

Droplet microfluidic iX-seq platform enables discovery of gut bacterial cross-feeding between *Phascolarctobacterium faecium* and *Eubacterium limosum*

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Abstract

Background

The human gut microbiota comprises hundreds of bacterial species that form a dynamic metabolic network, in which the exchange of microbial metabolites, known as cross-feeding, is integral to community function and host health. However, defining and isolating specific cross-feeding interactions remains challenging due to the limitations of conventional culture-based approaches. Droplet microfluidics enables high-throughput screening and isolation of bacteria from complex communities by encapsulating cells in water-in-oil droplets, allowing for parallel cultivation of thousands of stochastically contained microcultures. In this study, we developed a droplet microfluidics-based screening platform integrated with 16S rRNA gene amplicon sequencing, collectively termed iX-seq, to identify novel cross-feeding interactions directly from a human fecal sample.

Results

Using iX-seq, we identified *Phascolarctobacterium faecium* and *Eubacterium limosum* as potential unidirectional cross-feeders, wherein the growth of *P. faecium* was obligately supported by *E. limosum* in droplet co-cultures. The growth of *P. faecium* was modestly affected when co-cultured in vitro but notably required *E. limosum* co-inoculation for intestinal colonization in germ-free mice. Metabolomic profiling implicated 2-oxoglutarate (2-OG), an intermediate in *P. faecium*'s succinate metabolic pathway, as the key differentiating metabolite between *E. limosum*-inoculated mouse and control mouse feces. Supplementation of 2-OG in monoculture increased *P. faecium* growth in a concentration-dependent manner, confirming its supportive role.

Conclusions

Our results reveal a previously unrecognized, potential 2-OG-mediated cross-feeding relationship between *P. faecium* and *E. limosum*, offering new insight into gut microbial metabolic dependencies. By enabling high-throughput, culture-based screening directly from fecal samples, the iX-seq approach demonstrates a practical framework for studying cross-feeding interactions within the gut microbiota and represents a potential paradigm shift in how complex microbial communities are experimentally investigated.

Background

The human gut microbiota is a dense, complex network of trillions of microorganisms, largely dominated by bacteria. It has been implicated in numerous facets of human health, including nutrient absorption, immune system development, and mental function¹. The biological importance of the gut microbiota has thus been likened to the extent of an organ of the human body². Furthermore, abnormalities in the

balance of gut microbial species, referred to as dysbiosis, have been linked to disease states such as those associated with inflammatory bowel disease (IBD)³⁻⁵, type 2 diabetes^{6,7}, atherosclerosis^{8,9}, and colorectal cancer^{10,11}. The increasingly documented benefits of maintaining a healthy gut microbiota and the physiological repercussions of dysbiosis emphasize the need to better understand our microscopic companions and how we can ensure a mutually beneficial relationship.

The composition of the gut microbiota is both robust and dynamic, adapting to external influences such as diet and disease while preserving a relatively stable group of core taxa unique to each individual^{12,13}. Microbial communities in the gut are characterized by diverse interactions that collectively form a complex ecological network¹³. This ecological balance is critical for the maintenance of a healthy gut microbial community. One of the most noteworthy interactions between gut bacteria is referred to as cross-feeding, in which metabolic byproducts produced by a bacterium are utilized as a nutrient source for one or more others. Cross-feeding is a common and well-established phenomenon in microbial communities, as evidenced by the syntrophy observed in defined groups of intestinal bacteria¹⁴. As an example, in one co-culture study, *Anaerostipes caccae* DSM 14662 could grow by utilizing the fructose produced by *Bifidobacterium longum* BB536 in an otherwise nutritionally incompatible environment¹⁵. Moreover, the presence of bacterial genes for metabolizing substrates absent from the environment, along with adaptive shifts in their expression during cultivation in mixed bacterial cultures, have led researchers to infer cross-feeding in informatics-based studies of microbial communities¹⁶⁻¹⁸. However, due to factors such as nutritional preferences, secretion and metabolism of both waste products and intermediate substrates¹⁹, and shifts in macronutrient bioavailability due to fluctuations in host diet^{20,21}, the cross-feeding network is deceptively complex. This complexity poses a significant challenge to establishing a holistic framework for understanding the metabolic functionalities of a gut microbial community, both within the community itself and in its net output. Such characteristics profoundly impact host health and are promising targets for biomedical research. While attempts have been made to use *in silico* modeling of metabolic networks with metagenomic and metabolomic data to predict large-scale metabolic functionalities or disease associations within the gut microbiota²²⁻²⁷, further research is needed. Specifically, a reductionist approach exploring relationships between specific species coupled with wet-lab validation of *in silico* results will be necessary before these frameworks can reliably contextualize the complexity of gut microbial interactions.

In feces, which are commonly used as a proxy for the gut microbiota, investigating the interactions between thousands of gut bacterial species using conventional low-throughput methods is conceivably time-consuming and inefficient. Data-driven approaches have therefore been revolutionary in developing our understanding of the gut microbiota, allowing researchers to predict functional potential, composition, and other characteristics of microbial populations based on metagenomics and other “-omics” data. By studying nucleic acids, metabolites, and other molecules associated with bacterial life, these approaches can even circumvent common wet-lab hurdles such as “unculturable” bacteria. Yet, this information fails to provide mechanistic evidence of biological phenomena such as cross-feeding,

and interpretation of bioinformatics data largely relies on the painstakingly accumulated results of wet-lab research. Innovative strategies are thus necessary to bridge the gap between these approaches.

A promising approach developed in recent years involves the utilization of droplet microfluidics, in which bacterial cells are randomly encapsulated in oil droplets to form water-in-oil microcultures containing as few as one cell per droplet. This approach creates a stochastically contained environment within each droplet, enabling the parallel cultivation of hundreds or even thousands of bacterial microcultures. Several such droplet-based techniques for cell cultivation have been described in the literature^{28–30} and have been successfully utilized for the screening and analysis of complex microbial populations in recent years^{31–33}. Recognizing the potential of this approach, we decided to utilize this technology in our exploration of gut microbial cross-feeding, henceforth referred to as iX-seq. Our goal was to identify and isolate novel cross-feeding relationships between gut bacteria using this droplet-based method of anaerobic bacterial cultivation (Fig. 1).

