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Protocol

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High-throughput anaerobic screening for identifying compounds acting against gut bacteria in monocultures or communities

Patrick Müller[1,2,11], Jacobo de la Cuesta-Zuluaga[1,2,11], Michael Kuhn[3], Maral Baghai Arassi[3,4], Tim Treis[3,5], Sonja Blasche[6], Michael Zimmermann[3], Peer Bork[3,7,8,9], Kiran Raosaheb Patil[6], Athanasios Typas[3], Sarela Garcia-Santamarina[10] ✉ & Lisa Maier[1,2] ✉

Abstract

The human gut microbiome is a key contributor to health, and its perturbations are linked to many diseases. Small-molecule xenobiotics such as drugs, chemical pollutants and food additives can alter the microbiota composition and are now recognized as one of the main factors underlying microbiome diversity. Mapping the effects of such compounds on the gut microbiome is challenging because of the complexity of the community, anaerobic growth requirements of individual species and the large number of interactions that need to be quantitatively assessed. High-throughput screening setups offer a promising solution for probing the direct inhibitory effects of hundreds of xenobiotics on tens of anaerobic gut bacteria. When automated, such assays enable the cost-effective investigation of a wide range of compound-microbe combinations. We have developed an experimental setup and protocol that enables testing of up to 5,000 compounds on a target gut species under strict anaerobic conditions within 5 d. In addition, with minor modifications to the protocol, drug effects can be tested on microbial communities either assembled from isolates or obtained from stool samples. Experience in working in an anaerobic chamber, especially in performing delicate work with thick chamber gloves, is required for implementing this protocol. We anticipate that this protocol will accelerate the study of interactions between small molecules and the gut microbiome and provide a deeper understanding of this microbial ecosystem, which is intimately intertwined with human health.

Key points

- This is a high-throughput screening protocol that allows users to test the growth effects of diverse drugs on bacterial monocultures, synthetic communities and communities derived from stool samples.
- Compared with other methods, it provides increased throughput and cost-effectiveness and can be performed under anaerobic conditions.

Key references

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Maier, L. et al. *Nature* **599**, 120–124 (2021): https://doi.org/10.1038/s41586-021-03986-2

[1]Interfaculty Institute of Microbiology and Infection Medicine, University of Tübingen, Tübingen, Germany. [2]Cluster of Excellence 'Controlling Microbes to Fight Infections', University of Tübingen, Tübingen, Germany. [3]European Molecular Biology Laboratory, Heidelberg, Germany. [4]Department of Pediatrics I, University Children's Hospital Heidelberg, Heidelberg, Germany. [5]Institute of Computational Biology, Helmholtz Center München, Neuherberg, Germany. [6]Medical Research Council Toxicology Unit, University of Cambridge, Cambridge, UK. [7]Max Delbrück Centre for Molecular Medicine, Berlin, Germany. [8]Yonsei Frontier Lab (YFL), Yonsei University, Seoul, South Korea. [9]Department of Bioinformatics, Biocenter, University of Würzburg, Würzburg, Germany. [10]Instituto de Tecnologia Quimica e Biologica, Universidade Nova de Lisboa, Oeiras, Portugal. [11]These authors contributed equally: Patrick Müller, Jacobo de la Cuesta-Zuluaga. ✉e-mail: sarela.santamarina@itqb.unl.pt; l.maier@uni-tuebingen.de

Introduction

There is growing appreciation for cultivation-based approaches in the gut microbiome field because of the opportunities they offer to mechanistically investigate this complex ecosystem[1,2]. Over the past decade, an ever-increasing number of gut bacterial isolates have been collected worldwide[3–6]. These collections are invaluable for experimentally validating metagenomics-based predictions and for testing new hypotheses. Moreover, they provide a unique starting point for investigating poorly studied and newly isolated microbes as well as for characterizing their interactions with each other and their surroundings.

Decades of sequencing-based microbiome research have revealed a long list of small molecules that potentially interact with the human gut microbiome. These molecules include drugs, natural products, host factors and environmental pollutants[7]. Whether these compounds act directly on the gut microbiota by affecting bacterial physiology remains largely unknown. Yet, this is essential information toward preventing the collateral damage such compounds may cause to the gut microbiome or to repurpose them as microbiome therapeutics. To assess direct compound-microbiota interactions, high-throughput methods that allow the study of hundreds of small molecules on a broad spectrum of gut microbes are needed. Importantly, massively parallel testing requires cheap, simple and scalable yet robust assays, such as quantitative growth assays in multiwell plates. Measuring bacterial growth in monocultures by using OD measurements is a widely used method in microbiology, and protocols are well established for the analysis of microbes under regular laboratory conditions. However, for the phenotypic analysis of gut microbes, standard protocols need to be modified to account for the fact that the assays must be performed under anaerobic conditions.

By working with monocultures, the direct effects of drugs on individual gut bacteria can be studied. In contrast, microbial communities offer the advantage of accounting for inter-species interactions and thus better mimicking the in vivo effects of drugs on the microbiome. In the context of communities, the phenotypic responses reflect the combination of primary and higher-order effects resulting from direct inhibition of particular species as well as from altered ecological interactions. For example, novel niches could be created through an altered metabolic landscape due to drug bioaccumulation or drug detoxification, which in turn might affect the community composition. We and others have used various strategies to study the interactions of drugs with microbial communities[8–14], including the construction of defined, synthetic communities, as well as the use of complex communities derived from stool samples. Synthetic communities are bottom-up assembled from pure cultures; they have the advantage of consisting of defined members that can be characterized in monoculture. By comparing responses in pure monocultures with responses in synthetic communities, community-related characteristics such as cross-sensitivity and cross-resistance can be studied. The modular design of synthetic communities makes them experimentally amenable, because organisms can be selectively added, removed or genetically modified, thus facilitating the evaluation of causal relationships. Alternatively, communities can be derived from fecal samples of healthy or diseased donors. Stool-derived ex vivo communities are particularly useful to assess the genetic diversity and richness of a person's microbiome or to evaluate inter-individual variation such as in drug-microbiome interactions.

One limitation of using OD measurements when working with microbial communities is that they provide information only on the effect of a compound on the growth of the community as a whole. Therefore, sequencing-based compositional profiling is required to map the consequences of the disturbances on the individual community members. The inclusion of such analyses in the experimental design usually comes at the expense of sample throughput and cost.

Here, we describe a protocol for studying the effects of drugs on individual gut microbes or microbial communities in vitro. Consequently, we have subdivided this protocol into two major sections. In the first (Steps 1–28), we describe how to test the effects of drugs on the growth of individual anaerobic bacterial strains in a high-throughput manner. As an example, we describe a recently published effort, in which we tested the effect of ~1,200 Food and Drug Administration (FDA)-approved drugs (the Prestwick library) on a selection of 40 representative

bacterial isolates of the human gut microbiome, predominantly type strains[15]. In the second section (Steps 29–50), we extend our protocol to the analysis of microbial communities. As an example, we describe the steps to assess the effects of a set of drugs in both bottom-up assembled synthetic communities and stool-derived communities from different donors[8]. This comprehensive protocol can be adapted to other small molecules and to microaerophilic microbes and their communities. It can be further extended to maximize relevance for in vivo conditions, for instance, by including bacteria living in mucus. It can also be combined with several additional readouts, an example being the high-throughput investigation of the effects of drugs on bacteria in combination with bacterial metabolism and bioaccumulation of drugs[9,10,16].

Development of the protocol

Compared to working on a typical workbench, working in an anaerobic chamber is more time consuming and space limited. These factors are major obstacles to achieving high throughput. We considered the following points when developing the protocol to develop a reasonable workflow despite these constraints. Whenever possible, steps are performed or carefully prepared outside the anaerobic chamber. Work inside the chamber is automated as much as possible. This protocol uses 96-well plates, because this format reliably supports growth of a wide range of bacterial isolates. Using 384-well plates is possible, but in our experience, it can reduce growth of certain bacterial species because of differences in liquid-solid interaction surface and evaporation over time and demands more precise pipetting in the anaerobic chamber.

To facilitate microplate handling, we installed a microplate stacker coupled to a microplate spectrophotometer inside the anaerobic chamber. A stacker provides rapid loading, unloading and restacking and continuous feeding of microplates into the microplate reader while having a small footprint. To maintain a constant incubation temperature of all plates at 37 °C, we built an incubator around the stacker-plate reader combination (see Extended Data Fig. 1 and Box 1). We chose a modular design that allows the individual parts to be introduced through the equipment entry port of the anaerobic chamber. The incubator can then be assembled around the stacker-plate reader combination inside the chamber. This setup enables up to 20 microtiter plates to be routinely handled at once and allows nearly 2,000 anaerobic growth curves, with hourly measurement intervals, to be recorded simultaneously[15].

Similarly, we recommend the installation of a small liquid-handling robot (e.g., epMotion96) inside the anaerobic chamber to facilitate higher pipetting reproducibility.

When working with bacterial communities, especially when performing sequencing-based readouts, we use deep-well plates, which offer larger volumes, and use protocols with more complex steps, which decreases the throughput[8].

Applications of the method

We have successfully used this protocol to study the effects of FDA-approved drugs at various concentrations on multiple gut microbes, both in monoculture and in a small number of microbial communities[8,15]. Numerous adaptations, extensions and alternative applications of the protocol are conceivable. First, the drug concentrations in our protocol were chosen so that, on average, they correspond to physiologically relevant drug concentrations in the colon. However, the protocol can easily be adapted to test multiple drug concentrations in serial dilutions[15]. Second, the approach can also be used to test combinations of drugs across concentration ranges in so-called checkerboard assays[8]. Third, the protocol is not restricted to testing drugs; in principle, any compound that is stable under the experimental conditions can be tested. For instance, the setup can be used to study the inhibitory effect of natural compounds (such as polyphenols), xenobiotics (such as food additives or environmental pollutants) and bacterial or host-derived metabolites (such as bile acids[17] or antimicrobial peptides) on anaerobic bacteria. Furthermore, the protocol can be applied to perturbations such as pH, salinity[18], temperature[19] and osmotic stresses[20]. Fourth, the protocol is applicable to all facultative and strict anaerobic bacteria and other microbes (e.g., fungi) that grow robustly in multiwell plates in anaerobiosis. In addition to species collections, different isolates of

BOX 1

Recommendations for building a custom incubator around the plate reader and the stacker

Reproducible bacterial growth requires constant growth conditions, including stable temperature. Even a short temperature shift can lead to condensation effects on the screening plates, which result in artefacts in growth curves.

To ensure constant temperature during the acquisition of the growth curves, the EMBL mechanical workshop has constructed an incubator that surrounds both the plate reader and the stacker, as well as the interface between the two devices (Extended Data Fig. 1). Because of its modular design, it fits through the equipment entry port of the anaerobic chamber and can be assembled inside the chamber around the plate reader and stacker. The plate reader is only half integrated into the incubator, to have more working space for preparatory work.

The incubator consists of an aluminum profile frame with black PVC panels as insulation. The black color serves to protect light-sensitive substances and growth media. The panels are screwed to the frame, except for the two front panels, which can be fixed with magnets. This allows the front to be completely removed, making it easier to operate and load the stacker and reader. A shelf above the plate reader allows incubation of culture tubes and Petri dishes. All corners and edges must be rounded to protect the flexible vinyl wall of the chamber from possible damage. The base plate must be sufficiently robust so that precise alignment of the equipment is guaranteed.

Precise and constant temperatures inside the incubator are achieved by a digital temperature controller (Omron, E5CC-QX3D5M-000) together with a 400-W semiconductor fan heater (Stego, product number 02810.0-01). An adjustable axial compact circulation fan (4114 NH5 DC) is used for homogeneous temperature distribution in combination with a pulse width modulation control module (HDS, SN00009E20A) for speed control of the fan. The PT100 temperature sensor (Omega Engineering, P-U-MAGNET-A-TS-2) is placed next to the stacker to ensure that the set temperature at which bacterial growth takes place is reached.

Before the incubator is ready for use, temperature stability over time throughout the incubator must be checked. In addition, we recommend testing the growth curves in microtiter plates at different positions within the plates and within the stack for reproducible growth.

The prototype incubator of the EMBL workshop has been running reliably for years without interruption. In the meantime, various versions have been produced, for example, to integrate new product developments (Biotek EON, Epoch2 and Synergy H1). Other workshops independently of EMBL have replicated the incubator design with alternative solutions for heating (e.g., by using a circulating heated water bath for heating) or by working with different materials (e.g., by using galvanized iron frames).

the same species and genetically modified strains can be investigated. In our case, bacterial species selection and community design are intended to represent the gut microbiome of healthy adults[15,21]. Other collections may be characteristic, for example, of a given individual's geographic location, specific age group, host species, disease type or body site.

Limitations

In our assay, growth readout is based on OD measurements in a plate reader. Therefore, the main limitation of our protocol is that it is restricted to bacterial strains that robustly grow in multiwell plates and whose growth can be monitored by OD measurements. This can be problematic with spiral-shaped bacteria like *Spirochaeta*. Growth curves must be reproducible across batches and independent of the position within the 96-well plates. Under the selected growth conditions, the strains cannot show any characteristics that interfere with absorbance measurements, such as formation of large aggregates and/or severe precipitation. Likewise, test

compounds must be sufficiently soluble and chemically stable in the growth medium, and their intrinsic color should not interfere with the absorbance measurements.

Ideally, strains and communities should reach stationary phase within 24–48 h, because evaporation is a critical problem with longer incubation times due to the small volumes in multiwell plates. Adding water-filled wells or increasing the humidity of the anaerobic chamber may potentially help in minimizing evaporation. However, it is important to note that excessive humidity can lead to the accumulation of condensation on the plate's seals, leading to unreliable OD measurements, and of liquid droplets in the chamber and the sleeves of the gloves, increasing the risk for contamination. If extended incubation periods are required, other options such as using sterilized mineral oil to cover the wells could be considered, albeit with the potential drawback of reducing experimental throughput. Reliable OD measurements and bacterial growth should also be verified in this modified setup.

When interpreting the results, it is important to note that the protocol is primarily designed to detect growth inhibition, and it does not allow the determination of whether the tested molecule has a bactericidal or bacteriostatic effect. We use a complex and rich growth medium, mGAM, that allows cultivation of a wide range of gut bacteria and has been repeatedly shown to mimic the conditions in which bacterial growth occurs in vivo in the human gut[9,21]. Under conditions supporting strong growth, even minor inhibitory effects of test compounds are reliably detected, whereas growth promotion is rarely observed. When testing whether a compound can be used as a nutrient source for a particular bacterial strain, it is advisable to work with a minimal or defined medium[21], in which individual components can be replaced by the test compound.

Finally, our protocol is subject to the limitations of in vitro approaches. Testing relevant concentration ranges depends critically on the available data on physiologically occurring concentrations at the body site in question. In vitro cultures do not fully represent the complexity of microbial communities in the gut, where they interact with their host in a structured gut environment. As a result, important interactions can be missed. Key findings should therefore be complemented by in vivo methods to validate their relevance to human health.

Currently available alternative methods

In addition to broth-based approaches, disc diffusion methods are commonly used to assess drug susceptibility. In these techniques, antibiotic test discs or strips are placed on inoculated agar, and, after incubation, the resulting inhibition zone around the disc or strip indicates the sensitivity of the bacterial strain to the drug. We conducted a systematic comparison of the two methods for 815 combinations of antibiotics and human gut bacteria[8]. Despite experimental differences, the screening approach showed high specificity (97%) and sensitivity (97%) when benchmarked against the strip-based minimal inhibitory concentration (MIC) dataset. However, the diffusion-based approach has major drawbacks, such as a substantially lower throughput and a higher cost.

Various alternative methods for testing drug sensitivity, such as isothermal microcalorimetry or nanomotion-based measurements[22], have emerged. Currently, these methods still suffer from limitations such as lower throughput and insufficient testing under anaerobic conditions.

An increase in throughput can be achieved by reducing the test volume with the use of promising new techniques like droplet-based microfluidics[23]. Unfortunately, the complexity of mastering emulsion techniques and the necessity for specific microfluidic chips currently hinder the widespread adoption of droplet technology.

