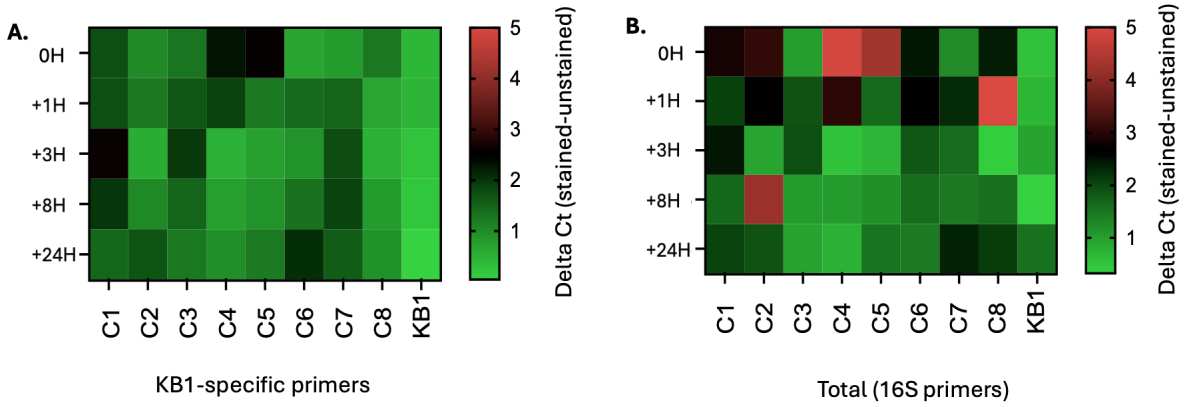

Figure 1: A) Live-dead staining of E. faecalis KB1 in different background consortia at different time points post-vancomycin exposure using Propidium Monoazide (PMA) dye, assessed via qPCR. (B) Live-dead staining of background microbial communities co-cultured with E. faecalis KB1 at different time points post-vancomycin exposure using Propidium Monoazide (PMA) dye, assessed via qPCR. The heatmap plots ΔCt values (PMA-stained – unstained), where ΔCt values close to 4 indicate a predominantly dead population, while values close to 1 indicate a mostly live population. All measurements were performed with three biological replicates (N=3).

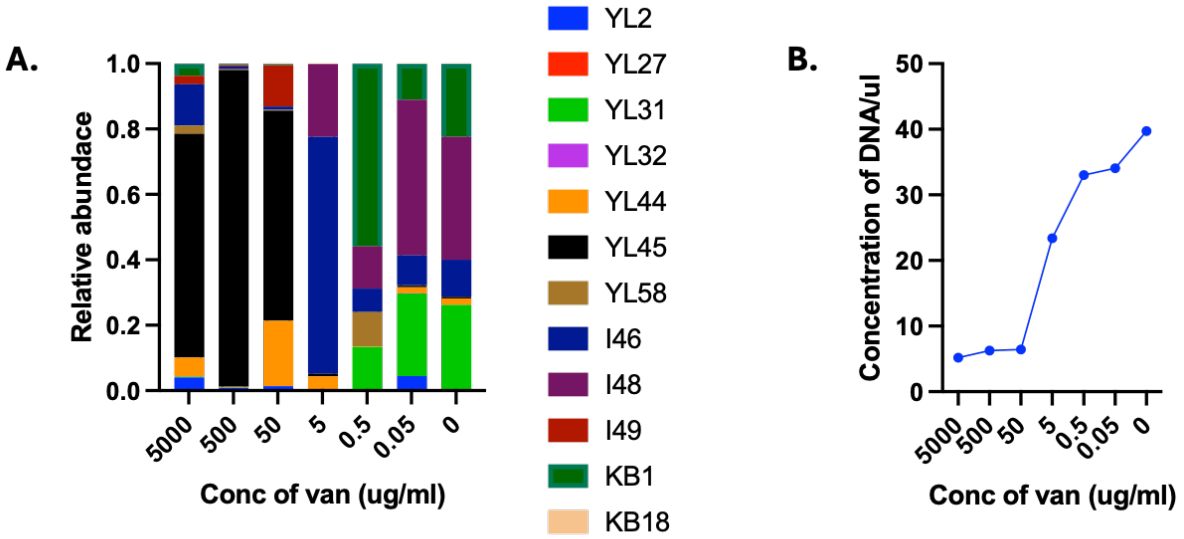

Figure 2: A) Relative abundance of OMM12 species after 48 hours of vancomycin exposure, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers in co-cultures. B) Concentration of DNA extracted from OMM12 co-culture after 48 hours of vancomycin exposure, represented as line graph. All measurements were performed with three biological replicates (N=3). (*Enterococcus faecalis*–KB1, *Bacteroides caecimuris*–I48, *Clostridium innocuum*–I46, *Flavonifractor plautii*–YL31, *Blautia coccoides*–YL58, *Lactobacillus reuteri*–I49, *Bifidobacterium longum subsp. animalis*–YL2, *Muribaculum intestinale*–YL27, *Acutalibacter muris*–KB18, *Akkermansia muciniphila*–YL44, *Turicimonas muris*–YL45, and *Clostridium clostridioforme*–YL32).