To establish proof of concept that iX-seq can be used to screen for potential cross-feeders in a complex microbial community, we aimed to identify potential cross-feeding bacterial pairs from fecal samples. These pairs would consist of a “receiver” strain, whose survival depends on metabolic substrates supplied by another strain, and a “sender” strain, capable of providing said substrates. We optimized our process to maximize 1-to-2-cell cultures, wherein a receiver strain would be identified by its ability to proliferate in the presence of a sender strain and failure to survive on its own. Identified sender-receiver pairs would then be subjected to further analyses, including metabolomics analysis and *in vivo* transplantation, to confirm cross-feeding relationships and potential mechanisms. This unidirectional cross-feeding approach provided a basic framework for testing our methodology and evaluating its feasibility in investigating the diverse mechanisms of cross-feeding within the gut microbiota.

Results

Droplet microculture screening for unidirectional cross-feeding in the human gut microbiota

Fresh human feces were collected from a healthy Japanese volunteer and processed for microfluidic droplet preparation, maximizing 1-2-cell microcultures. Representative fluorescence images of bacterial cell growth are shown in Fig. 2B. The premise of our investigation was as follows: if a given bacterium was undetectable in one-species droplets but detectable in two-species droplets—in which the accompanying species was also detectable in one-species droplets—this was interpreted as indicative of a potential unidirectional cross-feeding relationship. Droplets that contained no cells or more than two species were excluded from the analysis due to the complexity of interpreting relationships in higher-order combinations of senders and receivers.

By analyzing the droplet species compositions, we found that our droplet protocol was successful in focusing droplet compositions to 1 ~ 4 species (Fig. 2C). After excluding the droplets that did not meet

the aforementioned criteria, approximately ~ 65% of droplets remained as viable candidates for analysis.

Surprisingly, we found that the one-species droplets consisted of only three different species: unidentified members of *Eubacterium* (809/932 one-species droplets), *Escherichia-Shigella* (122/932), and Ruminococcaceae UBA1819 (1/932). In all, 22 different bacteria were identified in the mono- and two-species droplets (Fig. 2B). Using an observation rate threshold of 0.9 and requiring presence in at least three distinct droplets, a total of twelve potential receivers were identified (Table 1). Of these, we decided to focus on the pairing of two bacteria belonging to the genera *Phascolarctobacterium* and *Eubacterium*, for which a 16S rRNA sequence search using BLASTn identified them as *Phascolarctobacterium faecium* ACM 3679 (99.70% nt sequence identity, E-value = 7e-173) and *Eubacterium limosum* JCM 6421 (99.09% nt sequence identity, E-value = 6e-169). According to the literature, *Phascolarctobacterium* species can only utilize a narrow range of carbon sources and display a nutritional preference for succinate, which leads to the production of propionate^{34–37}. There is medical interest in this taxon due to its reported ability to suppress growth of *C. difficile* in the human gut by reducing the luminal availability of succinate, which is also a utilizable energy source for *C. difficile*³⁵.

Receiver	Sender	Rel. freq.	No. of droplets
<i>Enterobacter</i> unclassified	<i>Escherichia/ Shigella</i> unclassified	1	247
<i>Eggerthella</i> uncultured	<i>Eubacterium</i> unclassified	0.9885	86
<i>Catabacter hongkongensis</i>	<i>Eubacterium</i> unclassified	1	62
<i>Phascolarctobacterium</i>	<i>Eubacterium</i> unclassified	1	43
<i>Bacteroides cellulosilyticus</i> CL02T12C19	<i>Eubacterium</i> unclassified	1	31
<i>Bacteroides fragilis</i>	<i>Eubacterium</i> unclassified	0.9355	29
<i>Raoultibacter timonensis</i>	<i>Eubacterium</i> unclassified	1	20
Enterobacteriaceae unclassified	<i>Escherichia/ Shigella</i> unclassified	1	19
<i>Bacteroides thetaiotaomicron</i>	<i>Eubacterium</i> unclassified	0.9375	15
<i>Alistipes</i> uncultured	<i>Eubacterium</i> unclassified	1	10
Lachnospiraceae NK4A136 uncultured	<i>Eubacterium</i> unclassified	1	3
<i>Bacteroides</i> unclassified	<i>Eubacterium</i> unclassified	1	3

Going further, the *Phascolarctobacterium faecium* species specifically was reported to utilize succinate produced by *Bacteroides thetaiotaomicron*³⁸, implying that there is an established cross-feeding

relationship between these two gut inhabitants. Coincidentally, many *Eubacterium* species are also known to produce succinate as a metabolic byproduct³⁹, so we hypothesized that a similar metabolic exchange was occurring in the pairing of *E. limosum* and *P. faecium*. For these reasons, we elected to test these species for the presence of cross-feeding and validate the iX-seq methodology.

In vitro examination of *P. faecium* and *E. limosum* as possible cross-feeding bacteria

Our initial focus was to determine whether the observed cross-feeding phenomenon in the droplet cultures could be reproduced *in vitro*. We measured the growth of *P. faecium* co-cultured with *E. limosum* in mYCFA medium over a two-day time course. As a negative control, *P. faecium* was cultivated in monoculture with no additional substrates, while 1% (w/v) succinate-supplemented growth medium was also tested in recognition of the species' aforementioned ability to metabolize succinate^{35,37}.

To measure growth, we recorded CFU/mL and DNA copy number. The CFU count represents the viable cell count (Fig. 3A), while the DNA copy number estimates relative cell biomass (Fig. 3B). The control *P. faecium* monoculture exhibited a steady decline in CFU count and almost no increase in DNA copy number over time, indicative of limited cell proliferation and gradual cell death. In contrast, the succinate group showed a marked increase in CFUs and biomass by Day 1, followed by a sharp decrease in both metrics by Day 2. This growth-collapse trajectory likely reflects nutrient depletion or rapid accumulation of toxic metabolic byproducts, both of which would result in the precipitous loss of viability observed on Day 2. The timeline aligns with a previous study of *P. faecium*'s growth kinetics, reporting that OD₆₀₀ peaked between approximately 24 and 42 hours after inoculation in GAM supplemented with 1% (w/v) succinate, during which the succinate was also fully depleted³⁸. Notably, that study did not observe a collapse, but rather a stationary phase after reaching the peak by 42 hours. This may have been due to the presence of alternative growth substrates or protective cofactors in GAM that mitigated the metabolic stress caused by succinate depletion or toxic metabolite buildup. This would not be possible in the less-complex mYCFA medium, as it lacks many of the cell-derived extracts and complex nutrients found in the richer GAM. Overall, we hypothesize that the observations in the succinate-supplemented cultures were indicative of rapid growth, given that succinate serves as a preferred energy source for *P. faecium*^{35,37}.