Experimental design

Overall workflow

The entire workflow of this protocol is divided into five sections (Fig. 1). In the first section (Steps 1–4 and 29–31), drug master plates are prepared; this includes the optimization of the plate layout with possible rearrangement of some compounds (Fig. 2). In the second section

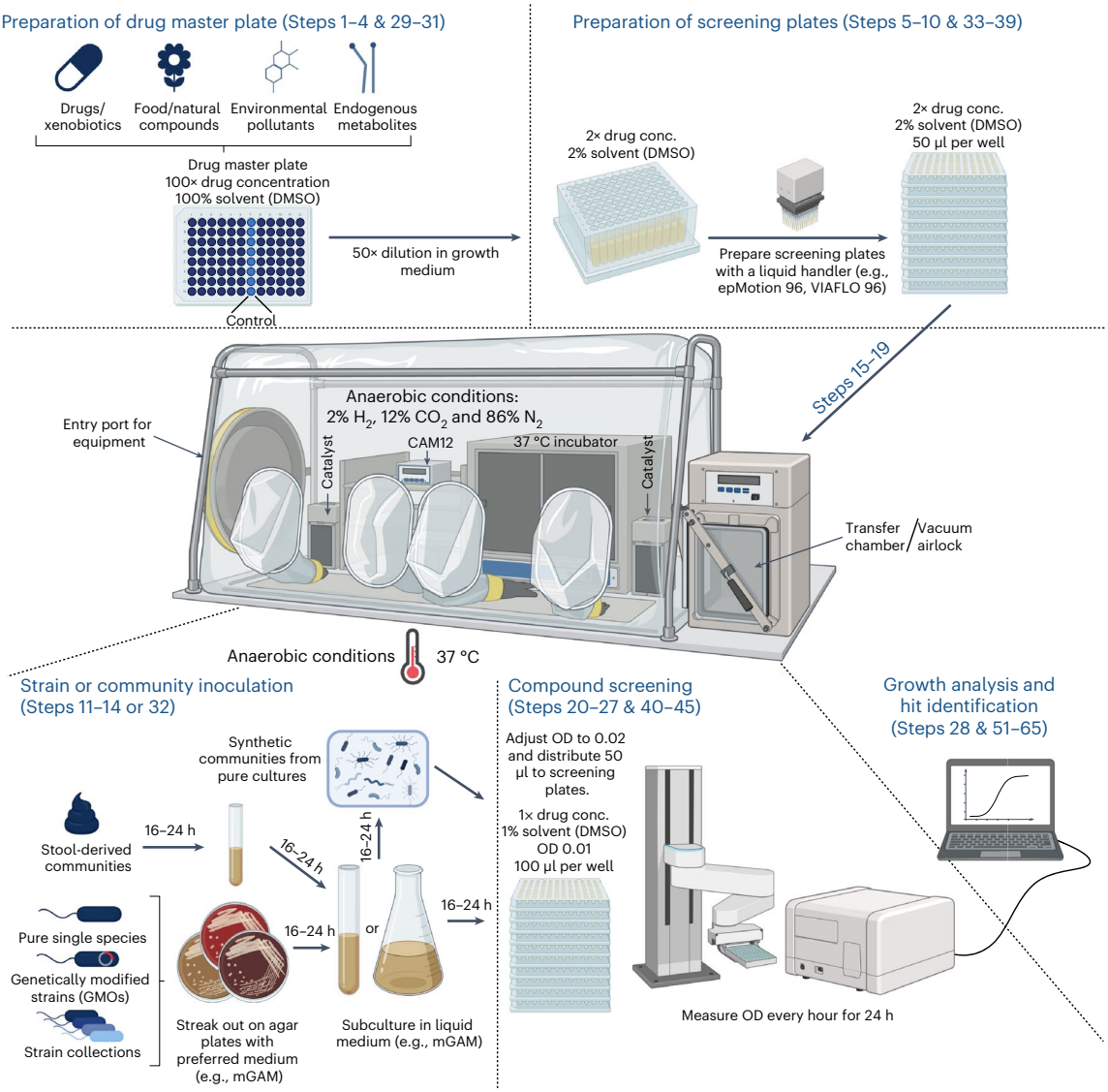


Fig. 1 | Overview of the workflow. The high-throughput screening is conducted in an anaerobic chamber. Various compounds, including drugs, natural compounds, environmental pollutants and endogenous metabolites, can be screened. For preparation of drug master plates, compounds of interest are dissolved in a solvent (e.g., dimethyl sulfoxide (DMSO)) and arrayed in a 96-well plate at 100× the desired assay concentration. For preparation of screening plates, sufficient replicates of 96-well screening plates (50 µl) with 2× assay concentration are prepared from drug master plates, allowing testing of all strains in replicates. For strain or community inoculation, this protocol is suitable for bacterial monocultures and synthetic- and stool-derived communities. For compound screening, the screening plates are inoculated with 50 µl of bacterial culture, and growth curves are acquired by hourly measurement of OD with the help of a microwell plate reader and a matching stacking device. For growth analysis and hit identification, resulting growth measurements are then analyzed with the R package 'neckaR'. Solvent concentrations in percent (e.g. 100% solvent (DMSO)) were determined as vol/vol. conc., concentration; GMO, genetically modified organism. Figure created with BioRender.com.

(Steps 5–10, 15–19 and 33–39), screening plates are prepared by diluting the drug master plates in the respective growth medium. In the third section (Steps 11–14 and 32A and B), bacterial monocultures, synthetic communities or stool-derived communities are prepared from frozen stocks, adapted to the growth medium of the screen and diluted to the starting OD. In the fourth section (Steps 20–27 and 40–45), the screening plates are inoculated with bacterial cultures, and growth curves are recorded, with the possibility of sampling for subsequent 16S rRNA gene amplicon or metagenomic sequencing, in the case that bacterial communities are being evaluated (Fig. 3). The fourth section is repeated in sufficient numbers of replicates for all

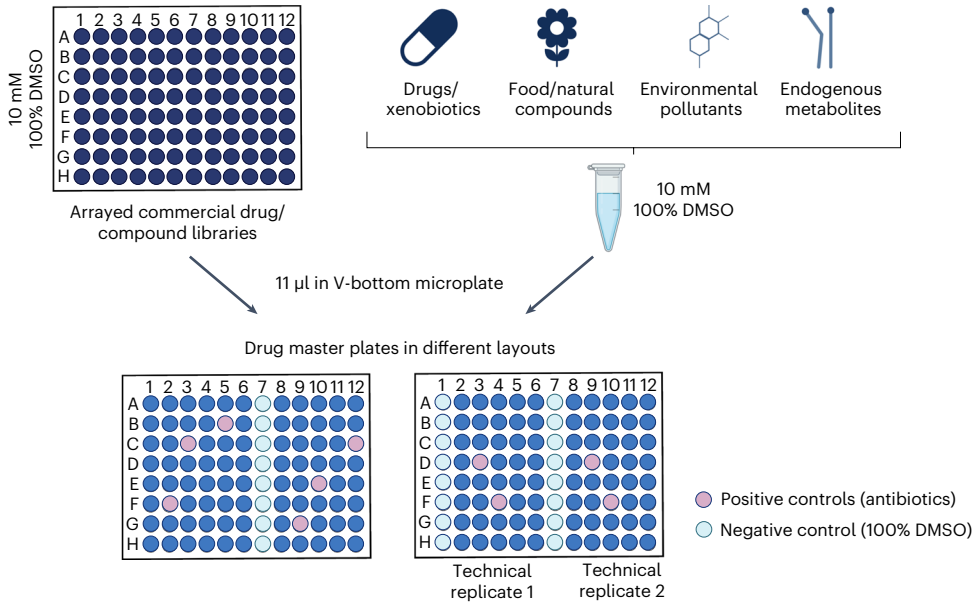


Fig. 2 | Preparation of drug master plates. Drug screens can be based on commercially available compound libraries or be custom-made by using a selection of compounds of interest. In both cases, compounds need to be soluble (e.g., in DMSO) at 100× assay concentration. Commercial libraries need to be diluted accordingly. Once all compounds are dissolved at the right concentration, a 96-well drug master plate, including negative and positive controls, can be designed. For commercial compound libraries, a rearrangement of the compound within the plate might be necessary. Solvent concentrations in percent (e.g. 100% solvent (DMSO)) were determined as vol/vol. Figure created with BioRender.com.

strains and communities to be studied. Finally, in the fifth section (Steps 28 and 51–65), bacterial growth curves and sequencing reads are analyzed, and hit compounds are identified by using appropriate statistical tests (Fig. 4).

Design of drug master plates

Drug master plates must be designed to minimize experimental variability, maximize throughput and facilitate data analysis. We therefore recommend that all wells in a plate are inoculated with the same bacterial strain or community to simplify liquid handling, avoid cross-contamination and optimize the number and position of control wells. To ensure the stability of the compounds, drug master plates are stored in solvents rather than in the growth medium. They should be prepared at 100-fold the working concentration to reduce the impact of the solvent on bacterial growth. We recommend avoiding pipetting steps with small volumes (i.e., <5 µl), to prevent inaccuracies and imprecisions leading to variation in drug concentrations. In this protocol, antibiotics are used as positive controls to validate the assay, while solvent controls provide a baseline for unperturbed growth. In addition, plates should contain positions with sterile medium controls that are not inoculated with bacteria but serve as contamination controls.

Depending on the planned readouts and the specific research question, the design of the master drug plates may require additional considerations (Fig. 2). In the monoculture setup, we investigated the effect of ~1,200 drugs from the Prestwick library, a commercial compound library, which required fourteen 96-well drug master plates. The library was supplied in 96-well format, and all compounds were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. To facilitate further aliquots, the library was re-arranged on 14 plates, where compounds were diluted in DMSO to 100-fold the assay concentration of 20 µM (i.e., to 2 mM). We re-arrayed compounds to minimize the total number of 96-well plates while still having sufficient control wells (all wells of column 7). From each master drug plate, we prepared 10 replicates of screening plates (2× assay concentration, in mGAM). In each experiment, we tested all drugs on one strain by inoculating all 14 different screening plates with one overnight (O/N) culture.

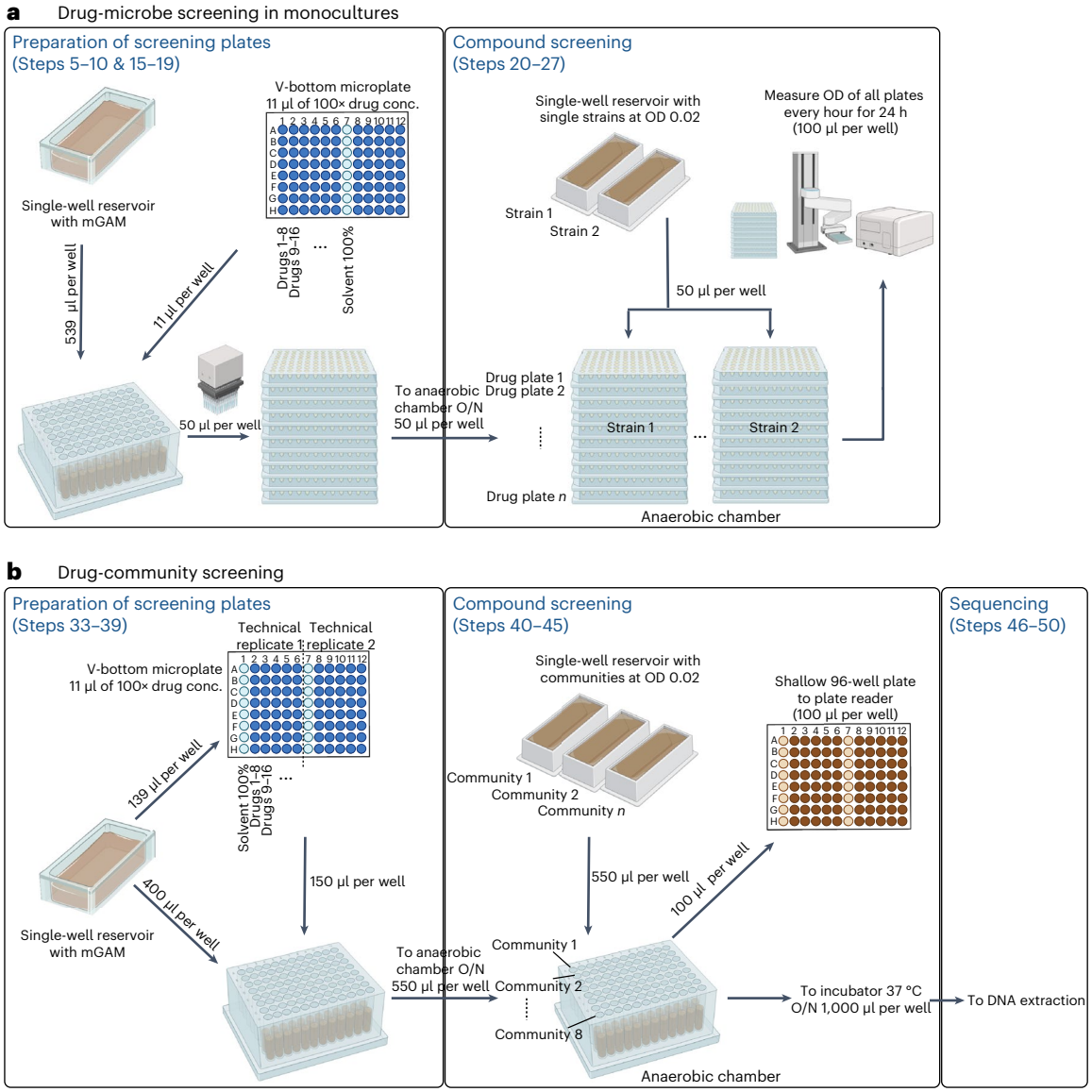


Fig. 3 | Workflow for compound screening assay in monoculture and community setups. a, Left: To prepare screening plates (containing drugs at 2× assay concentration in mGAM), 11 µl of the drugs from drug master plates (at 100× assay concentration, in DMSO) are transferred to a deep-well plate containing 539 µl of mGAM. After mixing, the diluted drugs are divided by transferring 50-µl aliquots into 96-well round-bottom plates. Plates can be stored at −20 °C until usage. In the figure, the process is shown for one drug master plate. However, it must be carried out for each drug master plate (i.e., for the 14 plates of the Prestwick library 14 times). Right: For compound screening, the overnight (O/N) cultures of the strains (Steps 13 and 14) are adjusted to an OD_{578} of 0.02 and filled into a single-well reservoir, and 50 µl per well is transferred into the pre-reduced screening plates. The plates are sealed with a breathable membrane by using a roller and loaded into the stacker for parallel growth curve measurements. **b**, Left: On the day before the compound screening assay, screening plates are prepared from the drug master plates. The drug plate is designed to contain two replicates of a control column with only solvent and four columns with drugs. First, mGAM is added to both a deep-well plate (400 µl per well) and the drug master plate (V-bottom plate, containing drugs at 100× assay concentration, 100% DMSO) (139 µl per well) by using a liquid handler or a multichannel pipette. The drug master plate was previously prepared by following Steps 29–31. The mGAM added to the drug master plates is mixed well with the drugs, and the full contents (150 µl per well) are transferred to the deep-well plate. The latter is sealed with a breathable membrane by using a roller and placed overnight in the anaerobic chamber for pre-reduction (Steps 33–39). Middle: On the day of the compound screen, communities (Step 32A for bottom-up assembled communities and Step 32B for stool-derived communities) are diluted in mGAM to reach an OD_{578} of 0.02. Diluted communities are added to each row of the deep-well plate (550 µl per well) and mixed thoroughly with the drugs in the plate. 100 µl of the contents of this deep-well plate is transferred to a 96-well round-bottom plate, which is used to monitor the growth of the community during 24 h at 37 °C in the plate reader. The remaining cultures of the deep-well plate are incubated at the same temperature (Steps 40–45). Right: After an incubation of 24 h, the deep-well plate is removed from the anaerobic chamber to harvest the bacterial pellets. The pellets are then further processed for DNA extraction and sequencing (Steps 46–50). Figure created with BioRender.com.

