

The reverse β -oxidation pathway in *Megasphaera indica* links valerate and hexanoate biosynthesis to the suppression of gut-colonizing fungi

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Abstract

The gastrointestinal expansion of opportunistic fungal pathogens has been shown as an important reservoir for severe invasive infections in immunocompromised population. In healthy individuals, such expansion is constrained by short chain fatty acids (SCFAs) which are linked with the presence of gut commensal bacteria. Although the antifungal activities and the biosynthesis of acetate, propionate, and butyrate have been well-characterized, the gut bacterial producers and biosynthetic pathways of valerate and hexanoate remain largely unknown. Here, we systematically profiled SCFA production across a phylogenetically diverse collection of healthy human gut bacterial isolates and identified *Megasphaera indica* as a prominent producer of valerate and hexanoate. We found that the reverse β -oxidation pathway genes were highly expressed in *Megasphaera indica* and knockout of the key pathway thiolase genes *thlA1-thlA2* abolished biosynthesis of both valerate and hexanoate. In mice experiments, wild-type and reverse β -oxidation-deficient *Megasphaera indica* colonized the intestine at comparable levels, but the mutant strain failed to produce valerate or hexanoate *in vivo*. Finally, we showed that disruption of the reverse β -oxidation pathway markedly attenuated the inhibitory activity of *Megasphaera indica* against diverse fungi, including *Candida albicans*, *Candida parapsilosis* and *Nakaseomyces glabratus*. Together, our findings elucidate the gut bacterial metabolic pathway that links valerate and hexanoate biosynthesis to the suppression of opportunistic gut fungi, providing critical insights for microbiota-based antifungal strategies.

Keywords: Short-chain fatty acids (SCFAs); Valerate; Hexanoate; Gut fungi; Reverse β -oxidation pathway; *Megasphaera*

Introduction

The gastrointestinal tract can serve as an important reservoir for opportunistic fungal pathogens that cause invasive infections in immunocompromised individuals¹⁻⁴. Several fungal priority pathogens, including *Candida albicans*, *Candida parapsilosis* and *Nakaseomyces glabratus*, can colonize the human gut before translocation and systemic dissemination⁵. Other gut-associated yeasts, including *Meyerozyma guilliermondii*, *M. caribbica*, *Kodamaea ohmeri*, *Candida lusitaniae*, *Pichia kudriavzevii* and *Wickerhamomyces anomalus*, have also been recovered from patients with bloodstream or other invasive fungal infections⁶⁻⁹. In healthy individuals, fungal expansion is constrained in part by metabolites produced by the commensal bacterial microbiota. Among these metabolites, the major short-chain fatty acids acetate (C2), propionate (C3) and butyrate (C4), together with the less-characterized C5 fatty acid valerate, have been shown to directly inhibit opportunistic fungi^{4,10}. Defining how microbiota-derived fatty acids constrain opportunistic fungi is therefore important for understanding resistance to fungal expansion and invasive disease.

This colonization resistance depends strongly on the commensal bacterial microbiota. In the human gut, the absolute abundance of bacteria is 10²- to 10⁴-fold higher than that of fungi¹¹. Depletion of commensal bacteria increases intestinal *C. albicans* burden and promotes systemic dissemination, whereas transplantation of a healthy fecal microbiota can restore colonization resistance^{12,13}. One mechanism underlying this protection involves bacteria-derived fatty acids. Acetate, propionate and butyrate accumulate at high millimolar concentrations in the colon¹⁴⁻¹⁶ and directly inhibit *C. albicans* by impairing hexose uptake, disrupting central carbon metabolism and inducing intracellular acidification¹⁷. More recently, valerate was shown to suppress *C. parapsilosis* through a related acidification-dependent mechanism¹⁰, extending the known antifungal activity of microbiota-derived fatty acids beyond the major C2–C4 metabolites.