Interestingly, the *P. faecium* and *E. limosum* co-cultures did not show an increase in CFU count over time as we expected. The DNA copy number did not differ significantly from the negative control on Days 1 and 2 either, though it was modestly higher. However, there was a statistically significant attenuation of CFU reduction between the co-culture and negative control, suggesting that while *E. limosum* could not effectively sustain growth of *P. faecium* by itself, it attenuated the decrease in viable cell count as a result of succinate deficiency in the growth medium. From these results, it appeared that *E. limosum* was able to provide some metabolic benefit to *P. faecium*, though it is unclear whether succinate production was the causal mechanism.

Mouse intestinal colonization model of *P. faecium* and *E. limosum* cross-feeding

It is widely recognized that the intestinal environment, where gut microbes colonize, produces vastly different outcomes compared to *in vitro* research conditions. Nutrient availability, shifts in pH, abundance of host-derived molecules, and countless other factors create an environment that cannot easily be replicated outside of the body. Since cross-feeding occurs within the context of the intestines, the next logical step in our study was to validate the predicted cross-feeding relationship through transplantation into an *in vivo* mouse model.

Germ-free (GF) BALB/c mice were initially inoculated with either sterile PBS or *E. limosum* and were observed for two weeks (Fig. 4A-B). Then, both groups were further inoculated with *P. faecium*. Fecal samples from each mouse were collected each week and measured for CFU/g feces of *P. faecium* and *E. limosum*. Interestingly, it was observed that *P. faecium* was only able to successfully colonize the mouse intestines when co-inoculated with *E. limosum*. At Week 3, the *E. limosum* group showed high *E. limosum* and *P. faecium* viable cell counts at about 10^{10} and 10^6 CFUs per gram of feces, respectively (Fig. 4B). These numbers remained consistent throughout the fourth and final week of measurement. It was apparent that some characteristic(s) of *E. limosum* conferred *P. faecium* the ability to colonize the mouse intestines.

Hypothesizing that cross-feeding underlies this phenomenon, we performed metabolome analysis of fecal samples from both PBS and *E. limosum*-inoculated mouse groups at Week 2 (prior to *P. faecium* inoculation) using principal component analysis (PCA) (Fig. 4C-D). As expected, we found that the metabolome profiles of each group were compositionally distinct. A number of distinguishing features were identified, including several amino acids.

However, the metabolite with the greatest influence on group separation was 2-oxoglutarate (2-OG), a molecule that is involved in the succinate metabolic pathway as a precursor to succinyl-CoA. Specifically, glutamate is hypothesized to be an alternative substrate to succinate in that it is intracellularly converted to 2-OG and subsequently to succinyl-CoA, a common intermediate predicted to be shared between succinate and glutamate in the succinate metabolic pathway of *P. faecium*³⁸ (Fig. 5A). Indeed, 2-OG was found in high quantities in the feces of *E. limosum*-inoculated mice, while mice without *E. limosum* did not excrete any detectable 2-OG (Fig. 4E). As we know that *P. faecium* can metabolize succinate, presumably by this pathway, we predicted that 2-OG was also implicated in the ability of *P. faecium* to colonize the mouse through metabolic cross-feeding from *E. limosum*.

Analysis of 2-OG as a cross-fed metabolite promoting *P. faecium* growth and colonization

We next performed *in vitro* growth experiments using *P. faecium* and 2-OG in order to investigate what effects it would have on *P. faecium* growth in defined conditions. 2-OG showed a significant increase in *P. faecium* growth that scaled with higher concentrations, reflected in both increased DNA copy numbers and CFUs by Day 2 (Fig. 5B-C). As 2-OG supplementation successfully promoted *P. faecium* growth, the data suggests that 2-OG production facilitating cross-feeding by *E. limosum* is at least one mechanism that may have contributed to colonization of *P. faecium* in the mouse intestines.

Discussion

In this study, we demonstrated the effectiveness of using iX-seq to supplement traditional methods of streak plating and colony isolation for screening of bacteria in complex environmental samples such as fecal samples. By applying this high-throughput methodology to bacterial communities, researchers can efficiently and rapidly identify bacteria of interest for their purposes.

As shown in the results, we were able to identify a number of potential cross-feeding organisms in fecal samples by virtue of the ability of the benefactor “sender” strain to support the growth of a beneficiary “receiver” strain. Furthermore, we were able to support the existence of this interaction through *in vitro* culturing and further evidenced their relationship in a mouse model. This culminated in the identification of what we believe to be a previously unreported cross-feeding relationship between *P. faecium* and *E. limosum*, thereby demonstrating the potential of iX-seq in exploring the relationships of complex microbial communities. Through the progression of high-throughput screening, 16S rRNA gene sequencing, *in vitro* culture, *in vivo* colonization, and metabolomic analysis, we were able to discover the pairing of *P. faecium* and *E. limosum* as cross-feeding members of the gut microbiota that are hypothesized to exert growth-supportive effects through the production of 2-OG.