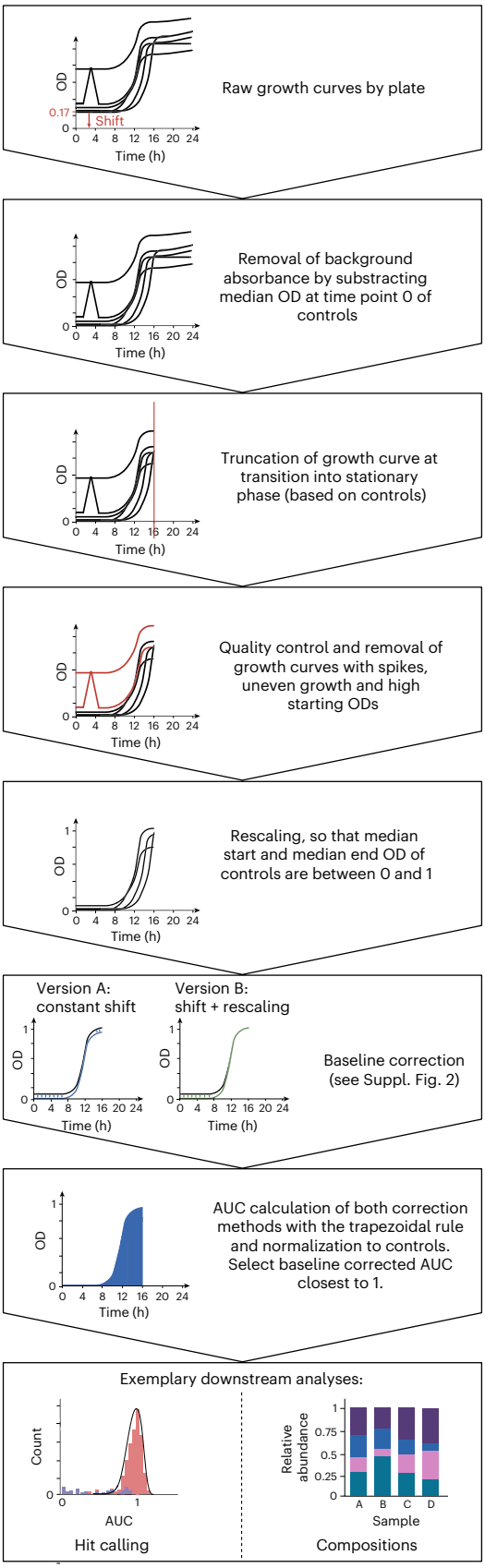


Fig. 4 | Schematic view of the data-analysis workflow of the R package neckaR. To account for inter-plate variation, each plate is analyzed separately. Curves are background-corrected by subtracting the median value of the controls at the first time point from all values. Then, curves are automatically truncated at the transition from exponential to stationary phase under control conditions and quality-controlled by discarding curves with sudden spikes and missing time points. Control growth curves are rescaled to a final OD_{578} of 1. This step is necessary to compare strains with different growth characteristics. We implement two methods to correct for baseline variations: assuming either a constant or a decreasing offset; the more conservative estimate is used (Extended Data Fig. 5). The area under the curves (AUCs) are calculated to account for effects on lag phase, growth rate and stationary phase plateau. Finally, median control AUCs within each plate are used to normalize AUCs. Further downstream analysis depends on the setup. In the case of the Prestwick library screen in monocultures, we identified hits from normalized AUC measurements by fitting heavy-tailed distributions, specifically the scaled Student's *t*-distribution[29], to the wells containing controls. These distributions capture the range of AUCs expected for compounds that did not affect growth and served as the null hypothesis for determining if a drug caused a growth defect in a given strain. *P* values for each drug and strain were then combined across replicates by using Fisher's method. We calculated the false discovery rate (FDR) by using the Benjamini-Hochberg method[30] over the complete matrix of *P* values, which consisted of 1,197 compounds by 40 strains. After evaluating representative AUCs for compound-strain pairs at different FDR levels, we selected a conservative FDR cutoff of 0.01. In the case of the community setup, the analysis of the growth curve is complemented by an analysis of the composition by 16S rRNA sequencing (beyond the scope of this protocol). Figure created with BioRender.com.

For smaller compound libraries that can be arranged on fewer master drug plates, several strains can be tested in one experimental batch. However, the total number of screening plates should not exceed 20 plates per experiment so that the OD can be measured hourly. In our assay, many compounds did not have an effect on bacterial growth and could therefore be used to calculate a distribution of areas under the growth curves (AUCs) for normal growth and to calculate *P* values on the basis of this distribution. If the library is enriched for compounds that inhibit growth, more negative control wells are needed to be able to distinguish slight growth inhibition from random variations in the AUC.

In the community setup, with expensive sequencing-based readouts, drug master plates required a more condensed layout at the expense of control wells. In this case, less stringent ways of hit-calling need to be used, for example, by setting a threshold for relevant growth reduction. We usually select a cutoff of 0.75 (i.e., 25% growth reduction compared to controls). Here, stringency and significance need to be carefully balanced to minimize the risk of false positives while still detecting real effects.

Before proceeding with drug screening, validating the master plate design by conducting pilot experiments is recommended. These small-scale pilot tests help to verify the overall procedure (e.g., sufficient number of mixing steps), identify potential problems (e.g., with logistics, precipitation of compounds in media and contamination risks) and refine the statistical analysis (e.g., number of control wells). Clear documentation of the layout and organization of the master plates, including the positions of samples, controls, replicates and any other relevant information, is required for data analysis and interpretation.

Good practice when working anaerobically

Prior experience working in an anaerobic chamber is necessary for users to effectively carry out this protocol. Users must adapt to and become comfortable with the confined space of the anaerobic chamber, which may involve standing extended periods of time without much movement or dealing with glove sleeves at an uncomfortable height. In addition, users must navigate the challenges of working with bulky gloves, which diminish the sense of touch and make precise movements difficult. We recommend that users familiarize themselves with the chamber by performing minor tasks and gaining practice with the bulky gloves before undertaking this high-throughput method.

This is a protocol designed to study the growth of strict anaerobes; thus, it is important that all molecular oxygen associated with materials and media is eliminated before being used in an experiment. Glassware should be placed inside the anaerobic chamber for a minimum of 24 h, whereas plastic consumables and large volumes of liquids should be pre-reduced for a minimum of 48 h before being used in experiments. Ensure that enclosed containers (e.g., Schott bottles, Falcon tubes and drug plates) are opened after being brought into the anaerobic chamber and are not tightly closed afterwards, to allow for gas exchange. Large volumes (>200 ml) should be stirred. It is highly recommended that the time needed for materials and liquids to become anaerobic be assessed by using redox indicator dyes (e.g., resazurin). Times might differ from the ones stated here.

Although we have not tested it systematically ourselves, it is conceivable that after plates have been inoculated with bacteria in the anaerobic chamber, bacterial growth could be assessed in a plate reader located outside the anaerobic chamber. This would require sealing the plates with optically transparent, gas-impermeable seals. This approach should be tested beforehand, because strict anaerobes might not grow, and lack of gas exchange might affect the reliability of OD measurements.

The atmosphere within the anaerobic chamber is a high-efficiency particulate air–filtered semi-sterile environment (it is not a clean bench). It is mandatory to disinfect the working area, including all equipment and pipettes, with a disinfectant suitable for vinyl anaerobic chambers (see Reagent setup). When working with different strains and communities, all surfaces should be disinfected between experiments to prevent contamination of media or cross-contamination of strains. All experiments should include a blank medium control from the same medium batch to rule out bacterial contamination of the medium. Ideally, all drug plates should contain

blank medium control wells (i.e., not inoculated with bacteria); at a minimum, one plate per experiment should contain such wells.

Considerations for bacterial strains

Bacterial growth can vary strongly between different experimental setups, plate format and strains. Before the experiments and for each strain, growth conditions must be optimized to ensure that growth curves are robust, reproducible and independent of plate and stack position. Optimization should be done in the same setup as used for screening (e.g., using the same plates, volume and solvent). The adjustable parameters include the growth medium, shaking time and intensity before OD measurement and starting ODs. For human gut bacteria, we recommend mGAM as growth medium because it best mimics bacterial growth conditions in vivo[9,21] and supports growth of phylogenetically diverse species. In our experiments, the strains were shaken for 1 min at a moderate intensity (567 cycles per minute (cpm)) immediately before measuring the OD. This duration and intensity were effective in maintaining a uniformly distributed bacterial culture within the well. However, for strains that tend to sediment or form clumps, alternative shaking conditions are required, such as continuous shaking or shaking at shorter intervals. With our shaking conditions, it was possible to measure the OD of twenty 96-well plates on an hourly basis. Once conditions are optimized for a strain, the incubation times in the protocol should be adjusted accordingly.

Considerations for bacterial communities

The members of synthetic communities are usually limited to a small, manageable number and are defined at the strain level (Extended Data Table 1 and Extended Data Fig. 2). The number of strains to be included depends on the specific research question and can range from as few as 2 to >100 strains[24]. Merely pooling of a certain number of strains does not guarantee that all of them will be present in the community, at least to detectable levels. For example, we constructed a community of 31 different strains, and after 24 h of growth, only 21 of them were detectable in a reproducible way[12]. Thus, community assembly needs to be checked beforehand. The choice of strains to include also depends on the research objectives. Communities can be tailored to have high diversity, spanning various taxonomic domains, or low diversity, such as focusing on bacteria from a specific genus. Ideally, they differ sufficiently in their 16S rRNA gene sequence to unambiguously determine their abundance after drug treatment by 16S rRNA gene amplicon sequencing (>3% in the V4 region in our case). For taxonomic assignment, we recommend the use of bespoke databases containing only reference sequences of community members. In cases in which the strains do not exhibit sufficient variation in their 16S rRNA sequences, alternative methods like metagenomics must be used to determine their abundance and taxonomic identification.

To investigate the effect of drugs on bottom-up assembled gut bacterial communities, multiple community assembly approaches can be used, including letting the community stabilize and only then beginning drug treatment or mixing the community members and starting drug treatment immediately. Community stabilization allows the determination of how perturbations affect bacteria-bacteria interactions that are already established[14]. Here, we used the second option, that is, to mix the community and start drug treatment immediately. This allows assessment of the treatment effect over low-abundant species that might be outcompeted during the stabilization phase of the untreated community[8,12].

Stool-derived communities represent undefined and diverse microbial ecosystems. Compared to the composition of fresh stool, stool-derived communities have lower alpha diversity; still, they are more complex than synthetic communities (Extended Data Fig. 3). To minimize the loss of diversity, we collected samples in house and swiftly transported them to the anaerobic chamber. This minimized oxygen exposure and the risk of overgrowth by aerotolerant organisms. In other settings, such as when samples are collected from patients, it is highly recommended that users optimize their collection methodology; collecting stools in a container equipped with an anaerobic generating sachet is a suggestion. Ready-to-use microbiome sampling kits, such as GutAlive (Microviable Therapeutics)[25] are commercially available. We underscore that all sampling steps, including sample storage temperature and

time until processing, should be carefully assessed for their consequences on bacterial viability and on maintaining the composition and diversity of the microbiome.

Stool samples can be collected only with the approval of an ethics board, by following a defined protocol and study design; informed consent must be obtained from all donors. The approval process may differ between countries, and the guidelines from the appropriate authorities must be followed. Stool samples should be treated by following the regulations for biosafety level 2 (BSL2) organisms.

Before testing drug effects on communities, their baseline characteristics should be assessed in pilot tests. This includes their exact composition after assembly, the time until stabilization, the possibility of working with glycerol stocks and the long-term stability of the glycerol stocks. Based on these characteristics, the screening protocol should be adapted (Extended Data Figs. 2 and 3). For example, in this protocol, the synthetic communities are cultured with drugs for 24 h. Depending on the synthetic community tested, treatment over several passages might increase reproducibility but might also select for resistant mutants, as we have verified with experiments in monoculture (S.G.-S., unpublished observations). We recommend pilot experiments for each synthetic community.

Regardless of the exact setup, we recommend monitoring the community growth by measuring the OD or alternative methods that provide similar information (e.g., weight of pellets and colony forming units (CFU) counting). This step allows transformation of the relative strain abundances obtained by sequencing into absolute abundances.

Considerations on the number of technical and biological replicates

Replicates can serve two purposes: first, to guard against technical variation, and second, to tease out the signal over the biological variation. When designing a screen, the number of biological and technical replicates should be carefully considered to ensure accuracy and reliability of results.

In this context, biological replicates refer to independent experiments starting from freshly taken samples or cryostocks and testing the effects of all compounds on a particular strain/community. The number of biological replicates required depends on the degree of biological variation within the samples, which may be strain dependent.

Technical replicates refer to repeated measurements within an experiment, for instance, quantifying the effect of the same drugs at the same concentration on the same plate on the same strain/community. The number of technical replicates required depends on the precision of the measurement and the variability of the sample, which is readout dependent (OD measurement versus 16S rRNA gene sequencing). OD measurement is a relatively simple approach with little variation, but rare events such as pipetting errors will occur and lead to apparently altered growth.

A greater number of biological and technical replicates can improve the ability to detect significant differences but must be weighed against practical considerations such as time, cost and feasibility. A large number of replicates will make it possible to detect slight growth inhibition, but in most cases, it will be preferable to increase the number of experimental conditions (e.g., tested drug concentrations) rather than increase the sensitivity for only one condition. For the Prestwick library screen, we tested three biological replicates. For the community experiments, we tested two biological replicates with two technical replicates per plate, because of the increased experimental effort in setting up the microbial community compared to the single-strain analysis.

Controls

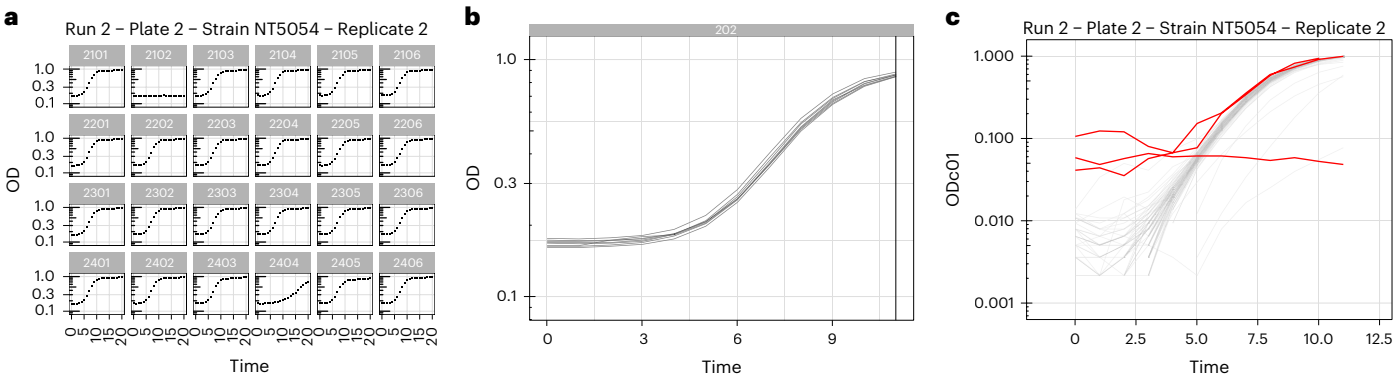
Throughout the protocol, controls have been designed to test for the robustness of the results and to aid in the identification of experimental issues.

- *Negative (solvent) controls*. They are designed to test the effect of the compounds independently, disregarding any potential effect of the solvent on the growth of gut bacteria. These are prepared in the 100× drug plates, by adding the same volume of solvent as that of the test compounds.

- *Blank medium controls*. They are designed to test any potential contamination of the medium. These are prepared the same way as the negative (solvent) controls, except that bacterial cultures will not be grown on them. The medium controls should not turn turbid in the course of the experiment.
- *Positive test compound controls*. These wells contain test compounds for which the growth behavior of the bacteria is known a priori. These are also prepared in the 100× drug plates and randomly distributed on the plate.
- *Community assembly controls*. They are designed to evaluate community composition at time 0. These are especially useful to characterize the starting community composition of bottom-up assembled communities and to verify that all members have been added at similar ratios. Their preparation is part of the protocol for assembling a bacterial community (see Step 32), bearing in mind that ≥100 ml of excess volume must be prepared to obtain sufficient material for sequencing.

Growth curve analysis

After acquisition of the growth curves by the software of the plate reader, thousands of growth curves need to be quality checked, baseline corrected, normalized and analyzed to detect treatment effects on the growth of microbial strains or communities (Fig. 4). This initial part of the analysis pipeline is independent of whether curves are from monocultures or communities and is included here as an R package called 'neckaR'. The software is based on our previously developed analysis pipeline[15]. It provides an open-source collection of functions for microbial growth-curve analysis designed to handle large data sets, allowing users to analyze multiple growth curves simultaneously and extract growth characteristics. Currently, the functionality of 'neckaR' includes loading and plotting OD measurements that were acquired by the Gen5 software (Fig. 5a), normalizing ODs to a user-specified control, calculating the



d

RRPPRCC	Drug	Drug_name	Replicate	Max_OD	Max_ODc01	cutoff_time	lag_time	normAUC
101102	Prestw-1	Azaguanine-8	1	0.739	0.81831290555155	11	4	0.728094850668894
201102	Prestw-1	Azaguanine-8	2	0.739	0.815770609318996	11	4	0.711757763398778
301102	Prestw-1	Azaguanine-8	3	0.729	0.802150537634409	12	5	0.708760932822353
110101	Prestw-1478	Rifaximin	1	0.179	0.009154929577465	12	11	0.003329160980677
210101	Prestw-1478	Rifaximin	2	0.167	0.002103786816269	12	11	0.004446849286777
310101	Prestw-1478	Rifaximin	3	0.164	−0.007741027445461	13	12	0.001834588049077
108708	Prestw-627	Folic acid	1	0.894	1.02471751412429	12	4	1.04021956153295
208708	Prestw-627	Folic acid	2	0.893	1.01118881118881	12	4	1.04216743430179
308708	Prestw-627	Folic acid	3	0.882	1.02582496413199	12	5	1.05484939061611

Fig. 5 | Graphs and tables produced by the 'neckaR' package for the analysis of microbial growth curves. The figure shows the different outputs produced by the 'neckaR' package during the growth curve processing workflow. **a**, Unprocessed OD values as obtained from the plate reader can be visually inspected. **b**, The transition from exponential to stationary growth phase is automatically determined and plotted. Growth curves are truncated to this transition point on the basis of the DMSO controls of a given plate. **c**, Curves with anomalous behavior (red) are detected and discarded. **d**, Users can export tables with the raw and adjusted maximum OD values, the end of lag and exponential phases and the normalized AUCs for each of the samples processed. The curves shown in **a**–**c** correspond to the same set of experiments from the same run, plate, strain and replicate. Source data for these panels are provided as Supplementary Information.

end of lag and exponential phases (Fig. 5b), detecting and removing anomalous growth curves (Fig. 5c) and calculating AUCs (Fig. 5d), maximum ODs and inhibitory concentrations. We strongly encourage users to browse the example vignettes and the documentation for the package, where detailed explanations of each of the steps and function parameters are provided.