Despite these advances, the gut bacterial producers and biosynthetic pathways of the less-characterized C5/C6 SCFAs valerate and hexanoate remain poorly understood. This contrasts with the comparatively well-characterized microbial origins of the major C2–C4 SCFAs¹⁸. Acetate is produced by a broad range of

intestinal anaerobes¹⁹, whereas propionate is generated mainly through the succinate, 1,2-propanediol and acrylate pathways^{16,19,20}. Butyrate is commonly synthesized through butyryl-CoA transferase- or butyrate kinase-dependent pathways, as well as through peptide and amino acid fermentation^{16,21,22}. Branched-chain fatty acids, including isobutyrate, isovalerate and 2-methylbutyrate, can be generated from branched-chain amino acids through the *porA* pathway^{23,24}. By comparison, the intestinal producers and genetic basis of straight-chain valerate and hexanoate biosynthesis remain largely unknown. In *Clostridium aminovalericum*, valerate formation has been linked to proline conversion and 5-aminovalerate degradation^{25,26}, whereas hexanoate can be generated through carbon-chain elongation using butyrate as a precursor²⁷. Because reverse β -oxidation extends acyl-CoA intermediates by two-carbon units, it provides a plausible route for converting propionate into valerate and butyrate into hexanoate. Whether human gut bacteria produce these metabolites through this pathway, and whether pathway-dependent production of valerate and hexanoate contributes to fungal suppression, remain unknown.

Here, we systematically profiled the antifungal activities of fatty acids from acetate to hexanoate across diverse gut-associated fungi and screened a phylogenetically diverse collection of human gut bacterial isolates for valerate and hexanoate production. We identified *Megasphaera indica* as a prominent producer of both metabolites. Propionate and butyrate supplementation selectively enhanced valerate and hexanoate production, respectively, while reverse β -oxidation pathway genes were highly expressed relative to other genes in *M. indica*. Knockout of the key pathway thiolase genes, *thlA1-thlA2*, abolished biosynthesis of both metabolites. Mouse colonization experiments further demonstrated that this pathway is required for valerate and hexanoate production in vivo. Finally, comparisons of wild-type and reverse β -oxidation-deficient strains showed that valerate and hexanoate biosynthesis contributes to the suppression of diverse opportunistic fungi. Together, our findings define a genetically validated gut bacterial pathway linking valerate and hexanoate production to the suppression of opportunistic fungi.

Results

Valerate and hexanoate inhibit gut opportunistic pathogenic fungi at physiological concentration

Acetate, propionate, and butyrate inhibit *Candida albicans* growth at high millimolar concentrations through mechanisms like reducing protein translation and regulating ribosomal protein acetylation¹⁴. The inhibitory effects of longer-chain fatty acids, such as valerate (C5) and hexanoate (C6), on *Candida* species and other commensal fungi remain poorly understood. To investigate this, we assessed the inhibitory effects of a gradient of SCFAs (acetate, propionate, butyrate, valerate, isovalerate and hexanoate) on three common opportunistic fungal pathogens: *Candida albicans*, *Candida parapsilosis*, and *Nakaseomyces glabratus*. The inhibitory effect of these fatty acids increased along with the carbon-chain length and concentration (**Fig. 1A, Supplementary Fig. 1, and Supplementary Table 1**). This pattern aligns with previous findings that propionate and butyrate inhibit *C. albicans* more effectively than acetate¹⁵. Among the fatty acids tested, valerate and hexanoate showed the strongest antifungal activities, with inhibitory concentrations in the low-millimolar range.

To determine whether SCFAs reach antifungal concentrations in the human gut, we quantified fecal SCFAs in 100 healthy individuals from the SIAT and Westlake cohorts (n = 50 per cohort). Median acetate, propionate and butyrate concentrations were 28.2, 13.8 and 14.1 $\mu\text{mol/g}$ wet feces, respectively, consistent with previous reports²⁸. Valerate had a median concentration of 1.7 $\mu\text{mol/g}$ and ranged from undetectable to 5.6 $\mu\text{mol/g}$, overlapping with the concentrations that inhibited fungal growth *in vitro* (**Fig. 1B and Supplementary Table 2**). Notably, however, hexanoate exhibited antifungal activity at lower concentrations than valerate, indicating greater antifungal potency. Thus, although valerate is more abundant in the human gut, the higher potency of hexanoate may compensate, at least in part, for its lower fecal concentration.