However, it should be noted that the involvement of 2-OG is largely speculative, as we have not yet explored the mechanisms of 2-OG production and whether the metabolite was directly involved in *P. faecium* colonization of *E. limosum*-colonized GF mice. Nutrient supplementation via cross-feeding is only one of several possible explanations, as *E. limosum* may have modified the gut environment to favor *P. faecium* through alterations in luminal pH, redox potential, modulation of host-derived compounds, or other adjustment of physiochemical conditions. Furthermore, while *E. limosum* did appear to support *P. faecium* growth in droplet cultures, *E. limosum*'s growth-supportive effects were comparatively limited when cultured in larger culture volumes *in vitro*, raising further questions as to what conditions are necessary for *P. faecium* to receive benefits from *E. limosum*. To elucidate the exact nature of their relationship, further investigation and mechanistic studies are necessary. However, as the initial goal of this study was to test the viability of iX-seq for research on cross feeding, further investigation into the mechanisms underlying this predicted cross-feeding relationship is a topic of future research.

One obvious limitation of iX-seq that we realized was the limited diversity of bacteria that could be cultivated in single-species droplets, as well as the overall diversity of cultured bacteria. A key advantage of droplet microfluidics is believed to be the ability to isolate low-abundance bacteria from complex communities⁴⁰. However, with only 22 species identified and very few able to grow in single-species droplets in our case, further development of our methodology is necessary to maximize species diversity and cell viability. As applications of this technology to microbiology are still in their infancy, there are various factors to consider such as medium composition, droplet stability, anaerobicity, and sample handling, for which there are no universal standards. While droplet microfluidics is a powerful and increasingly popular technique in microbial ecology, the wide range of available microfluidic devices and methods necessitates methodological development on a case-by-case basis⁴¹. And regrettably, as with

traditional isolation methods, we must concede that certain bacteria may be unculturable, condemning these species to the confines of metagenomic data until advancements in bacterial culturing techniques facilitate their cultivation. Nevertheless, we aim to continue refining our own methodology to maximize the diversity of cultivable species and enable the observation of unknown cross-feeding interactions within the gut microbiota.

In order to narrow our focus to the most straightforward approach to droplet microfluidics-based screening, we only conducted tests to identify unidirectional cross-feeding bacteria and excluded other possibilities in this study. However, droplet microfluidics may theoretically be applied to various cross-feeding scenarios as we continue to develop our methodology. At higher levels of complexity, cross-feeding can also be bidirectional (both parties provide substrates to each other) or multidirectional (multiple parties and exchanges of substrates are involved), illustrating the ecological interconnectedness of the gut metabolic network. There are also a number of possible mechanisms for cross-feeding, such as the secretion of small molecules and degradation of complex molecules by the sender strain²⁰. While technically more complicated, exploration of such interactions may be achievable by using innovative approaches such as the development of a metabolite-detection pipeline integrated into the iX-seq method.

Conclusion

High-throughput methods such as iX-seq are invaluable for studying the complex microbial interactions within the human gut, enabling the evaluation of the thousands of species that constitute the gut microbiota. Using the iX-seq platform, we were able to isolate and provide evidence for a previously unknown cross-feeding relationship between *P. faecium* and *E. limosum*, also demonstrating the utility and potential of droplet microfluidics. Due to the increasing accessibility and efficiency of metagenomic sequencing, the mechanistic insights needed to contextualize the vast amounts of generated data are increasingly necessary, particularly for translating gut microbiota research into applications beyond the lab, such as in health and medicine.

Methods

Culture conditions

Modified YCFA (mYCFA) medium was prepared as described in a previous study⁴². The medium was sparged with an anoxic gas mixture of 85% nitrogen, 5% hydrogen, and 10% carbon dioxide while heating, supplemented with L-cysteine hydrochloride monohydrate, and dispensed into 30 mL serum bottles at 10 mL volumes under anoxic gas flow. The bottles were sealed with butyl rubber stoppers and aluminum crimp seals to preserve anaerobicity and autoclaved at 121°C for 20 minutes. To initiate bacterial cultures, bottles were inoculated with the respective inocula via syringe and incubated at 37 °C without agitation. In the case of well plate incubation, well plates were incubated partially uncovered in a vinyl

anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI, USA) at 37 °C. The chamber contained the same anoxic gas mixture as used for medium preparation.

Fecal sample collection and preparation

A fecal sample from a healthy Japanese volunteer was collected on the day of testing. Subjects with intestinal diseases, skin diseases, those who had taken antibiotics, or were allergic to the specified diet were excluded from consideration. All steps following fecal sample collection were performed in an anaerobic chamber containing an anoxic gas mixture of 85% nitrogen, 5% hydrogen, and 10% carbon dioxide. The fecal sample was homogenized in mYCFA as described in a previous study⁴² at a concentration of 10% (w/v), filtered through a 40 µm cell strainer to remove large debris, and further diluted in mYCFA medium supplemented with 0.5% SeaPlaque agarose, resulting in a final concentration of 0.01% (v/v). To prevent the agarose from solidifying, the diluted sample was maintained at 37 °C using a heat block.

Microfluidic droplet generation

A Droplet Generator (On-chip Biotechnologies Co., Ltd, Tokyo, Japan) was used to generate water-in-oil microfluidic droplets from the fecal suspension with a dispensation rate of 0 ~ 9 cells per droplet. The oil used was a 5% 008-FluoroSurfactant formulation (008-FluoroSurfactant-5wtH-10mL (2003001); On-chip Biotechnologies Co., Ltd.). Each droplet had a diameter of approximately 60 µm and was generated following the manufacturer's instructions. The droplets were collected in 1.5 mL microcentrifuge tubes and incubated at 37°C overnight in an anaerobic chamber. Following incubation, an equal volume of oil was added to each sample, and the tubes were cooled at 4°C for 15 minutes to allow the agarose to solidify. Subsequently, an equal volume of 1H,1H,2H,2H-perfluoro-1-octanol was added and mixed with the suspension to disrupt the oil layer. Then, 50 µL of T buffer (On-chip Biotechnologies Co., Ltd.) was added, and the mixture was centrifuged at 200 × g for 3 minutes at room temperature. The supernatant was collected, and the resulting gel beads (GBs) were recovered. These GBs were then dispensed into 384-well plates containing 100 µL of mYCFA per well using the Single-Particle Isolation and Sequencing (SPiS) instrument (On-chip Biotechnologies Co., Ltd.) and once more incubated overnight at 37°C in an anaerobic chamber.