The growth curves used in the present example and bundled with the 'neckaR' package correspond to a subset of the data from the supporting primary research article[15], specifically, to the assessment of the effect of a panel of 1,197 FDA-approved compounds on the single bacterial isolate *Bacteroides ovatus*. The step-by-step procedure as detailed here (Steps 51–65) encompasses the loading of the experimental design and plate reader files, the quality control of bacterial growth curves, the visualization of the curves and the calculation of the AUC.

Manually generated input tables. To analyze the optical density data obtained from the plate reader, the user needs to manually create two tables that allow the experimental design and the treatment layout to be linked with the actual OD measurements. These are the *Runs* table and the *Layout* table:

- The *Runs* table provides a means of matching the experimental design with plate reader data by assigning each plate an identifier and associating it with the corresponding bacterial strain and technical or biological replicate number. This process is performed for each experimental run or set of experiments, with each variable stored in its own column. The example data included in the 'neckaR' package comprises three runs (listed under the Runs column, labeled '1' through '3'), with each run consisting of 14 plates (listed under the Plates column, labeled '1' through '14'). Each plate features a single strain (listed under the Strain column, labeled with the internal laboratory ID NT5054) and an identifier of the layout of treatments across the wells (listed under the Design column, labeled 'Plate 1' through 'Plate 14'). Experiments were conducted in triplicate (indicated in the Replicate column, labeled '1' through '3').
- The *Layout* table contains the information concerning the allocation of treatments both across and within the plates. The data bundled with the 'neckaR' package contains 14 plate designs (listed under the design column, labeled as 'Plate 1' through 'Plate 14'). Within each plate design, each well features a specific treatment (listed under the drug and drug_name columns, labeled with the Prestwick library ID and common names, respectively). The well positions are denoted by a three-digit numeric code, with the hundreds digit indicating the row number (ranging from 1 to 8, rather than A to H) and the tens and unit digits denoting the column number (ranging from 01 to 12). For example, the well positioned at row B and column 11 is denoted as 211.

Materials

Reagents

Chemicals

- DMSO (Carl Roth, cat. no. A994.1; Chemical Abstracts Service (CAS) no. 67-68-5)
- *Drugs/compounds to test*. They can be purchased either from chemical vendors (e.g., Sigma and TCI Chemicals) or from commercially available arrayed drug/compound libraries (e.g., Prestwick Chemical Libraries and MedChemExpress Screening Libraries).
  ▲ **CRITICAL** Depending on the drug/compound, solvents other than DMSO might be required (e.g., sterile distilled water, ethanol or sodium hydroxide). It is important to check drug/compound solubility in the desired solvent and upon dilution in growth medium.
- Glycerol (Carl Roth, cat. no. 3783.1; CAS no. 56-81-5)
- Palladium black (Sigma-Aldrich, cat. no. 520810; CAS no. 7440-05-3)
- Methanol (Honeywell, cat. no. 32213; CAS no. 67-65-1)

- Benzalkonium chloride solution (50% vol/vol) (Sigma-Aldrich, cat. no. 63249; CAS no. 63449-41-2)
- Cysteine hydrochloride (Sigma-Aldrich, cat. no. C7880; CAS no. 7048-04-6)
- Microcidal/sporicidal peracetic acid (PAA) wipes (Schuelke, cat. no. 70003205)
- Bleach (sodium hypochlorite concentration between 5% and 9% vol/vol; Sulpeco, cat. no. 105614; CAS no. 7681-52-9)

Media

- GAM broth modified (HyServe, cat. no. 5433)
- BD Columbia agar with 5% (vol/vol) sheep blood (COLS) plates (Thermo Scientific, cat. no. 10463833)
- Brain heart infusion agar (Oxoid; Thermo Scientific, cat. no. CM1136)
- BD BBL IsoVitaleX Enrichment BD 211876 (Fisher Scientific, cat. no. 11798163)
- Agar (Becton Dickinson, cat. no. 257353)
- 100 ml of sheep blood, defibrinated (Oxoid; Thermo Scientific, cat. no. SR0051C)

Kits (for sequencing-based analysis)

- DNeasy UltraClean 96 microbial kit (384) (Qiagen, cat. no. 10196-4) for the isolation of DNA from bacterial cultures
- DNeasy 96 PowerSoil Pro kit (384) (Qiagen, cat. no. 47017) for the isolation of DNA from fecal samples

Biological materials

Bacterial strains and communities

▲ **CRITICAL** When using single species or synthetic communities, purity and identity should be regularly checked by 16S rRNA gene sequencing or MALDI-TOF mass spectrometry[26].

- All strains used in this protocol were acquired from public culture collections such as the German Collection of Microorganisms and Cell Cultures (www.dsmz.de), the American Type Culture Collection (www.atcc.org) and the Biodefense and Emerging Infections Research Resources Repository (www.beiresources.org)[27]. However, other facultative or obligate anaerobic microorganisms can be tested, including their genetically modified derivatives.
- Synthetic communities can be assembled from these strains (Step 32A(i–iv)).
- Stool-derived communities can originate from healthy donors or patient cohorts.
  ▲ **CRITICAL** Such work has to be performed in accordance with the Declaration of Helsinki and must be approved by the responsible ethics committee. Informed consent must be obtained from all human research participants. Stool material collection was approved by the EMBL Bioethics Internal Advisory Committee, and informed consent was obtained from all donors (BIAC2015-009).

Equipment

- Vinyl anaerobic chamber (COY)
  ▲ **CRITICAL** To fit both the Epoch2 multi-plate reader and the BioStack 4 and to ensure enough space for preparatory work, the Type B anaerobic chamber is strongly recommended. The Type A anaerobic chamber can also fit the reader and the stacker, although the preparatory workspace is considerably reduced.
  ▲ **CRITICAL** Note that it is possible to use thinner gloves instead of those supplied by the chamber manufacturer. However, we do not recommend doing so, because even with thick gloves, the fingertips are prone to tears and small holes. The gloves must therefore be replaced regularly.
- 2× catalyst fan box (COY) with palladium catalyst and desiccant
- Anaerobic monitor (CAM12; COY)
- CO_2 controller (Model AC100; COY)
- Hydrogen sulfide removal column with trilayer (COY)
- Recirculating high-efficiency particulate air–filter–based atmosphere filtration system (COY)

- Incubator (37 °C)
  ▲ **CRITICAL** The incubator needs to fit both the stacker and the reader with space for additional incubation of bacterial cultures. Use a dark-colored incubator for light protection (Extended Data Fig. 1 and Box 1).
- (Semi-) automated liquid-handling systems with corresponding tips (e.g., epMotion 96 (Eppendorf), VIAFLO 96 (Integra) and Biomek i5/i7 (Beckman Coulter))
- Epoch2 with BioStack 4 (both Agilent)
  ▲ **CRITICAL** Optionally, any other microplate spectrophotometer that fits inside the anaerobic chamber and is able to run at 37 °C could be used. To increase throughput, a compatible stacking device is required.
- Cuvette spectrophotometer (a device that can measure multiple cuvettes simultaneously is optimal; e.g., GENESYS 140 UV-Vis (Thermo Scientific))
- Multichannel pipettes, sterile (5–50 and 20–200 µl)
- Pipettes, sterile (1–10, 10–100, 20–200 and 200–1,000 µl)
- Pipette controller
- Scissors and scalpel
  ▲ **CAUTION** Both should not have pointed ends, to prevent damaging the anaerobic chamber by accident.
- Plate centrifuge (e.g., Megafuge 16 (Thermo Scientific) and Heraeus Multifuge 3SR Plus (Thermo Scientific))
- Centrifuge for Falcon tubes (e.g., Heraeus Multifuge 3SR Plus (Thermo Scientific))
- Drying cabinet
- 13-mm manual crimper (Chromatography Research Supplies, cat. nos. 313990 and 313991)
- Polar Safe freezing block (PCR-tube rack and 1.5/2-ml rack; Carl Roth, cat. nos. EKT2.1 and EKT0.1, respectively)
- Precision balance (e.g., VWR, cat. no. PBP2202-15)
- Disperser system (ULTRA-TURRAX tube drive; VWR, cat. no. 431-2897)

Plasticware

- Breathe-Easy sealing membrane (Sigma-Aldrich, cat. no. Z380059)
  ▲ **CRITICAL** The membranes should be cut at the long end so that they fit onto the test plates without overlapping (size: 15.24 cm × 7.85 cm). Overlapping membrane parts must be removed and not glued to the sides of the plates. When the plates are stacked, excess membrane parts often cause the plates to stick together.
- Nunclon 96-well U-bottom microplate, sterile (Thermo Scientific, cat. no. 168136)
- Microplate, 96-well, polypropylene (PP), V-bottom, sterile (Greiner Bio-One, cat. no. 651261)
- Reservoirs, 25 ml (Carl Roth, cat. no. HT66.1)
- Falcon tubes, sterile (CELLSTAR, 15 and 50 ml; Greiner Bio-One, cat. nos. 188 271-N and 227 261, respectively)
- Pipette tips, sterile (1,000 µl: Ratiolab, cat. no. 2100610; 200 µl: Sarstedt, cat. no. 70.3030; 10 µl: Greiner Bio-One, cat. no. 771250)
- Stripettes (5, 10, 25 and 50 ml; Corning, cat. nos. 4051, 4101, 4251 and 4501, respectively)
- Cuvettes, plastic transparent (Sarstedt, cat. no. 67.742)
- SingleCap 8-SoftStrips, 0.2 ml (Biozym, cat. no. 710988)
- Petri dishes (Greiner Bio-One, cat. no. 633180)
- 96-well deep-well plates, sterile (Costar, cat. no. 3959)
- Aluminum foil seals (Beckman Coulter, cat. no. 538619)
- Sterile breathable sealing film (AeraSeal; Sigma-Aldrich, cat. no. A9224)
- Single-well reagent reservoir (Axygen, cat. no. RESSW1LP)
- Fisherbrand commode specimen collection system (Fisher Scientific, cat. no. 02-544-208)
- Disposable plastic sterile spatula (VWR, cat. no. 331-0371)
- Tube with rotor/stator element for ULTRA-TURRAX tube drive disperser (DT-50-M-gamma; VWR, cat. no. 531-0492)

Glassware

- Glass culture tubes (VWR, cat. no. 42775251), sterile, with loose-fitting metal cap (Schuett-Biotec, cat. no. 3.620 613; 16 mm × 30 mm) for bacterial culturing
- Serum vial, clear, 2 ml, neck size: 13-mm crimp (Ziemer Chromatographie, cat. no. 1.300012), to be closed with butyl stoppers R 13 (Ziemer Chromatographie, cat. no. 2.301192) and silver Alu Cap R 13-L (Ziemer Chromatographie, cat. no. 2.301012) for cryostocks, sterile
- Schott bottles, sterile
- Cylinders, sterile

Computer hardware

- A 64-bit computer with ≥8 GB of random-access memory running Windows, Linux or MacOS
- Adequate storage capacity (≥10 GB) to store the program, the input optical density data and the output data and graphs generated during processing
- An internet connection to download the required R packages

Computer software

- Spreadsheet software such as Microsoft Excel (https://www.office.com), Google Sheets (https://docs.google.com) or LibreOffice Calc (https://www.libreoffice.org)
- Base R version 4.0 or higher (https://www.r-project.org/) and an integrated development environment such as RStudio (https://posit.co/products/open-source/rstudio/) or Jupyter (https://jupyter.org/)
- Essential R library: the 'neckaR' package, available on GitHub (https://github.com/Lisa-Maier-Lab/neckaR)
- Multiple packages required by the 'neckaR' package as dependency. All dependencies of 'neckaR' are retrieved from the comprehensive R archive network (https://cran.r-project.org/) and are automatically installed together with 'neckaR'. The package devtools is needed for the installation of 'neckaR'. For the code executed in the present protocol, the R package 'dplyr' is explicitly required.
- Gen5 (version 3.05 or higher, Agilent) for Epoch2 with the BioStack 4
  ▲ **CRITICAL** If a different reader and stacker combination is used, the recommended software for the multiwell plate reader should be used instead.

Reagent setup

mGAM growth medium for gut anaerobes

Weigh 20.85 g of GAM broth modified powder into a 500-ml glass Schott bottle. Fill up with 500 ml of distilled water. Sterilize by autoclaving at 115 °C for 15 min. Prepared mGAM medium is an amber-colored clear solution.
▲ **CRITICAL** Do not autoclave at temperatures above 115 °C, because this will affect the medium. Protect from light. The medium should be used within 2 weeks.

mGAM agar plates

Weigh 20.85 g of GAM broth modified powder and 7.5 g of agar into a 500-ml glass Schott bottle. Fill up with 500 ml of distilled water. Sterilize by autoclaving at 115 °C for 15 min. Stir and pour plates.
▲ **CRITICAL** Do not autoclave at temperatures above 115 °C, because this will affect the medium. Protect from light. Agar plates should be stored at 4 °C and used within 2 weeks.

Brain heart infusion + 5% sheep blood agar plates (BH+ plates)

Suspend 47 g of brain heart infusion agar in 1 liter of distilled water and dissolve completely. Sterilize by autoclaving at 121 °C for 20 min. Cool to 73 °C and add 5% (vol/vol) sheep blood under sterile conditions. Stir and heat at 74 °C for 1 min. Let it cool to 50 °C and add one bottle of IsoVitaleX Enrichment (dissolved in 12 ml of diluent) under sterile conditions. Stir and pour plates.
▲ **CRITICAL** Agar plates should be stored at 4 °C and can be used for 2 weeks.

Freezing medium for anaerobes (glycerol (50% (vol/vol)) with palladium black)

Mix 25 ml of sterile distilled water with 25 ml of 100% (vol/vol) glycerol. Add two scoop tips of palladium black. Mix and sterilize by autoclaving at 121 °C for 15 min. This solution can be stored at room temperature (20–25 °C) for ≥6 months if the sterility is maintained.

Disinfectant for the anaerobic chamber

Mix 667 µl of benzalkonium chloride (50% solution) with 500 ml of sterile distilled water. The solution is stable under room temperature conditions for ≥6 weeks. Alternatively, bleach (sodium hypochlorite concentration between 5% and 9% vol/vol) can be used.

▲ **CAUTION** Use protective gloves, suitable clothes and chemical goggles for handling the benzalkonium chloride.

▲ **CAUTION** Use protective gloves for hand protection. Note the expiry date of the PAA wipes.

▲ **CAUTION** Do not use any disinfectant that is based on ethanol. Ethanol can damage the vinyl (polyvinyl chloride, PVC) tent of the anaerobic chamber.

▲ **CRITICAL** In addition to the disinfectant, sporicidal PAA wipes can be used to regularly disinfect the chamber.

Equipment setup

Anaerobic chamber

The anaerobic chamber provides an oxygen-free working atmosphere to allow for the cultivation of facultative and obligate anaerobes. Refer to the manufacturer's instructions for installation and operation of the anaerobic chamber, including the anaerobic monitor, atmosphere filtration system and catalyst fan boxes. It should always be ensured that the atmosphere comprises 86% nitrogen, 12% carbon dioxide (CO_2) and 2% hydrogen. Check the CO_2 controller and anaerobic monitor daily to ensure that CO_2 is at 12% and that hydrogen is ~1.9–2.3%. Note that the anaerobic monitor's displayed hydrogen value depends on the CO_2 concentration.