To determine whether these activities extend across the gut mycobiota, we further examined dose-dependent inhibition by valerate and hexanoate against 16 gut-associated fungal species (**Supplementary Fig.**

2, and Supplementary Table 3). Both valerate and hexanoate effectively inhibited the growth of emerging opportunistic yeasts (Fig. 1C, Supplementary Fig. 3, and Supplementary Table 3), including *Meyerozyma guilliermondii*^{9,29}, *Meyerozyma caribbica*⁹, *Kodamaea ohmeri*⁶, *Candida lusitanae*⁷, and *Wickerhamomyces anomalus*⁸. Additionally, *Candida parapsilosis* and *Pichia kudriavzevii*, listed on the WHO Fungal Priority Pathogen List, were also significantly inhibited. Across the fungal panel, hexanoate generally exerted comparable or stronger inhibition at lower concentrations than valerate, whereas valerate was present at higher concentrations in human feces. Together, these results reveal complementary abundance–potency profiles for valerate and hexanoate and suggest that both fatty acids may contribute to microbiota-mediated resistance to opportunistic fungal colonization.

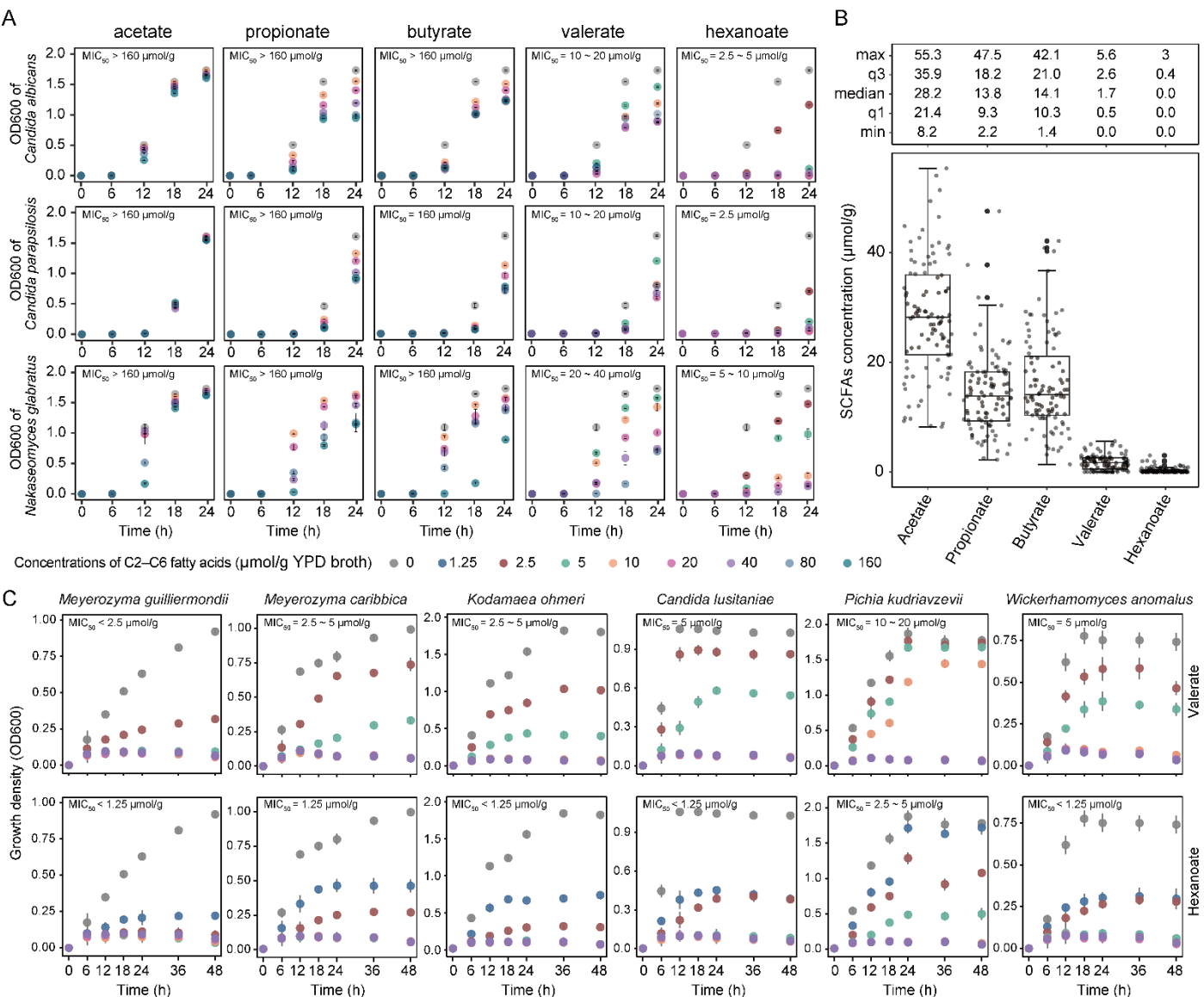


Figure 1. Valerate and hexanoate inhibit diverse gut-associated opportunistic fungi. A) The growth curve