DNA barcoding and 16S rRNA gene sequencing

After cultivation in the 384-well plates, 20 µL of each well culture were transferred to a new well plate, mixed with an equal volume of 20% (v/v) glycerol in PBS for a final concentration of 10% (v/v) glycerol, and stored at -80°C for future analysis. The remaining culture volume was used for unique 16S rRNA gene barcoding of bacterial DNA using the Echo 525 Liquid Handler. The 16S rRNA gene library was then sequenced using the Illumina MiSeq platform, and bacterial amplicon sequence variants (ASVs) were assigned using QIIME2. Droplet composition data was manually analyzed using R.

Strain isolation from droplets

The bacteria of interest were collected from their wells in the 384-well plate 10% (v/v) glycerol stocks and revitalized in liquid mYCFA medium. The bacteria were then cultured for two days at 37°C in an anaerobic chamber and subsequently passaged onto Gifu Anaerobic Medium (GAM; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) agar plates with or without vancomycin (20 µg/mL) to culture for four additional days until distinct colonies were visible. The purpose of vancomycin was to isolate *P. faecium* from two-species droplets, utilizing *E. limosum*'s inherent sensitivity to the antibiotic and *P. faecium*'s resistance. Individual colonies were then isolated and cultured in liquid mYCFA medium for an additional two days before mixing with an equal volume of 20% (v/v) glycerol in PBS for storage at -80°C at a final glycerol concentration of 10% (v/v).

Mouse experiments

Germ-free (GF) BALB/c mice (female, 6–20 weeks of age) were housed in gnotobiotic isolators and fed an autoclaved, standard rodent chow diet (CMF, Oriental Yeast Co., Ltd., Tokyo, Japan). *P. faecium* was cultured in GAM medium supplemented with 1% (w/v) succinate for 48 hours. After cultivation, the cultures were centrifuged at 10,000 × g for 5 minutes at room temperature, and the supernatant was discarded. The resulting cell pellets were resuspended in freshly prepared GAM medium. *E. limosum* was cultured in GAM medium for 24 hours and was processed in the same manner as *P. faecium*. The mice were inoculated with *P. faecium* or *E. limosum* cells by oral gavage of 200 µL of 1×10⁸ colony-forming units (CFUs). During the 4-week experiment, feces were collected at weekly intervals for additional testing. All experiments were conducted in accordance with protocols approved by the University of Tsukuba Animal Experiment Committee.

CE-TOFMS-based metabolome analysis

Fecal metabolites were extracted from the samples and detected by capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) using methods described in previous studies^{43,44}. Briefly, fecal metabolites were extracted from 10 mg of freeze-dried feces by adding 500 µL of methanol containing 20 µM each of methionine sulfone (A17027; Alfa Aesar, Ward Hill, MA, USA) and D-camphol-10-sulfonic acid (CSA) (4987481429680; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) as internal standards. and shaking vigorously with 100 mg of 0.1-mm zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK, USA) for 5 min at 1500 rpm using a Shake Master Neo (Bio Medical Science, Inc., Tokyo, Japan). Next, this mixture was mixed with 200 µL of ultrapure water and 500 µL of chloroform and shaken again. The suspension was centrifuged at 4,600 × g for 15 min at 4°C, and the resulting supernatant was transferred to a 5-kDa-cutoff filter column (Ultrafree MC-PLHCC 250/pk for Metabolome Analysis; Human Metabolome Technologies, Tsuruoka, Japan). The flow-through was dried under a vacuum, and the residue was then dissolved in 50 µL of Milli-Q water containing reference compounds (200 µM each of 3-aminopyrrolidine and trimesate). The levels of extracted metabolites were measured in both positive and negative modes by CE-TOFMS as described previously⁴⁵. All CE-TOFMS experiments were performed using an Agilent capillary electrophoresis system (Agilent

Technologies, Santa Clara, CA, USA). Raw data was analyzed using our proprietary automatic integration software MasterHands (ver. 2.16.0.15)⁴⁵. Principal component analysis (PCA) was performed using the software MetaboAnalyst 6.0^{46,47}. Metabolome data was normalized by sum, log₁₀-transformed, and Pareto-scaled prior to analysis.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committees of Keio University Shonan Fujisawa Campus (No. 355). All subjects were informed of the purpose of this study, and written consent was obtained from all subjects. This study was conducted with strict consideration for privacy.

Consent for publication

Not applicable.

Availability of data and material

Microbiome data has been deposited to DDBJ (accession number PRJDB37686), and metabolome data has been deposited to MetaboBank (accession number MTBKS266).

Competing interests

The authors declare no competing interests.

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Authors' contributions

SF, KT, and IS conceived the study and designed the experiments. KT, NT, and NO conducted animal experiments. NT and MK performed microfluidics experiments, metagenome and metabolome analyses. IS and NT conducted informatics analysis. SF, NO, JY, GN, CI, and KT provided critical review and commentary of the manuscript. All authors contributed to the article and approved the submitted version.