Many gut bacteria produce hydrogen sulfide (H_2S), which poses a threat to any electrical device inside the anaerobic chamber. Because high-throughput screening requires large amounts of bacterial cultures, a substantial amount of H_2S is produced. In particular, for communities of unknown composition (stool-derived communities), H_2S production can vary greatly between samples. It is critical to remove H_2S with a removal column (see Equipment), which should be installed inside the anaerobic chamber and be readily replenished[28].

The palladium catalysts of the two catalyst boxes are required to eliminate oxygen inside the chamber to 0–5 parts per million. They should be changed regularly, and the used catalysts should be restored in a drying cabinet at 200 °C for ≥5 h.

▲ **CAUTION** Under normal atmospheric pressure, hydrogen gas forms flammable (from 4.0–75%) or explosive (18–59%) mixtures with oxygen.

▲ **CRITICAL** Do not place anything on top of the catalyst boxes within the chamber; otherwise, the oxygen removal in the chamber will be hampered.

Plate reader and stacker for high-throughput screening

To perform high-throughput screening, a multiwell plate reader compatible with a stacking device is required (e.g., Epoch2 reader and BioStack 4; see Equipment). Both devices should be housed within an incubator (Extended Data Fig. 1 and Box 1). As an alternative, additional plate readers that allow for parallel screening can be installed inside the anaerobic chamber.

Before the first experiment, the settings of the plate reader should be configured according to the specifications in Table 1. Proper alignment of the reader and the stacker and stable temperature throughout the incubator should be verified.

Installation of the R package 'neckaR'

To install 'neckaR' and the required dependencies, follow the steps outlined below. Depending on the R packages already installed, the installation process can take up to 10 min. The following blocks of code can be executed directly from the R console, an R script and an RMarkdown

Table 1 | Gen5 3.12 settings for measuring growth curves and data export

Options		Settings
Startup options	Standard	–
Task manager	Protocols	Create new
	Select protocol type	Standard protocol
Define the plate brand in the system		
Select System (tab menu)	Plate types	Select your plate brand
Settings for measuring bacterial growth by using the stacker coupled to the plate reader		
Select protocol (tab menu)	Procedure	
Select steps	Set temperature	Incubator: On
		Temperature: 37 °C
		Gradient: 0 °C
		Select preheat before continuing with the next step
Select steps	Shake	Shake mode linear
		Duration: 01:00 (min:s)
		Linear frequency: 567 cpm
Select steps	Read	Detection method: absorbance
		Read type: endpoint/kinetic
		Optics type: monochromators
	Read step	Step label: <default>
		Wavelength: 578 nm
		Read speed: normal
Select steps	Options	Select discontinuous kinetic procedure
		Estimated total time: 1:00:00 (d:h:min)
		Estimated interval: 0:01:00 (d:h:min)
		Select eject plate when the procedure is finished
Settings for exporting the data		
Select protocol (tab menu)	Report/export builders	–
	New export to Excel	–
	Properties	Scope: plate
	–	Content: automatic
	Content	Select system description
		Select plate description
		Select procedure
		Select plate layout matrix
		Select raw data
		Select calculated data
		General format: matrix
		Select regroup data in one matrix/table when possible
		Kinetic/spectrum/scanning data format: column-wise table
	Workflow	Select all plates in the same workbook
		Select as a new worksheet
	Format	Select formatted text
		Select export row and column headers

notebook or from a Jupyter notebook with an R kernel. Start R and install 'neckaR' from GitHub with the following commands:

```
install.packages("devtools")
devtools::install_github("Lisa-Maier-Lab/neckaR")
```

To install the dplyr R package, execute the following command:

```
install.packages("dplyr")
```

▲ **CRITICAL** After the installation of the 'neckaR' package, restart the R session.

Procedure

▲ **CRITICAL** We have subdivided the Procedure into two major sections. In the first (Steps 1–28), we describe how to test the effects of drugs on the growth of individual anaerobic bacterial strains in a high-throughput manner. In the second section (Steps 29–50), we extend our protocol to the analysis of microbial communities. Steps 51–65 then cover the processing and analysis of microbial growth curve data, which is common to both sections.

Drug-microbe screening in monocultures

Preparation of drug master plates and screening plates (sterile conditions)

● **TIMING 2 d (1 h of hands-on time per drug master plate)**

1. Dissolve all compounds, if possible, in DMSO at 100× of the desired assay concentration (e.g., if a compound is to be screened at 20 µM, prepare a stock of 2 mM). In case a compound library in DMSO is used, dilute the whole plate to 100× of the desired assay concentration by using a liquid handler.
   ▲ **CRITICAL STEP** Arrange the plate so that the center of the plate (i.e., column 7) is free for the negative (solvent) controls. One of these (e.g., A7) can be dedicated to test for contamination of the medium (i.e., blank control). If positive controls are available, they should be randomly distributed across wells and plates (Fig. 2).
2. Use a 96-well V-bottom polypropylene plate and add 120 µl of the 100× drug stock solution to the corresponding wells and 120 µl of solvent (e.g., 120 µl of 100% DMSO vol/vol) to column 7. For pre-arrayed library plates, this step can be performed by a liquid handler. For custom-made libraries, this can be done manually.
3. To prepare 10 replicates of screening plates in V-bottom polypropylene plates, use a liquid handler or a multichannel pipette to transfer 11 µl from the wells of the multiwell plate from Step 2 into each of the 10 plates. This process enables the preparation of experimental batches of 10 replicates of screening plates at 2× drug concentration (see Step 7), a batch size that allows rapid use of these screening plates within a maximum of 3 weeks.
4. Seal the drug plates with an aluminum foil seal. Use a roller to ensure that each well is tightly sealed. Freeze the plates at −20 °C.
   ■ **PAUSE POINT** Drug master plates in DMSO are stable for a maximum of 3 months at −20 °C if not thawed.
5. Prepare a 96-well deep-well U-bottom plate with 539 µl of mGAM in each well by using a liquid handler.
6. Thaw the drug master plates from Step 4 and centrifuge them in a plate centrifuge (400*g*, 5 min, room temperature) to eliminate potential drops on the aluminum seal, which could lead to cross-contamination. Remove the aluminum foil seals.
   ▲ **CAUTION** Avoid cross-contamination by liquid splashes. Make sure that no drops remain on the aluminum seal after centrifugation. The plates should be pressed down firmly when removing the seal, and abrupt movements should be avoided. A pair of tweezers, a blade or other tools can be helpful.

7. Using a liquid handler, mix the drug master plate by pipetting and transfer 11 µl into the deep-well plate from Step 5 (50-fold dilution). The drug concentration is now 2× the assay concentration.
8. Mix the entire deep-well plate 5–10 times by using a liquid handler.
   ▲ **CRITICAL STEP** Ensure sufficient mixing. Otherwise, the final drug concentration will vary between different screening plates. The mixing process can be calibrated by diluting a dye in water, by using the same volumes and solvents described here.
9. Repeatedly distribute 50-µl aliquots from the deep-well plate into Nunclon 96-well U-bottom plates by using a liquid handler (a deep-well plate with 550-µl volume per well will allow the users to prepare 10 plates).
10. Seal the Nunclon 96-well U-bottom plates with an aluminum foil seal. Use a roller to ensure that each well is tightly sealed and put the lid back on. Freeze the plates at −20 °C.
    ▲ **CAUTION** If drugs are not stable under these conditions, this can lead to false-negative results.
    ■ **PAUSE POINT** Drug plates in mGAM are stable for a maximum of 3 weeks at −20 °C if not thawed. They should be thawed only once, just before use.

Preparation of bacterial stocks (anaerobic chamber)

● **TIMING 2 d (1 h of hands-on time per strain)**

11. Using a sterile pipette tip, select a single colony from an agar plate containing the strain of interest and inoculate it into 5 ml of pre-reduced mGAM medium (or other growth medium of choice) in a glass tube inside the anaerobic chamber. Incubate at 37 °C O/N.
    ▲ **CRITICAL STEP** All consumables and reagents must be pre-reduced before use.
12. After 16–24 h of incubation, bring a freezing block into the anaerobic chamber. Check the turbidity of the culture by eye as an indication of bacterial growth. Mix 4.8 ml of bacterial culture with 1.2 ml of freezing medium for anaerobes (see Reagent setup). Vortex gently and distribute aliquots of the mixture into 2-ml sterile glass vials for long-term storage. Close the vials with a butyl rubber stopper and an aluminum cap. Crimp the aluminum cap around the upper edge of the vial by using the crimper. In addition, distribute 100-µl aliquots into 0.2-ml strip tubes for single-use during screening (prepare as many single-use stocks as required). Place the stocks directly into the freezing block. Bring them immediately out of the chamber and freeze them at −80 °C for long-term storage.
    ▲ **CAUTION** Do not use ice or dry ice to cool the cryostocks inside the anaerobic chamber. Ice will melt and release dissolved oxygen into the chamber, while dry ice will sublimate and release CO_2, which will bloat the anaerobic chamber, change the mixture ratio of gases and interfere with the H_2 sensor.
    ■ **PAUSE POINT** Glycerol stocks in glass vials are stable for years at −80 °C if not thawed often. Stocks in plastic tubes are stable for ≥6 months but designed for single-use and should be discarded after use and not refrozen.

Strain inoculation (anaerobic chamber)

● **TIMING 3 d (30 min of hands-on time per strain)**

▲ **CRITICAL** All times refer to the day on which the compound screening assay will take place (in Step 20 or 40).

13. On day −2, streak out the single-use bacterial stocks onto mGAM agar plates inside the anaerobic chamber. Should the strain(s) not grow on mGAM agar plates, use their preferred medium (alternatives could be COLS agar or BH+ agar; see Media and Reagent setup). Incubate at 37 °C O/N.
    ▲ **CRITICAL STEP** Keep the cryostock in a freezing block when bringing it into the chamber. Discard the stock after use. Slow-growing strains may need longer incubation periods (e.g., 48 h). Therefore, it is important to know the growth behavior of the strains to be used beforehand.
14. After 16–24 h of subculture (on day −1), transfer a single colony into 5 ml of fresh mGAM medium (or the preferred medium). Incubate O/N (16–24 h) again at 37 °C.
    ▲ **CRITICAL STEP** Slow-growing strains may need longer incubation periods (e.g., 48 h).

Compound screening in monoculture setup (anaerobic chamber)

● **TIMING 2 d (2 h of hands-on time for one strain with 14 plates)**

15. The day before the start of the screening, thaw the screening plates (2× assay concentration, in mGAM) from Step 10 that are to be tested. Remove the lid outside the anaerobic chamber and disinfect it.
16. Centrifuge all plates without the lid and just the aluminum foil seal at 400*g* for 5 min to eliminate potential drops on the aluminum seal.
17. Remove the aluminum foil seal under sterile conditions.
    ▲ **CAUTION** Avoid cross-contamination by liquid splashes.
18. Using a spray bottle with a shortened straw filled with a small amount of methanol, blow methanol vapor over the plates where air bubbles are visible in the wells.
    ▲ **CAUTION** Use adequate protective wear (laboratory coat, gloves and goggles) when working with methanol, because it is highly toxic. Note the resilience time of your gloves against methanol.
    ▲ **CRITICAL STEP** The methanol vapor will pop the air bubbles. Air bubbles will cause problems during OD measurements because they affect optical measurements. Prevent methanol from leaking from the bottle, because this will affect the growth and survival of bacteria.
19. Close all plates with the lid and bring them into the anaerobic chamber ≥1 d (minimum: 16 h) before the screening assay to allow gas exchange. Cover all plates with standard aluminum foil to protect light-sensitive compounds.
20. On the next day (the screening day), measure the OD_{578} of the bacterial cultures from Step 14 by using a cuvette spectrophotometer. We recommend a cuvette spectrophotometer for this step, because the path length is typically standardized to 1 cm, whereas the path length in a microplate well is dependent on the volume and therefore more error prone.
    ▲ **CRITICAL STEP** Dilute the cultures if needed. Note the linear range of your photometer.
    ◆ **TROUBLESHOOTING**
21. Adjust the OD_{578} of each strain to 0.02 in mGAM. Note that for one plate, a volume of 5 ml is needed.
22. Transfer the bacterial dilution into a plastic reservoir and fill each well (except those dedicated to blank controls) of the pre-reduced screening plates from Step 19 with 50 µl of the diluted culture from Step 21. Fill the wells allocated for blank controls with 50 µl of mGAM. Ideally, this step should be performed by a liquid handler installed inside the anaerobic chamber. Alternatively, use multichannel pipettes. Note that the screening plates are now filled with 100 µl in 1× assay concentration, 1% DMSO (vol/vol) and a bacterial OD of 0.01.
    ▲ **CRITICAL STEP** Avoid introducing air bubbles.
23. Seal each Nunclon 96-well U-bottom plate tightly with a Breathe-Easy sealing membrane by using a roller to reduce evaporation and prevent cross-contamination.
    ▲ **CRITICAL STEP** Ensure the membrane fits over all wells but does not overhang at the long edges of the plate, so that it does not get caught when stacking and moving plates (see Extended Data Fig. 4). Prevent any bending or wrinkling of the membrane, because this will dramatically affect optical measurements.
    ▲ **CRITICAL STEP** For experimentalists who are getting used to working inside the anaerobic chamber and are still slow in working with the thick gloves, a division of labor between two people is recommended, with one person taking on the pipetting tasks and the other sealing the plates with the breathable membrane. The aim is to keep the time between the inoculation of the strain and measurement of the first OD value in the plate reader as short as possible.
24. Cut off the short ends of the sealing membrane with scissors so that the membrane is not overhanging any edge of the plate and ensure that it fits tightly. Use the roller again on the cut edges to ensure that the seal is properly attached to the plate. Otherwise, the plates in the stack can easily stick together (Extended Data Fig. 4).
25. Stack all plates.
    ▲ **CAUTION** Ensure that plates are not swapped or rotated inadvertently, and when analyzing growth curves, check plate-wise correlations between replicates to detect such swaps that can happen in a high-throughput screen that runs over weeks.

▲ **CRITICAL STEP** Note the order of your stack. The bottom plate will be the first plate to be measured, and the order will also dictate the order of your output file (see Step 51 and Manually generated input tables).

26. Load the plate stack into the BioStack4.
27. Start the measurement with the Gen5 software (version 3.05 or higher). Use a kinetic assay that measures each plate every hour for 24 h at 37 °C, preceded by 1 min of linear shaking before each measurement (Table 1).
    ▲ **CRITICAL STEP** Shaking is necessary to homogenize clumps and sediments before measurement and to prevent excessive aggregation of bacteria. With this protocol and setup, a maximum of 20 plates can be screened simultaneously to ensure hourly measurements.
    ◆ **TROUBLESHOOTING**
28. Visually inspect the raw growth curves in the Gen5 software to ensure that bacteria in solvent control wells grew as expected. Verify that the blank medium control wells are still sterile. Export your measurement data to an Excel file in which each sheet contains the data of one plate (e.g., sheet one contains data of plate one and so on). Continue with data analysis (see Steps 51–65) or follow Steps 29–50 for screening of bacterial communities.
    ◆ **TROUBLESHOOTING**

Drug-community screening

Preparation of drug master plates (sterile conditions)

● **TIMING 1 d (2 h of hands-on time per drug master plate)**

29. Dissolve all drugs/compounds, if possible, in the same solvent (here: DMSO) at 100× the desired assay concentration (i.e., if a compound is to be screened at 20 µM, prepare a stock of 2 mM).
30. Use a 96-well Greiner V-bottom microplate and add 120 µl of the 100× drug-stock to the corresponding wells. Design the plate to allow two technical replicates per plate and fill columns 1 (A1-H1) and 6 (A7-H7) with the same volume of solvent (120 µl) for the controls. One of these (e.g., A7) can be dedicated to test for contamination of the medium (i.e., blank control).
31. Repeat Steps 3 and 4.

Preparation of microbial communities

32. Use option A to prepare a synthetic community or option B to prepare stool-derived communities. Synthetic communities are typically restricted to a predetermined number of members and can be customized to suit the user's needs, resulting in reduced complexity. Stool-derived communities have greater complexity and diversity compared to synthetic communities but are undefined. The choice of one over the other will depend on the user's research question and the overall feasibility of the experiment.

    (A) **Preparation of bottom-up assembled synthetic communities: strain inoculation and community assembly (anaerobic chamber)**

    ● **TIMING 3 d (30 min of hands-on time per strain)**

    (i) Two days before the screening (day −2), streak out the single-use bacterial stocks (from Steps 11 and 12) onto mGAM agar plates inside the anaerobic chamber. Should the strain(s) not grow on mGAM agar plates, use their preferred medium (alternatives could be COLS agar or BH+ agar; see Media and Reagent setup). Incubate at 37 °C O/N.
    ▲ **CRITICAL STEP** Keep the cryostock in a freezing block when bringing it into the chamber. Discard the stock after use. Slow-growing strains may need longer incubation periods (e.g., 48 h). Therefore, it is important to know the growth behavior of the strains to be used in the screening beforehand.