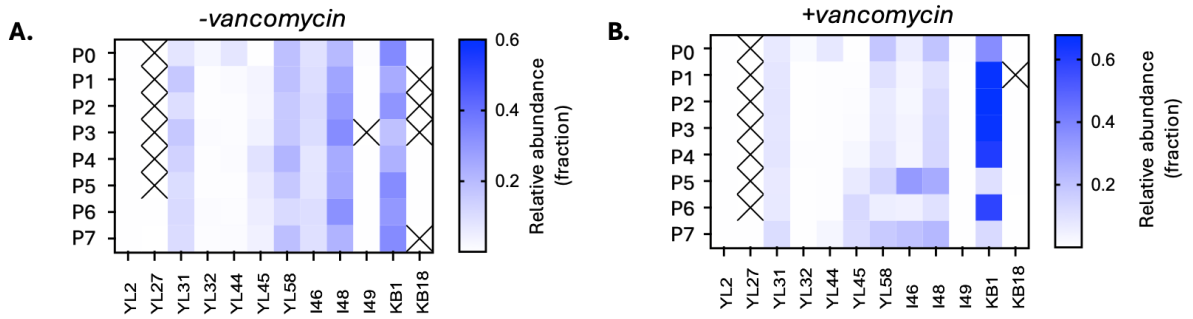

Figure 3: *Evolution 1 (concentration of vancomycin, 0.5 ug/ml). (A) Relative abundance of OMM12 strains in co-cultures over passages during evolution, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the absence of vancomycin, represented as heatmaps. (B) Relative abundance of OMM12 strains in co-cultures over passages during evolution, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the presence of vancomycin, represented as heatmaps. All measurements were performed with three biological replicates (N=3).* (*Enterococcus faecalis*–KB1, *Bacteroides caecimuris*–I48, *Clostridium innocuum*–I46, *Flavonifractor plautii*–YL31, *Blautia coccoides*–YL58, *Lactobacillus reuteri*–I49, *Bifidobacterium longum subsp. animalis*–YL2, *Muribaculum intestinale*–YL27, *Acutalibacter muris*–KB18, *Akkermansia muciniphila*–YL44, *Turicimonas muris*–YL45, and *Clostridium clostridioforme*–YL32).

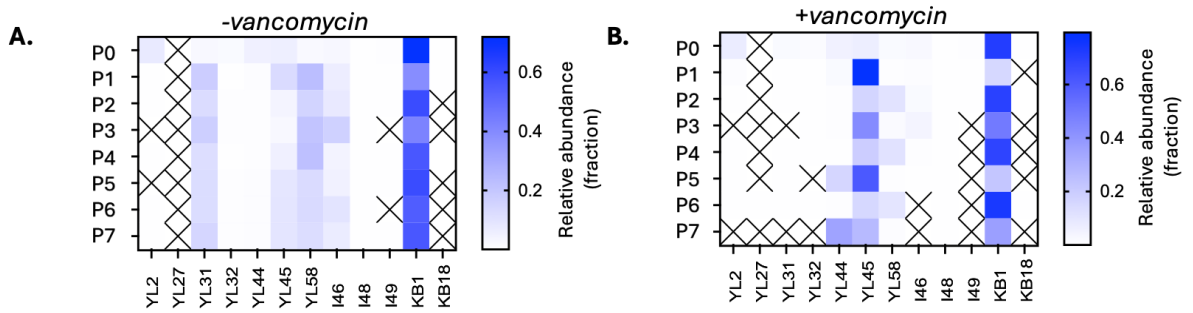

Figure 4: *Evolution 2 (concentration of vancomycin, 10 ug/ml). (A) Relative abundance of OMM12 strains in co-cultures over passages during evolution, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the absence of vancomycin, represented as heatmaps. (B) Relative abundance of OMM12 strains in co-cultures over passages during evolution, represented as a fraction (0-1), assessed via qPCR using OMM12-specific primers for co-cultures grown in the presence of vancomycin, represented as heatmaps. All measurements were performed with three biological replicates (N=3).* (*Enterococcus faecalis*–KB1, *Bacteroides caecimuris*–I48, *Clostridium innocuum*–I46, *Flavonifractor plautii*–YL31, *Blautia coccoides*–YL58, *Lactobacillus reuteri*–I49, *Bifidobacterium longum subsp. animalis*–YL2, *Muribaculum intestinale*–YL27, *Acutalibacter muris*–KB18, *Akkermansia muciniphila*–YL44, *Turicimonas muris*–YL45, and *Clostridium clostridioforme*–YL32).

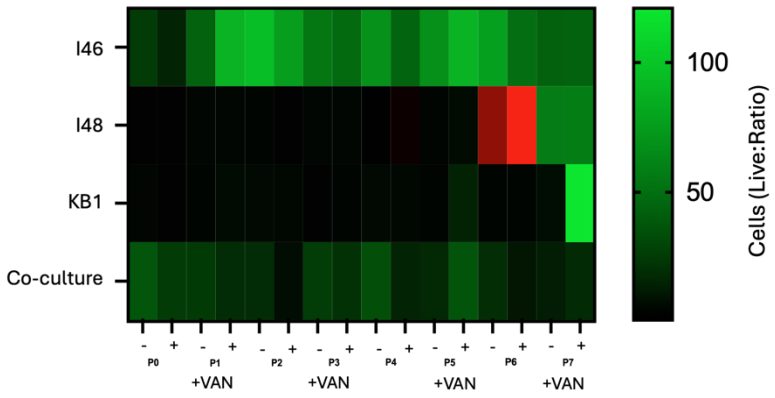

Figure 5: *Evolution 1 (concentration of vancomycin, 0.5 ug/ml). Live-to-dead cell ratio over passages in evolving Clostridium innocuum* I46, *Enterocloster clostridioformis* I48, *Enterococcus faecalis* KB1, *and* OMM12 *co-cultures. The heatmap represents the ratio of live to dead cells determined by flow cytometry using SYBR Green and propidium iodide (PI) staining. Measurements were taken across multiple passages during the evolution experiment. Green color indicate a higher live-to-dead cell ratio, while red colour indicate lower live-to-dead cell ratio.*