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Figures

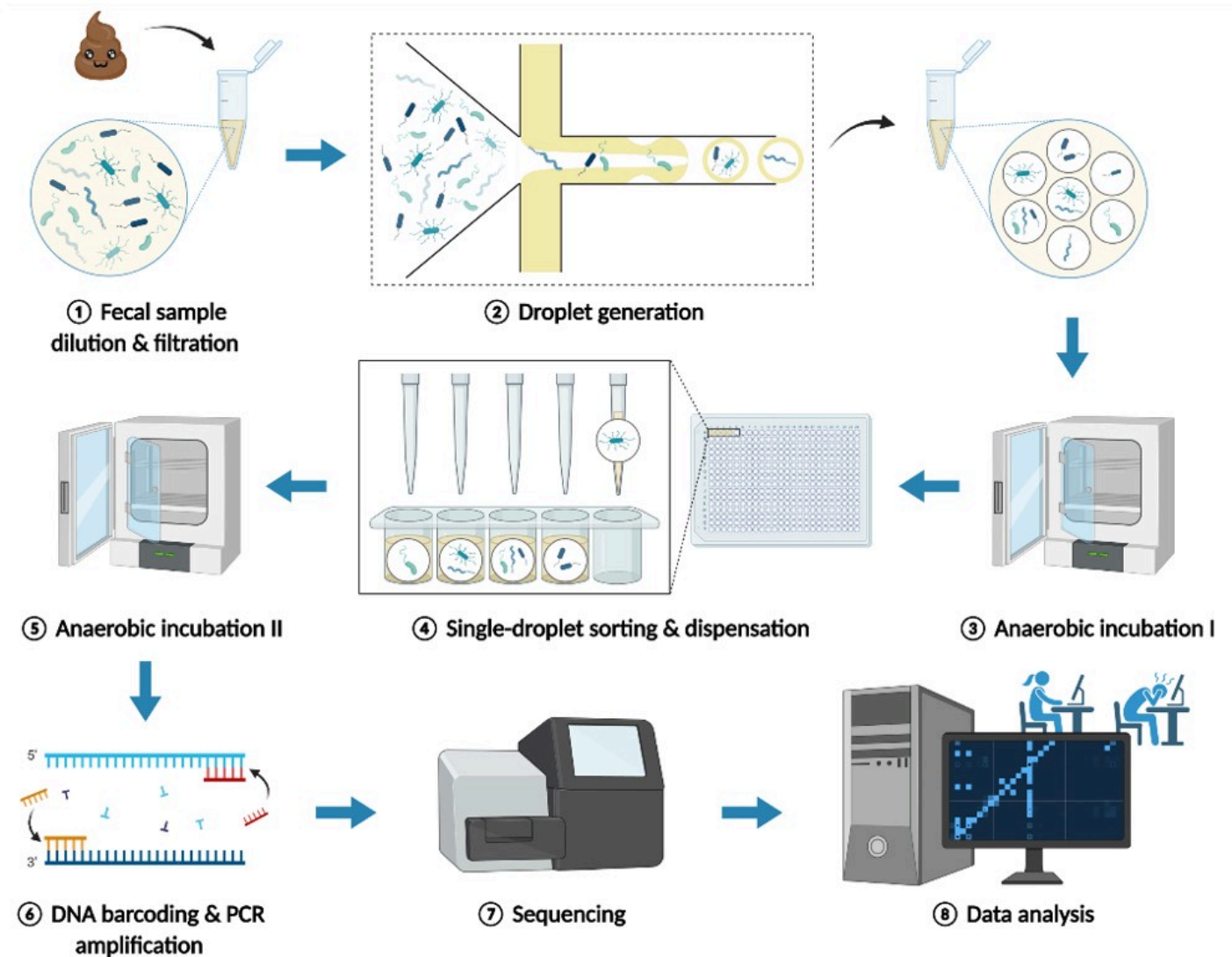


Figure 1

Overview of iX-seq. iX-seq enables high-throughput screening of bacterial interactions by combining anaerobic co-culture, single-droplet sorting, and sequence-based identification. Only droplets containing one or two species were retained for analysis in this study.

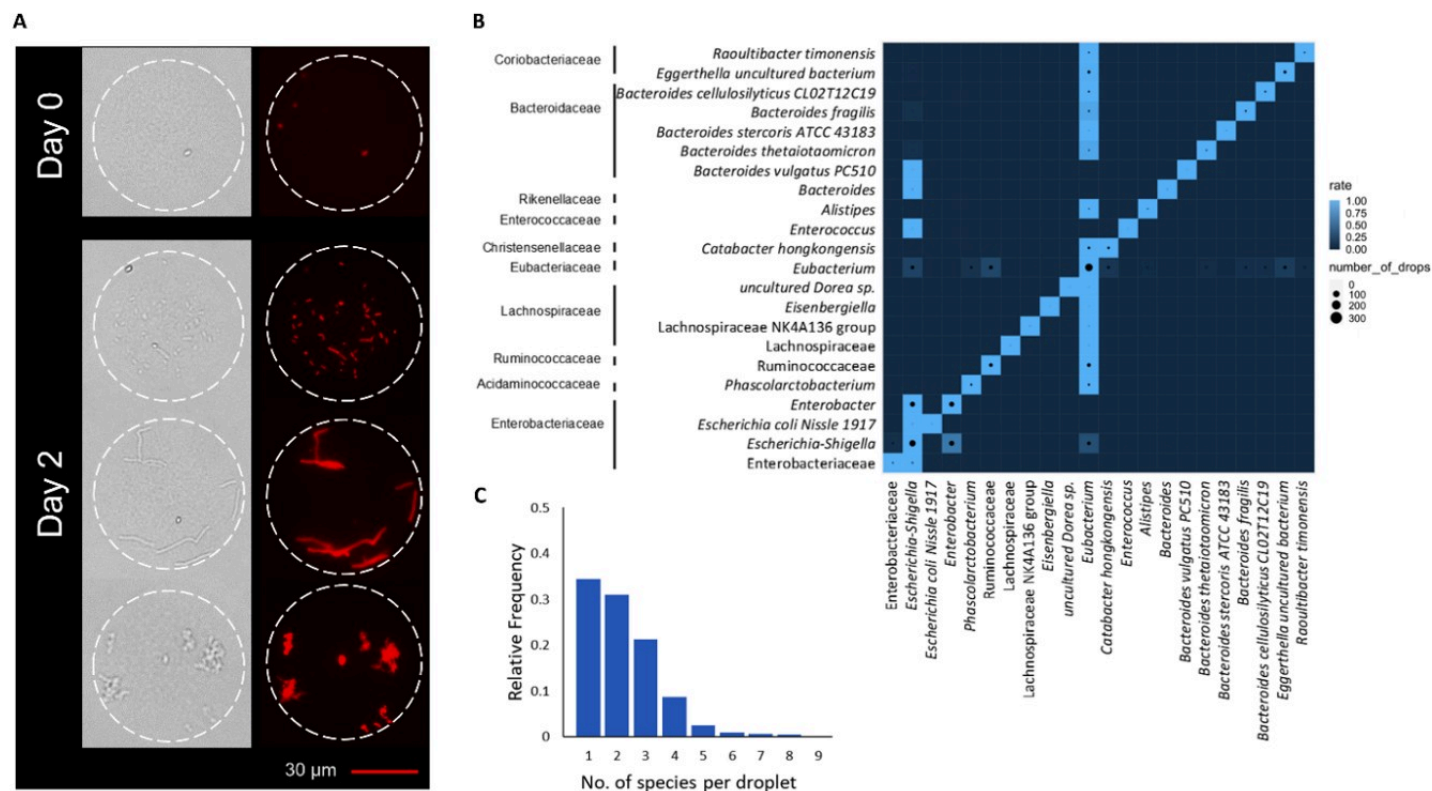


Figure 2

Bacterial compositions in droplet microcultures. (A) Fluorescence visualization of bacterial cells stained with FM4-64 dye after 0 and 2 days of incubation in microfluidic droplets. (B) Heatmap showing observed bacterial compositions in two-species droplet microcultures. For each y-axis species, the heatmap shows (1) the frequency of co-occurrence with x-axis species as a proportion of total droplets containing the y-axis species, and (2) the absolute number of droplets in which each pair was observed. (C) Relative frequency distribution of species counts per droplet (2707 total droplets).