    (ii) After 16–24 h of subculture (on day −1), transfer a single colony into 5 ml of fresh mGAM medium (or the preferred medium). Incubate O/N (16–24 h) again at 37 °C.
    ▲ **CRITICAL STEP** Slow-growing strains may need longer incubation periods (e.g., 48 h).

(iii) On the day of the compound screening assay, measure the OD_{578} of the bacterial cultures from Step 32A(ii) by using a cuvette spectrophotometer.
▲ **CRITICAL STEP** Dilute the cultures if needed. Note the linear range of your photometer.
◆ **TROUBLESHOOTING**

(iv) Assemble the communities by adding all the bacteria in equal amounts (according to OD_{578}) in one culture. For this, adjust the OD_{578} of each bacterium to reach a final community OD of 0.02 in mGAM. For example, for a community of 30 members, each member should be added at an OD_{578} of 0.0006. Note that for one row of a 96-well deep-well plate, 10 ml are needed. Prepare in sterile 150-ml Schott bottles.
▲ **CRITICAL STEP** Prepare ≥100 ml in excess to have enough volume for sequencing (community assembly control, Step 32A(vi)).

(v) Continue with Step 40 to perform screening of the bottom-up assembled-derived communities.

(vi) After filling the screening plates (Steps 40 and 41), spin down the excess volume of the communities in 50-ml Falcon tubes at 3,220*g* for 10 min at 4 °C in a centrifuge outside the anaerobic chamber, remove the supernatants and freeze the pellets for later DNA extraction and metagenomics/16S rRNA gene sequencing (i.e., the time point 0, community assembly control).
■ **PAUSE POINT** DNA pellets can be stored at −20 °C for ≥6 months, if not thawed in the meantime.

(vii) For DNA extraction, we recommend using a semi-automated protocol in 96-well plates[8] or commercial kits that allow for DNA extraction in 96-well plates (e.g., DNeasy UltraClean kit for bacterial cultures and DNeasy 96 PowerSoil kit for fecal samples (both Qiagen)). Process this time point 0 community-assembled control together with the screened samples in Step 49.

(B) **Preparation of communities derived from stool: stool sample collection and cultivation of communities (anaerobic chamber)**
● **TIMING 6 d (3 h of hands-on time per stool sample for collection; 2 h of hands-on time per day for the rest of steps)**

(i) Obtain one fresh stool sample specimen per donor deposited in an adequate collection device, immediately place it in an anaerobic chamber and homogenize it by stirring it with a sterile plastic disposable spatula. Weigh 25 g of this homogenized material into a tube with a rotor element, add 25 ml of 40% glycerol (vol/vol) in PBS + 0.5 g of cysteine/liter and blend at full speed for ~3 min with the disperser system. Trim the thin end of a sterile 1-ml pipette tip and use it to distribute ~700-µl aliquots into glass vials.
▲ **CAUTION** Work with stool samples collected from human donors has to be performed in accordance with the Declaration of Helsinki and must be approved by the responsible ethics committee. Informed consent must be obtained from all human research participants. Stool material collection was approved by the EMBL Bioethics Internal Advisory Committee, and informed consent was obtained from all donors (BIAC2015-009).
▲ **CAUTION** Cover the working surface of the anaerobic chamber with tissue and cover the gloves of the anaerobic chamber with disposable vinyl gloves to prevent contamination of the anaerobic chamber with stool material. Dispose of all residues contaminated with stool in BSL2 containers.
▲ **CRITICAL STEP** Immediately freeze the stock aliquots at −80 °C for long-term storage of ≥12 months.
◆ **TROUBLESHOOTING**

(ii) From the fresh material (after blending and before freezing), dilute 1 glycerol stock aliquot in 50 ml of mGAM medium in a 50-ml Falcon tube.
▲ **CRITICAL STEP** Homogenize the contents well by shaking the tube manually for 10 s and let the sediment settle for 5–10 min.

(iii) Once the sediment is settled at the bottom of the 50-ml tube, take 500 µl of the supernatant into a tube with 5 ml of pre-reduced mGAM. From this 10-fold dilution, perform two additional 1:10 serial dilutions in 5 ml of pre-reduced mGAM and culture the 10^{-3} dilution at 37 °C under anaerobic conditions O/N.
▲ **CRITICAL STEP** Note that dilutions are necessary to ensure enrichment of the viable fraction of bacteria from the stool sample. Optimal dilutions for samples may vary and must be determined in preliminary tests.

(iv) After 16–24 h of incubation, bring a freezing block into the anaerobic chamber. Mix 4.8 ml of bacterial culture (10^{-3} dilution) with 1.2 ml of freezing medium for anaerobes (50% (vol/vol) glycerol with palladium black). Vortex and distribute aliquots of the mixture into 2-ml sterile glass vials for long-term storage. Close the vials with a butyl rubber stopper and an aluminum cap. Crimp the aluminum cap around the upper edge of the vial by using the crimper. In addition, distribute 100-µl aliquots of the 10^{-3} dilution after mixing with freezing medium into 0.2-ml strip tubes for single use during screening (prepare as many single-use stocks as required). Place the stocks directly into the freezing block. Bring them immediately out of the chamber and freeze them at −80 °C for long-term storage.
■ **PAUSE POINT** Stocks can be stored at −20 °C for ≥6 months.

(v) The day before the screening, inoculate 50 µl of the single-use frozen stocks in 5 ml of pre-reduced mGAM (1 tube of mGAM per frozen stock, each corresponding to a different microbiome) and grow for 24 h at 37 °C in the anaerobic chamber.

(vi) On the day of the screening assay, measure the OD_{578} of the bacterial communities from Step 32B(v) by using a cuvette spectrophotometer.
▲ **CRITICAL STEP** Dilute the cultures if needed. Note the linear range of your photometer.
◆ **TROUBLESHOOTING**

(vii) Dilute the communities to a final OD_{578} of 0.02 in mGAM. Note that for one row of a 96-well deep-well plate, 10 ml are needed. Prepare in sterile 150-ml Schott bottles.
▲ **CRITICAL STEP** Prepare ≥100 ml in excess to have enough volume for sequencing (community control, Step 32B(viii)).

(viii) Repeat Step 32A(v–vii).

Compound screening in community setup (anaerobic chamber)

● **TIMING 2 d (2 h of hands-on time per drug plate per day with up to eight different communities)**

33. The day before the screening, thaw the drug master plates (100× assay concentration in DMSO) from Step 31 that are to be screened.
34. Centrifuge the plates with the aluminum foil seals at 400*g* for 5 min at room temperature to remove potential drops on the aluminum seal.
35. Remove the aluminum foil seals under sterile conditions.
▲ **CAUTION** Avoid cross-contamination by liquid splashes.
36. Fill a single-well reagent reservoir with 40 ml of mGAM. Transfer 139 µl of mGAM from this reservoir to each well of the thawed drug master plates by using a liquid handler.
37. Prepare 96-well deep-well plates with 400 µl of mGAM in each well. Prepare the same number of 96-well deep-well plates as drug master plates.
38. Using the liquid handler, transfer the total volume of 150 µl from the drug master plates to the 96-well deep-well plates prefilled with 400 µl of mGAM. Mix the entire deep-well plate 5–10 times with 200–300 µl by using the liquid handler.
▲ **CRITICAL STEP** Ensure sufficient mixing.
39. Close the deep well plates with breathable AeraSeal sealing membranes by using a roller and bring them into the anaerobic chamber 1 d before the screening assay to allow gas exchange.
40. On the screening day, you must have followed Step 32A if you want to screen synthetic communities or Step 32B if you want to screen stool-derived communities. Alternatively, you can perform Step 32B(v–viii) if you want to start with a frozen synthetic community. Transfer the diluted communities (OD_{578} of 0.02) from the 150-ml Schott bottles (from

Step 32A(iv) if you are screening synthetic communities and Step 32B(vii) if you are screening stool-derived communities) to single-well reagent reservoirs.
▲ **CRITICAL STEP** The reservoirs might not handle the whole volume of the diluted community. If this is the case, transfer smaller volumes and refill when used up.

41. Using a multichannel pipette, transfer 550 µl of the diluted bacterial communities to each well of the 96-well deep-well plates containing mGAM and the drugs (from Step 39). Note that the 96-well deep-well screening plates are now filled with 1,100 µl at 1× assay concentration, 1% DMSO (vol/vol) and a bacterial OD of 0.01.
42. Using the liquid handler, mix the entire deep-well plates 5–10 times with 200–300 µl.
▲ **CRITICAL STEP** Ensure sufficient mixing so that the drug concentration is homogeneous in the well.
43. Using the liquid handler, distribute 100-µl aliquots of the deep-well plates into empty Nunclon 96-well U-bottom plates.
44. Seal the deep-well plates with breathable AeraSeal sealing membranes by using a roller and incubate them for 24 h at 37 °C.
45. Repeat Steps 23–28.
46. Cover the community plates that have grown for 24 h with aluminum foil seals by using a roller and take them out of the anaerobic chamber.
47. Centrifuge the plates for 10 min at 3,220*g* at room temperature. Remove the aluminum foil seals.
▲ **CAUTION** Avoid cross-contamination by liquid splashes.
48. Remove the supernatants of the wells with a multichannel pipette and cover the plates again with aluminum foil seals by using a roller.
▲ **CAUTION** Work under a laminar flow hood to remove the supernatants, because these samples potentially contain BSL2 category organisms.
49. Freeze the plates at −20 °C for metagenomics/amplicon sequencing processing and analysis.
■ **PAUSE POINT** DNA pellets can be stored at −20 °C for ≥6 months, if not thawed in the meantime.
50. Repeat Step 32A(vii).

Processing of microbial growth curves

Analysis of bacterial growth curves

● **TIMING 30 min**

51. Prepare the *Layout* and *Runs* table by using a spreadsheet program (see Experimental design) and note the path to the location where they were stored.
▲ **CRITICAL STEP** The functions of 'neckaR' assume that the *Runs* file is sorted by run and by plate. Users can verify this by using a spreadsheet program.
52. Save the raw measurement files from the plate reader and note the path to the location where they were stored.
53. Open R and load all the necessary libraries:

```
library(neckaR)
library(dplyr)
```

54. Specify paths to the *Layout* and *Runs* tables and plate reader raw measurement files. Users need to customize the paths for their own directory structure.
▲ **CRITICAL STEP** The raw measurement files should contain the word *run* and the corresponding number in the file name (e.g., '2023_03_21_RUN01.xlsx'). In addition, the vector (i.e., the R data object) with the names of the raw measurement files should be sorted by run (e.g., c('2023_03_21_RUN01.xlsx', '2023_03_21_RUN02.xlsx', '2023_03_21_RUN03.xlsx')); to facilitate sorting the file names, use the 'Sort_by_run()' function, as follows:

```
#List file locations
data_dir = file.path(system.file("extdata", package = "neckaR"),
"Bovatus")
```

```
# List raw plate reader files
Raw_Data_files = list.files(data_dir, pattern = "RUN") %>%
Sort_by_run()
# Tab1
Runs = file.path(data_dir, "Tab1.xlsx")
# Tab2
Layout = file.path(data_dir, "Tab2.xlsx")
```

55. Build the master data frame, specifying that the column 'Design' contain plate design information in both the *Run* and *Layout* tables, as follows:

```
master_df = Make_master_df(Data_folder = data_dir,
                Data_files = Raw_Data_files,
                Runs_path = Runs,
                Layout_path = Layout,
                Design_tab_col = "Design")
```

▲ **CAUTION** The 'Make_master_df()' function is designed to work specifically with 96-well plates and output files from the Gen5 software (version 3.05 or higher). However, researchers have the option of using data that are saved in different formats and can load them into R by using alternative methods. For a detailed explanation of the format of the master data frame used in downstream steps, see Box 2.

56. Optionally, plot and inspect raw curves, specifying that the 'Replicate' column contain experiment replication information as follows:

```
Master_plots = Make_curve_plots(master_df,
     save_plots = FALSE,
     replicate_variable = "Replicate")
```

57. Baseline-adjust the optical (OD) measurements by subtracting the median OD of the controls from all other measurements by plate and determine the end of the exponential phase. For a given plate, the transition from exponential to stationary phase corresponds to the average time when controls reach maximum baseline-adjusted OD. Specify that

BOX 2

Generating the master data frame by using alternative methods

Note that the 'Make_master_df()' function is designed to work specifically with 96-well plates and output files from the Gen5 software (the data collection software used by the Epoch2 multiplate readers). However, researchers have the option of using data that are saved in different formats and can load them into R by using the functions from the built-in 'utils' package or install other libraries such as 'readxl', 'readr' or 'data.table'. Downstream processing steps require data in tidy format. Tidy data sets have each variable represented in a separate column, and each row corresponds to a single measurement. In the 'neckaR' package, the generated master data frame is in tidy format and includes the following columns:

- OD: optical density as measured by the plate reader
- Time: the time point at which OD was measured
- Position: numeric value of the well on the plate
- Run: the batch or number of the set of plates performed together
- Plate: the plate ID number
- Strain: name of the species or strain
- Replicate: the biological or technical replicate of the experiment
- Drug: ID of the compound tested
- Drug_name: common name of the compound
- ID: a four-digit numeric code that identifies a well on a specific plate. The tens of thousands and thousands digits indicate the plate number, the hundreds digit indicates the row number (which ranges from 1 to 8), and the tens and unit digits denote the column numbers (ranging from 01 to 12). The formula used to calculate this code is (Plate × 1,000) + (Row × 100) + Column.
- RRPPRCC: unique curve identifier, calculated by using Run and ID. The formula for RRPPRCC is RUN × 100,000 + ID. The acronym stands for 'Run-Run-Plate-Plate-Row Column-Column'.

the column 'Drug' contain the treatment information, with the controls labeled 'control', as follows:

```
adjusted_df = Adjust_OD(curves_df = master_df,
     control_factor = "Drug",
     control_level = "control",
     offset_control = 0.02)
```

58. Optionally, plot control curves of each plate and run combination and display the end of the exponential phase as follows:

```
cutoff_plot = Make_cutoff_plots(curves_df = adjusted_df,
     save_plots = FALSE,
     vline = "cutoff")
```

59. Optionally, manually adjust cutoff values on specific plates if the user thinks the automatic detection was not adequate. To do so, first determine which run/plate combination has a cutoff time that requires adjustment. In this case, use times under 12 h, as follows:

```
adjusted_df %>%
     filter(Control == TRUE, cutoff_time <= 12) %>%
     count(RRPP) %>%
     select(-n)
```

Then, manually set the cutoff time for the desired run/plate combinations as follows:

```
# Create list
replace_values = list(c(306, 11))
# replace values
readjusted_df = Fix_cutoff(adjusted_df, replace_values)
```

60. Optionally, cut curves at the end of the exponential phase and plot as follows:

```
# Cut all curves at the cut-off
cutoff_df = adjusted_df %>%
     filter(Time < (cutoff_time + 0.5)) %>%
     arrange(Time)
# Plot
cutoff_df %>%
     filter(Control == TRUE) %>%
     Make_cutoff_plots(save_plots = FALSE,
          plot_name = "Truncated_control_cutoff")
```

61. Perform quality control of growth curves as follows. Note that the values shown here are the defaults of the function; users may need to adjust these parameters to best suit their data.

```
marked_df = Mark_artefacts(adjusted_df,
     sum_inc = 3,
     increased_sd = 2,
     t0_sd = 3,
     p_delta = 1e-3,
     p_delta2 = 1e-3)
```

62. Optionally, plot low-quality curves, specifying that the column 'Replicate' contain experiment replication information, as follows:

```
filtered_plots = Make_filtered_plot(marked_df,
     save_plots = FALSE,
     replicate_variable = "Replicate")
```

63. Remove curves with anomalous behavior, as follows:

```
filtered_marked_df = marked_df %>%
     filter(discard_conc == FALSE)
```

64. Calculate AUCs normalized to the growth of the controls and corrected for possible deviations of optical density calculation at time 0 of individual wells, as follows:

```
AUC_df = Calculate_AUC(filtered_marked_df)
```

65. Build the final data frame, as follows:

```
# Create data frame with maximum OD and cut-off times of each curve
Max_OD_df = filtered_marked_df %>%
     group_by(RRPPRCC) %>%
     slice(which.min(cutoff_time - Time)) %>%
     ungroup() %>%
     select(RRPPRCC, Drug, Drug_name, Replicate,
            Max_OD = OD, Max_ODc01 = ODc01, cutoff_time)
# Create data frame with only normalized AUC
selected_AUC_df = AUC_df %>%
     select(RRPPRCC, normAUC)
# Combine the created data frames
final_df = left_join(Max_OD_df,
                     selected_AUC_df,
                     by = "RRPPRCC")
```

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
20, 32A(iii) and 32B(vi)	The optical density of a bacterial culture does not match the previously observed measurements	The optical measurement was not in the linear range of the photometer	The culture was not diluted properly. Adjust the dilution factor
		Bacterial contamination	Include blank medium controls in all plates. Thoroughly clean and disinfect the anaerobic chamber and all equipment used. Check that the single-use stocks are correct. Prevent mixing up or spilling of bacteria
27	Precipitate or pellet in a well	Precipitation or clumping of culture	Increase shaking time
		Compounds are not soluble in the tested concentration in mGAM	Reduce the concentration of the compound
	The plates identified by the reader do not match the plates in the stack	Plates might stick together because of bent membranes or sticky plate surfaces	Restack the plates and fix membrane issues. If necessary, remove troublesome plates
	The reading/run does not start	The reader/stacker is not communicating with the software	Make sure that the devices are properly connected and restart the software

Table 2 (continued) | Troubleshooting table

Step	Problem	Possible reason	Solution
	The reading/run stops despite having recognized and read all plates at least once	The plates might stick together because of bent membranes or sticky plate surfaces	Restack the plates and fix membrane issues. If necessary, remove troublesome plates
			For species that produced a lot of gas, pierce the seal with a sterile needle
		Check if a plate fell out of the stack	Restack the plates and restart. If necessary, realign the stacker with the reader. Make sure that the reader and the stacker are on a hard surface
	Bacteria do not grow in wells where growth is expected (i.e., control wells)	No bacteria added	Ensure that bacteria are added in the right amount to every well
		Methanol was accidently spilled into some wells	Ensure that only methanol vapor is blown over the plates and no liquid methanol
		Plate sizes do not match the settings of the plate reader	Make sure that your plate settings/brand are selected in the protocol of the software
28	Spikes in optical density or high starting optical densities	The membranes have wrinkles	Reseal plates with a new membrane
		Liquid droplets on the membrane	Remove the droplets from the membrane or reseal the plate with a new membrane. If only one time point is affected (especially at the start), exclude this time point from the computational analysis
		Air bubbles in the well	Remove all air bubbles with the methanol spray bottle before bringing the plates into the chamber. Remove air bubbles with a sterile pipette tip inside the chamber if needed
		Colored compounds	Use lower concentrations of the compound, which do not change the color of the medium
32B(i)	The rotor element in the tube used to homogenize the stool with a disperser system gets clogged	Stiff stool pieces are very hard and difficult to homogenize	Start the blending speed from low to high
			Use a disposable sterile spatula to remove those pieces or to help the blender resume the process

Timing

Drug-microbe screening in monocultures

Steps 1–10, preparation of drug master plates and screening plates: 2 d (1 h of hands-on time per drug master plate)
Steps 11–12, reparation of bacterial stocks: 2 d (1 h of hands-on time per strain)
Steps 13–14, strain inoculation: 3 d (30 min of hands-on time per strain)
Steps 15–28, compound screening in monoculture setup: 2 d (2 h of hands-on time for one strain with 14 plates)

Drug-community screening

Steps 29–31, preparation of drug master plates: 1 d (2 h of hands-on time per drug master plate)
Step 32A, preparation of bottom-up assembled synthetic communities—strain inoculation and community assembly (anaerobic chamber): 3 d (30 min of hands-on time per strain)
Step 32B, Preparation of communities derived from stool—stool sample collection and cultivation of communities (anaerobic chamber): 6 d (3 h of hands-on time per stool sample for collection and 2 h of hands-on time per day for the rest of the steps)
Steps 33–50, compound screening in community setup (anaerobic chamber): 2 d (2 h of hands-on time per drug plate per day with up to eight different communities)

Processing of microbial growth curves

Steps 51–65, analysis of bacterial growth curves: 30 min per batch

Anticipated results

Upon successful completion of the protocol, the overall result is a list of compounds with antimicrobial activity against a specific strain or microbial community of the human gut

microbiome. This activity is quantified by the normalized AUC values for each compound-strain or compound-community combination. To calculate AUCs, growth curves are cut at the time point when controls transition to stationary phase to capture effects on lag time, growth rate and yield. This transition point needs to be adjusted per species, strain and community. Low-quality AUCs from growth curves with spikes, uneven growth or high initial OD are removed from the dataset (Fig. 5c, red lines). AUC values are then normalized to unperturbed controls, with AUC values of 1 indicating no effect on growth and values below 1 indicating growth inhibition. In our experience, AUCs strongly correlate with final ODs, with a Pearson correlation of 0.95 across all compounds and replicates within the Prestwick library screen[15]. However, we recommend using AUCs as readout to reduce the influence of the final time point, which introduces more noise than a measure based on all time points. Normalized AUCs can then be used to assess the statistical significance of the observed antimicrobial activity by calculating *P* values by using the distribution of AUCs from negative control wells, possibly also including treatment conditions that do not inhibit growth. As in all high-throughput screens, correcting for multiple hypothesis testing is mandatory.

To provide context for interpreting these results, AUC values can be compared to the antimicrobial activity of the compounds being tested as positive control (e.g., a compound with known antimicrobial activity for a given strain/community).

In our hands, the monoculture assays of the Prestwick library screen were highly reproducible. The standard deviation of AUCs between replicates had a mean value of 0.04. Similarly, the shape of growth curves from different replicates of the same strain were congruent when overlaid, and Pearson correlation of AUCs between replicates of the same strain was very high (median: 0.88).

The Prestwick library screen was benchmarked with an independent set of experiments by using compounds sourced from other suppliers. We achieved high precision (94%) but slightly lower recall (85%). We identified some false negatives (i.e., drugs with antimicrobial activity that remained undetected in our screen), probably because of the instability of the specific compounds in the Prestwick library. Overall, we expect similar results in terms of reproducibility and hit rates when high-quality and well-maintained compound libraries and phylogenetically related species are tested with our protocol[15].

Growth curves measured from experiments in bacterial communities can be analyzed in the same way as those from monocultures by using the 'neckaR' package. When growth curve measurements are accompanied by an analysis of community composition (e.g., 16S rRNA gene sequencing), the ODs can be used to transform species abundances from relative to absolute. In our experience, treatment with an active drug allows reproducible shifts in the overall community composition. Similarly, the effects on individual taxa of stool-derived communities are consistent across replicates[8]. Drug effects on communities can often, but not always, be predicted on the basis of observations in pure cultures. This allows the identification of phenomena that can be observed only in the context of the community, such as cross-sensitivity and cross-resistance.

Data availability

The 16S rRNA gene amplicon sequence data generated during this study (Extended Data Figs. 2 and 3) have been deposited in the European Nucleotide Archive with accession ID PRJEB64209. Source data files detailing the underlying processed data set are provided for both figures. Plate reader output files for the example analysis with the R package 'neckaR' are included in the package. All other data are available in the supporting primary research articles[8,15]. Source data are provided with this paper.

Code availability

The 'neckaR' package is available on GitHub (https://github.com/Lisa-Maier-Lab/neckaR).

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Author contributions
M.Z., P.B., K.R.P., A.T., S.G.-S. and L.M. conceived the study. P.M., M.B.A., T.T., S.B., S.G.-S. and L.M. designed experiments and collected data. J.d.l.C.-Z. and M.K. analyzed the data. P.M., J.d.l.C.-Z., S.G.-S. and L.M. interpreted the data and wrote the manuscript. All authors approved the final version for publication.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Sarela Garcia-Santamarina or Lisa Maier.

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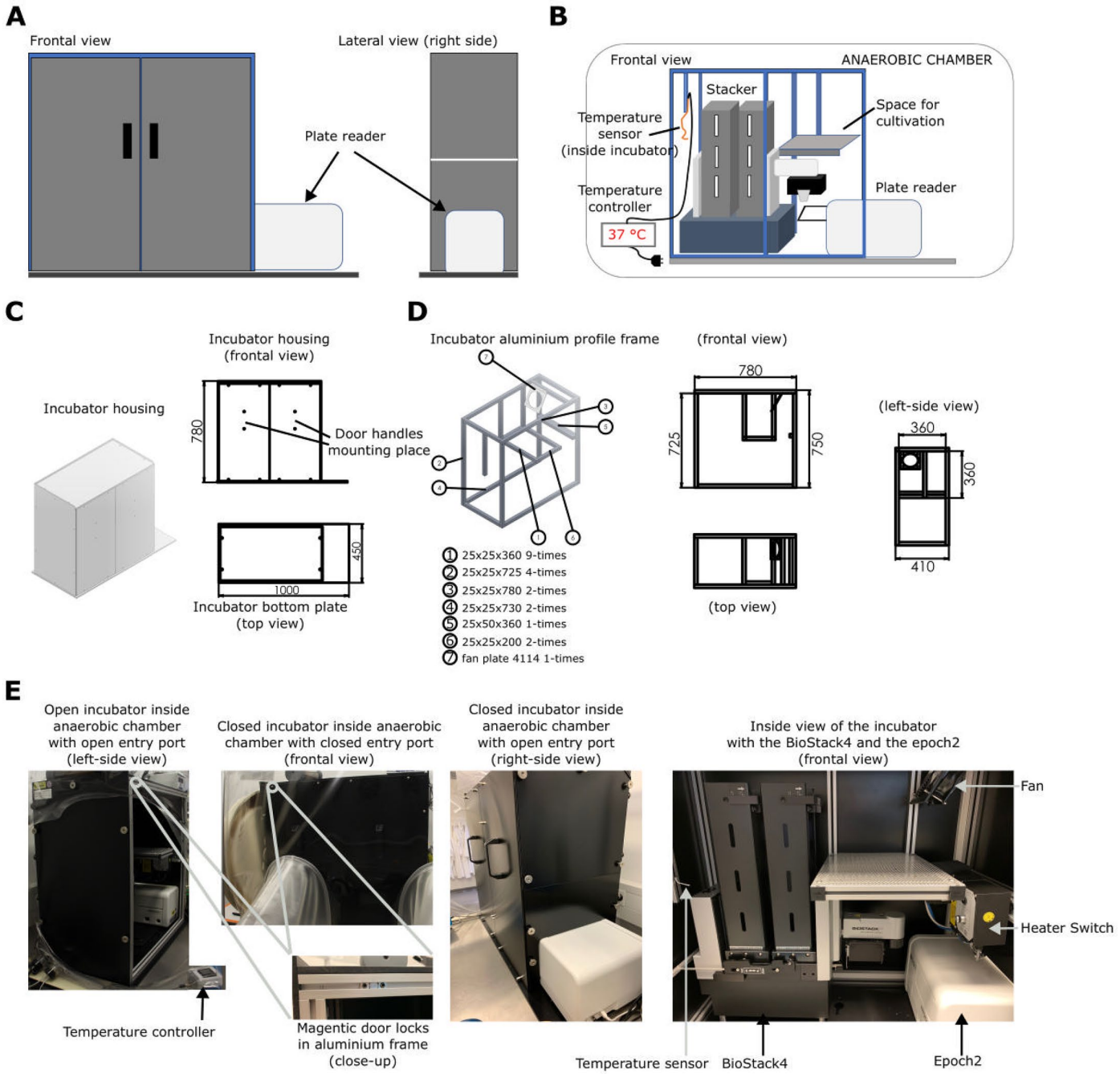
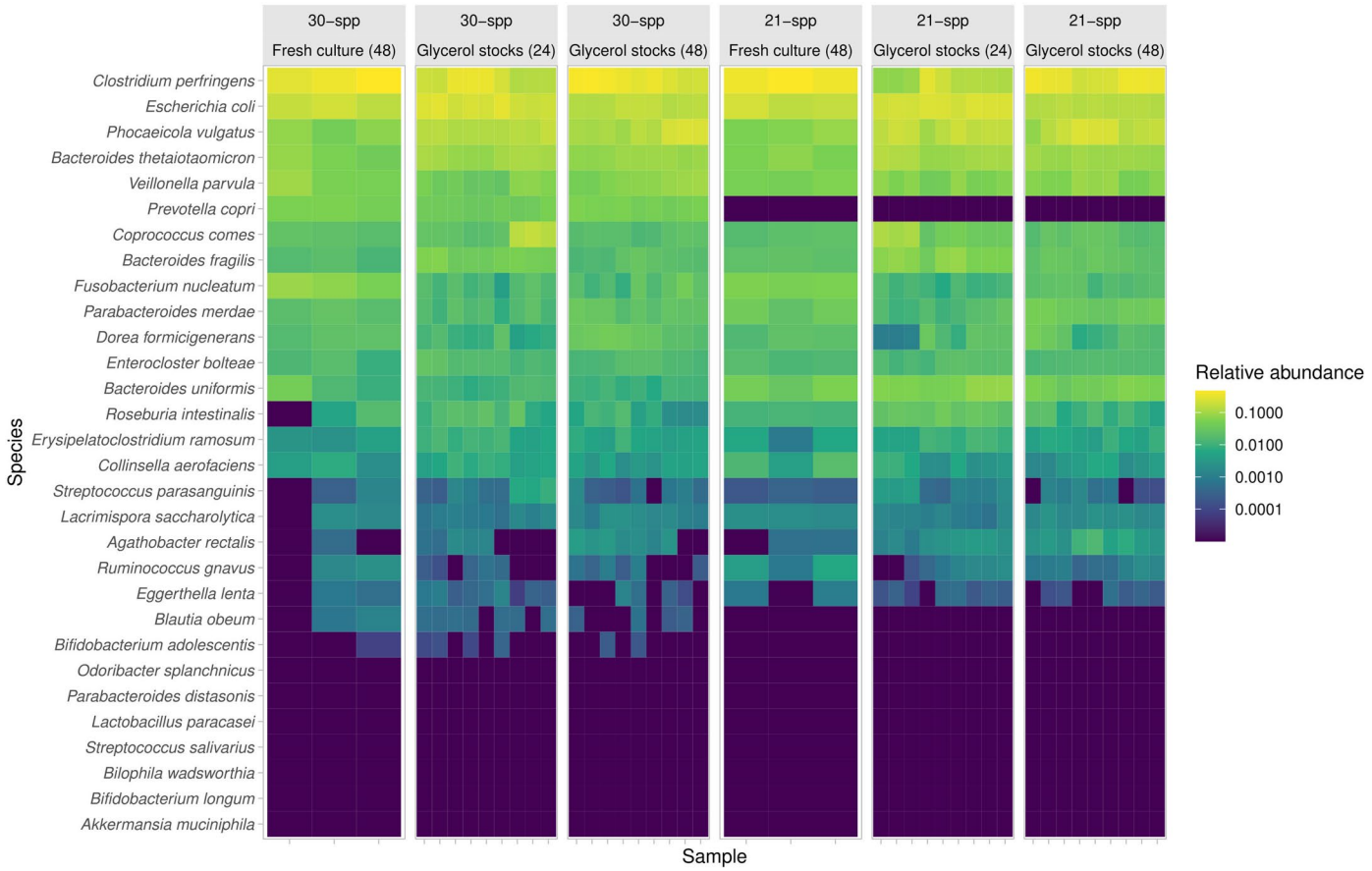
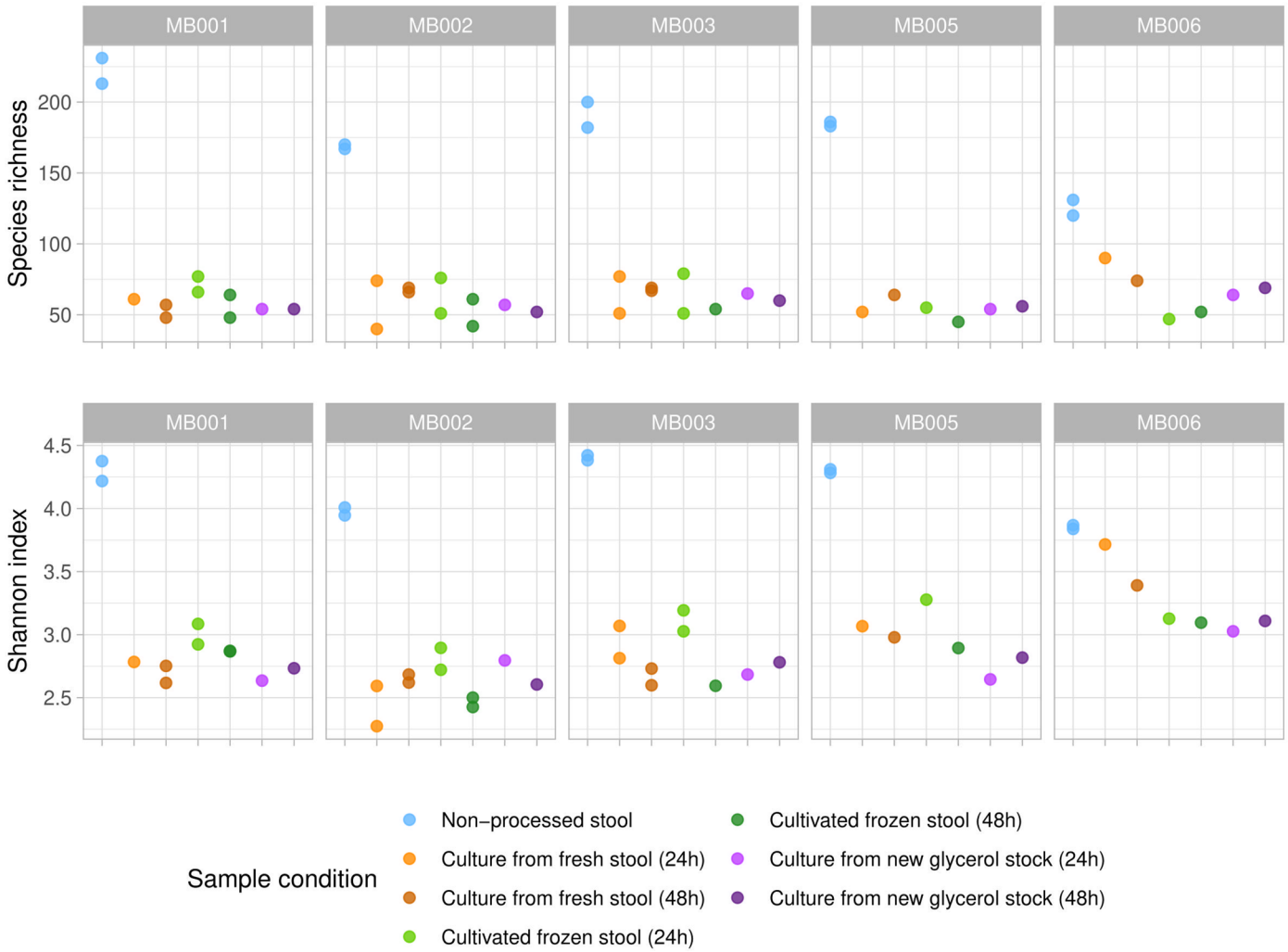
Related links
Key references using this protocol
Maier, L. et al. *Nature* **555**, 623–628 (2018): https://doi.org/10.1038/nature25979
Maier, L. et al. *Nature* **599**, 120–124 (2021): https://doi.org/10.1038/s41586-021-03986-2