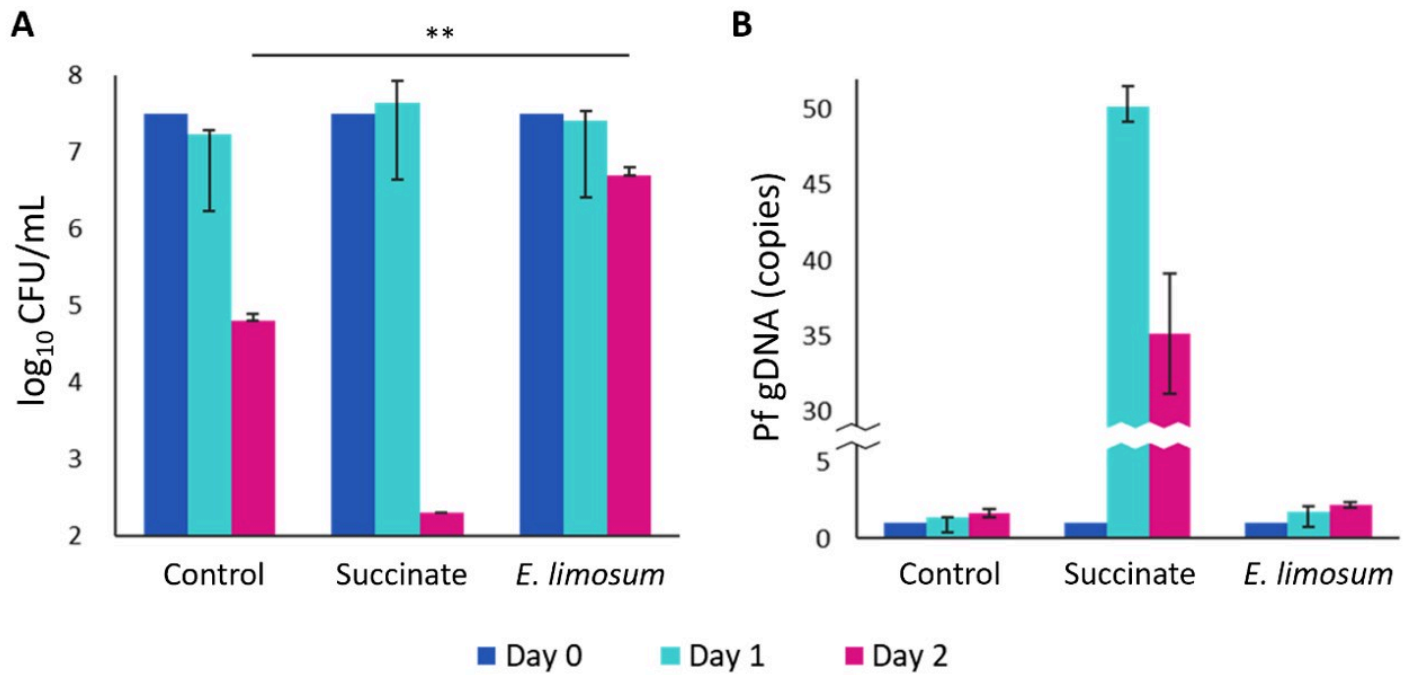


Figure 3

***In vitro* growth of *P. faecium* in succinate-supplemented medium or co-cultured with *E. limosum*.** Bar plots represent (A) $\log_{10}$ CFU/mL and (B) DNA copy number of *P. faecium* cultures over a 2-day period under different conditions: Control (non-supplemented *P. faecium* monoculture), Succinate (1% (w/v) succinate-supplemented *P. faecium* monoculture), and *E. limosum* (*P. faecium* + *E. limosum* co-culture). Data shows mean $\pm$ standard deviation ($n = 3$). Statistical significance was evaluated using Tukey-Kramer test (** $p < 0.01$).