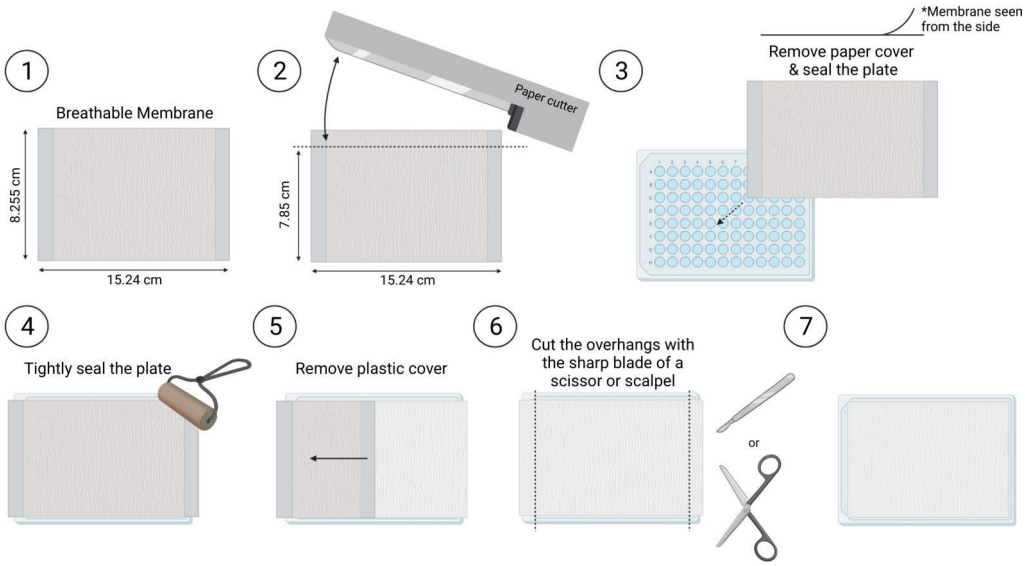
Extended Data Fig. 1 | Construction of the custom-made incubator around Epoch2 and BioStack 4. a, Illustration of the custom-made heatable incubator (closed) with microplate spectrophotometer. Frontal and lateral view. **b**, Illustration of the arrangement of the stacker and plate reader in the incubator in frontal view. **c**, Schematic drawings of the incubator housing (bottom, walls, roof and two magnetic doors). **d**, Schematic drawings of the aluminum profile frame of the incubator. The housing consists of black PVC plates that are screwed to the aluminium profile frame. **e**, Images of the incubator in the anaerobic chamber with the BioStack 4 and Epoch2.



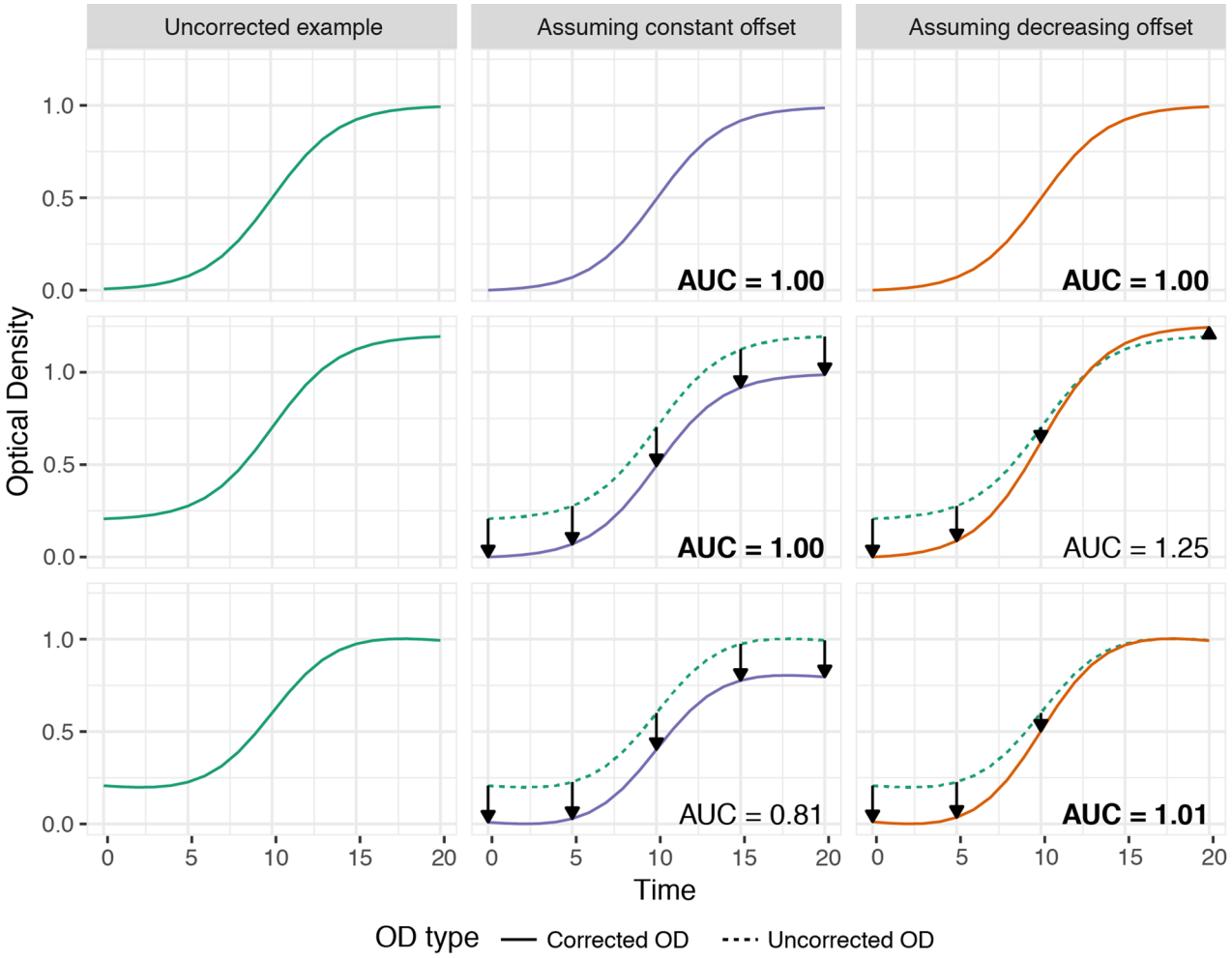
Extended Data Fig. 2 | The composition of a 21-member synthetic community is independent of the initial complexity, storage method and number of passages. The heatmap shows the relative abundances of strains within a synthetic microbial community. Each column depicts one replicate. For samples from fresh cultures, three biological replicates are depicted; for cultures from glycerol stocks, both biological and technical replicates are shown. The community was assembled in two different ways: in both cases, the bacterial strains were first grown individually, and then the community was mixed in mGAM according to Step 32A of this protocol. In the first case, the community was assembled from all 30 species (30-spp; see Extended Data Table 1); in the second case, species that were not consistently detectable within the 30-spp community were omitted (21-spp). The nondetectable species included *Akkermansia muciniphila, Bifidobacterium adolescentis, Bifidobacterium longum subsp. longum, Bilophila wadsworthia, Lactobacillus paracasei, Blautia obeum, Parabacteroides distasonis* and *Odoribacter splanchnicus*. We did not observe growth of *Prevotella copri* in the 21-member community. Alternatively, cultures were inoculated from frozen glycerol stocks. These glycerol stocks were prepared from freshly assembled communities after 24-h incubation at 37 °C (Step 32B(iv)). Community composition was determined either directly after 24 h of growth or after subculturing (1:50) for 48 h. In all cases, cell pellets of the cultures were harvested by centrifugation and further processed for DNA extraction and 16S rRNA amplicon sequencing. In the example of the 30-spp community, a stable community of 21 species is formed within the first 24 h. A community with similar composition can be obtained from glycerol stocks or by omitting the strains that cannot grow in the community. For this community, it is therefore possible to work from cryopreserved, pre-assembled communities, which can be tested directly after a single O/N culture, without further passages. However, it is strongly recommended that similar pilot tests are carried out for each community to be tested and that the stability of the cryo-stocks is checked over the duration of the test.



Extended Data Fig. 3 | Alpha-diversity indices of stool and stool-derived microbial communities. Stool samples from five healthy donors (MB001–MB003, MB005 and MB006) were collected and immediately placed in an anaerobic chamber. Samples were mixed with 40% glycerol in PBS + 0.5 g of cysteine/liter, divided into aliquots in ~700-µl glycerol stocks and frozen at −80 °C (non-processed stool). Before freezing, we performed a 1:1,000 dilution of one aliquot per donor in 50 ml of mGAM medium and grew it anaerobically at 37 °C for 24 h (culture from fresh stool (24h)). We then mixed 800 µl of the culture with 200 µl of 50% (vol/vol) glycerol with palladium black (glycerol stock); we also diluted another 5 µl of the culture in 5 ml of mGAM and cultured it anaerobically at 37 °C for 24 h (culture from fresh stool (48 h)). After 10–14 weeks, we inoculated the glycerol stocks in 5 ml of mGAM, cultured them anaerobically at 37 °C for 24 h (culture from glycerol stock (24 h)), passaged them (1:1,000) in fresh mGAM and cultured them at 37 °C for another 24 h (culture from glycerol stock (48 h)). We harvested cell pellets from the cultures by centrifugation, extracted DNA and performed 16S rRNA amplicon sequencing. After read count rarefaction and exclusion of samples with low sequencing depth, we calculated the species richness and Shannon index of the samples. Compared to fresh stool samples, the alpha diversity in the stool-derived communities was reduced. However, the number of passages and cryopreservation did not seem to have a major effect on the diversity of the communities. Similar pilot tests should be carried out for each community. It is also recommended to check the long-term stability of the cryopreserved communities. Each experiment should include an unperturbed (solvent) control from the same cryotube. Stool material collection was approved by the EMBL Bioethics Internal Advisory Committee, and informed consent was obtained from all donors (BIAC2015-009).



Extended Data Fig. 4 | How to seal the screening plates with a breathable membrane. 1, Breathe-Easy sealing membrane dimensions. 2, The sealing membranes should fit on a 96-well plate without overhanging. To ensure this, the membranes should be cut to size at the long end by using a standard paper cutter. 3, Once the screening plates are fully prepared, they are sealed with a Breathe-Easy membrane in the anaerobic chamber. This is achieved by removing the paper cover of the membrane and sealing the plate. Ensure that the long ends of the membrane do not extend beyond the edges of the plate. 4, A roller is used to seal the membrane firmly to the plate. 5, The plastic cover is removed from the breathable membrane. 6, By using the sharp blade of scissors or a scalpel, the short ends of the membrane are cut off so that no part of the membrane protrudes beyond the edge of the plate. Use the roller again over the cut edges to ensure that they stick to the plate. 7, Ensure that the membrane has no bends and is not sticking to plates above it in the stack. The sealed plate is now ready for the first OD measurement. Figure created with BioRender.com.



Extended Data Fig. 5 | Two methods for baseline correction. For each growth curve, two baseline correction methods are applied. This is illustrated here by using three examples (one per row). In the first method (second column), a constant shift is subtracted from all time points of the growth curve, setting the minimum value to zero. In the second method (third column), an initial perturbation that affects earlier times more than later times (e.g., due to condensation) is assumed; a constant shift is subtracted as described earlier. The curve is then rescaled so that an uncorrected OD value of 1 would also have a corrected OD value of 1. AUCs are calculated for both scenarios, with reference compounds used to set the AUC to 1 after rescaling. The baseline correction that resulted in an AUC closest to 1, indicative of normal growth (conservative approach), is selected for each compound (highlighted in bold). Note that the figure is presented as a simplified illustrative example and that the curves depicted do not correspond to actual bacterial growth curves.

Lab code	Species	Strain	Source
NT5001	*Phocaeicola vulgatus*	type strain	DSM No.: 1447
NT5002	*Bacteroides uniformis*	VPI 0061	DSM No.: 6597
NT5003	*Bacteroides fragilis* nontoxigenic	EN-2, VPI 2553	DSM No.: 2151
NT5004	*Bacteroides thetaiotaomicron*	E50(VPI 5482)	DSM No.: 2079
NT5006	*Erysipelatoclostridium ramosum*	type strain	DSM No.: 1402
NT5009	*Agothobacter rectalis*	A1-86	DSM No.: 17629
NT5011	*Roseburia intestinalis*	L1-82	DSM No.: 14610
NT5017	*Veillonella parvula*	type strain	DSM No.: 2008
NT5019	*Prevotella copri*	type strain	DSM No.: 18205
NT5021	*Akkermansia muciniphila*	type strain	DSM No.: 22959
NT5022	*Bifidobacterium adolescentis*	type strain	DSM No.: 20083
NT5024	*Eggerthella lenta*	type strain	DSM No.: 2243
NT5025	*Fusobacterium nucleatum* ssp. *nucleatum*	type strain	DSM No.: 15643
NT5026	*Enterocloster bolteae*	type strain	DSM No.: 15670
NT5028	*Bifidobacterium longum* ssp. *longum*	type strain	DSM No.: 20219
NT5032	*Clostridium perfringens*	C36	DSM No.: 11782
NT5036	*Bilophila wadsworthia*	type strain	ATCC No.: 49260
NT5037	*Lacrimispora saccharolytica*	type strain	DSM No.: 2544
NT5038	*Streptococcus salivarius*	type strain	DSM No.: 20560
NT5042	*Lactobacillus paracasei*	LPC-37	Dupont Health & Nutrition
NT5046	*Ruminococcus gnavus*	type strain	ATCC No.: 29149
NT5048	*Coprococcus comes*	type strain	ATCC No.: 27758
NT5069	*Blautia obeum*	type strain	DSM No.: 25238
NT5071	*Parabacteroides merdae*	VPI T4-1, CIP 104202T	DSM No.: 19495
NT5072	*Streptococcus parasanguinis*	type strain	DSM No.: 6778
NT5073	*Collinsella aerofaciens*	type strain	DSM No.: 3979
NT5074	*Parabacteroides distasonis*	ATCC 8503	DSM No.: 20701
NT5076	*Dorea formicigenerans*	VPI C8-13	DSM No.: 3992
NT5078	*Escherichia coli*	ED1a	Denamur Lab (INSERM)
NT5081	*Odoribacter splanchnicus*	type strain	DSM No.: 20712

Extended Data Table 1 | Strains used in this study