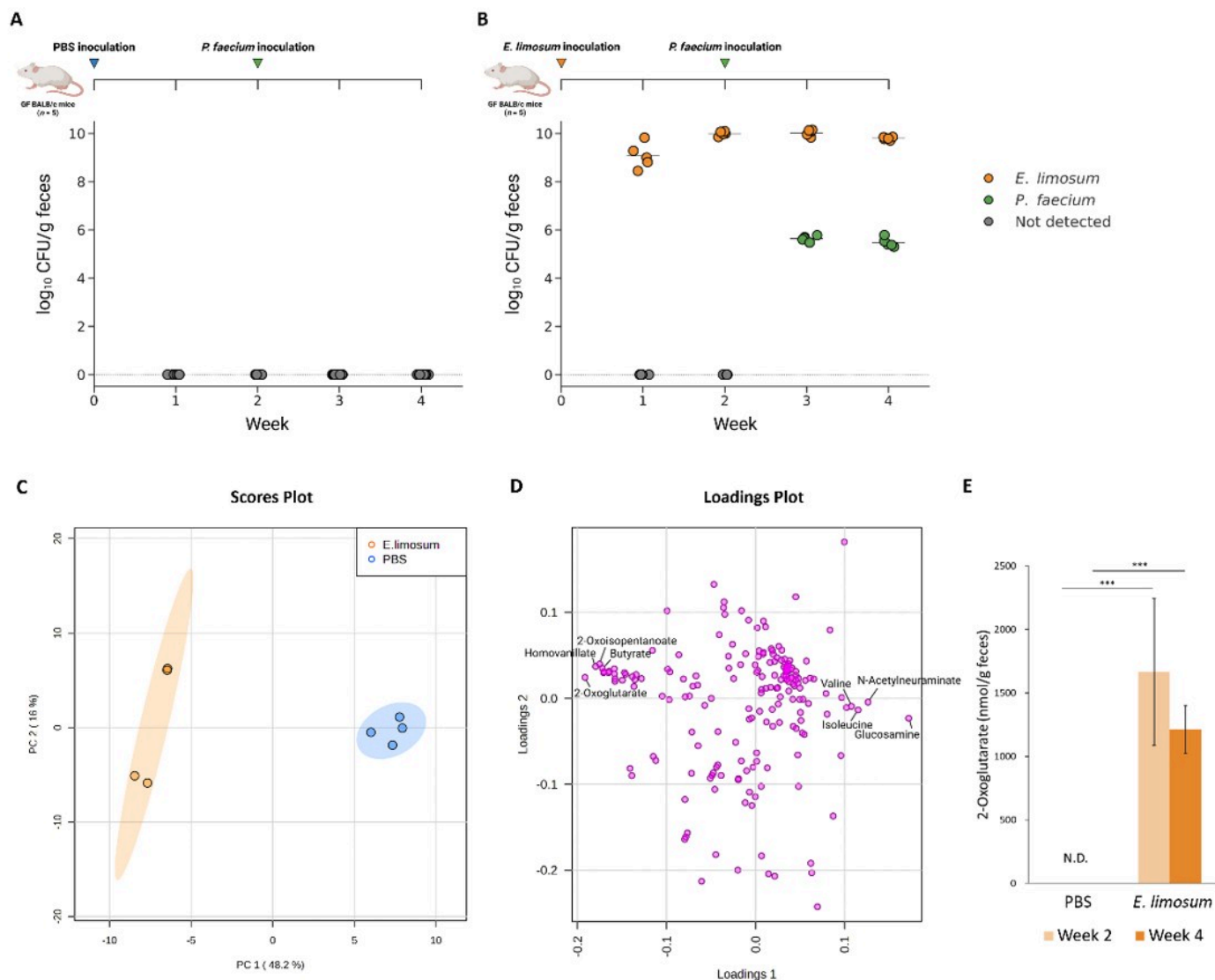


Figure 4

***In vivo* growth of *P. faecium* in GF mice co-inoculated with *E. limosum*.** Overview of 4-week mouse experiment and log₁₀ CFU/g feces of *E. limosum* and *P. faecium*. Germ-free (GF) BALB/c mice were initially inoculated with either **(A)** PBS or **(B)** *E. limosum* at Week 0 and then were subsequently inoculated with *P. faecium* after 2 weeks ($n = 5$). **(A-B)** CFUs of *E. limosum* and *P. faecium* in feces were measured weekly. **(C-D)** PCA of fecal metabolome data. **(C)** Scores plot showing separation of metabolic profiles in feces from *E. limosum*-inoculated GF mice and PBS-inoculated GF mice after two weeks and prior to *P. faecium* inoculation. **(D)** Loadings plot illustrating the contribution of individual metabolites to the principal components. The most strongly discriminating metabolites between groups are located toward the left and right ends of the plot, and the top-4 discriminatory metabolites on each side are labeled. **(E)** Concentrations of 2-oxoglutarate in feces from control and treatment group. Statistical significance was evaluated using Tukey-Kramer test ($***p < 0.001$).

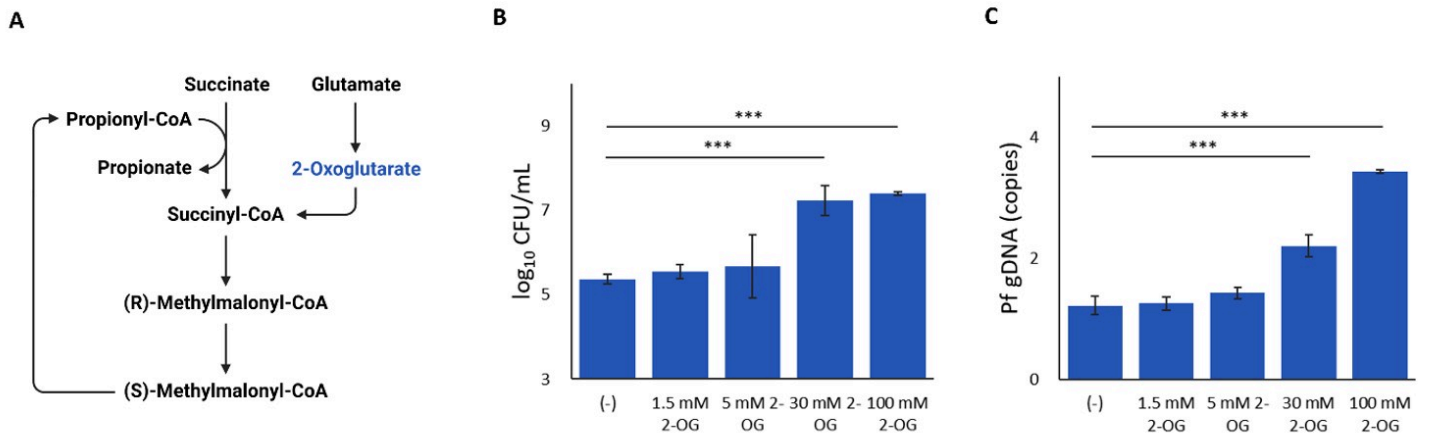


Figure 5

***In vitro P. faecium* growth in 2-oxoglutarate (2-OG)-supplemented medium.** Bar plots show **(A)** $\log_{10}$ CFU/mL and **(B)** DNA copy number of *P. faecium* in cultures supplemented with increasing concentrations of 2-OG. Statistical significance was evaluated using Tukey-Kramer test (**p < 0.01). **(C)** Schematic overview of the succinate metabolic pathway in *P. faecium* and the predicted involvement of 2-OG.