shows the changes in optical density at 600 nm (OD₆₀₀) over time for three common opportunistic pathogens, *Candida albicans*, *Candida parapsilosis*, and *Nakaseomyces glabratus*, in response to a two-fold gradient of short-chain fatty acid concentrations. The concentration ranges tested were 10-160 $\mu\text{mol/g}$ for acetate, propionate, and butyrate; 5-80 $\mu\text{mol/g}$ for valerate; and 2.5-40 $\mu\text{mol/g}$ for hexanoate. MIC₅₀ values, defined as the minimum concentration that reduces fungal growth by 50% relative to the untreated control, are indicated on each curve. Data are presented as mean values $\pm$ SD. Assays were performed in technical triplicates (n = 3). **B)** The boxplot displays the concentrations of short-chain fatty acids (acetate, propionate, butyrate, valerate, and hexanoate) in 100 healthy human feces, expressed in $\mu\text{mol/g}$. The concentrations were determined using ethyl acetate extraction and GC analysis (**Methods section**). The minimum, 1st quartile (q1), median, 3rd quartile (q3), and maximum values for each fatty acid are shown in the upper region of the plot. **C)** The growth curve shows changes in optical density at 600 nm over time for 6 common gut fungi at 37°C. All strains were cultured in YPD medium, in response to a two-fold gradient of valerate concentrations (2.5-40 $\mu\text{mol/g}$) and hexanoate concentrations (1.25-40 $\mu\text{mol/g}$). MIC₅₀ values are marked on the curves. Data are presented as mean values $\pm$ SD. Assays were performed in technical triplicates (n = 3).

Systematic profiling of human gut bacteria to identify key producers of valerate and hexanoate

Based on the concentration-dependent inhibitory effects of SCFA against gut opportunistic pathogenic fungi, it is crucial to profile the SCFAs production of gut bacteria to identify high SCFA-producing strains. However, experimental data on SCFA production from monocultures of gut bacteria remain limited. To address this gap, we conducted a comprehensive screening of SCFA production across 557 gut bacterial isolates (**Supplementary Fig. 4, and Supplementary Table 4**). Among the top 20 producers of acetate, propionate, butyrate, and valerate, 45 strains were selected for whole-genome sequencing and subsequent validation through triple-replicate experiments (**Fig. 2A, and Supplementary Table 5**). Our SCFA profiling results showed that 34 out of 45 gut bacteria strains could produce acetate, consistent with previous reports that acetate is produced across various phyla¹⁹. The top acetate producers were *Bacteroides dorei* DA26 and *Anaerostipes caccae* DA534. Moreover, 23 gut bacteria strains were able to produce propionate, predominantly members of the *Bacteroidetes* and *Firmicutes* phyla. The top propionate producers were

Bacteroides dorei DA26 and *Parabacteroides johnsonii* DA701. 16 gut bacteria strains produced butyrate, with high producers primarily in the *Firmicutes* phylum. The top butyrate producers were *Emergencia timonensis* DA94 and *CAG-81 sp900066785* DA50. 16 gut bacteria strains produced branched-chain fatty acids (isobutyrate and isovalerate), including *Bacteroides*, *Parabacteroides*, and *Alistipes* species. 9 strains were capable of producing valerate, with high production observed in certain *Firmicutes* strains, such as *Megasphaera indica* DA18, *Clostridium_AQ innocuum* DA537. 3 gut bacteria strains produced hexanoate, with *Megasphaera indica* DA18 being the most notable high producer (**Fig. 2B**).

Phylogenetic analysis revealed that species within the same genus, such as *Bacteroides* spp., *Parabacteroides* spp., and *Blautia* spp., exhibited similar short-chain fatty acid (SCFA) production profiles (**Fig. 2A**), suggesting functional conservation of SCFA production within the genera. Similarly, beyond *M. indica*, other *Megasphaera* species, including *M. elsdenii*³⁰, *M. massiliensis*³¹, and *M. hexanoica*³², have also been reported to produce valerate, suggesting that genus- or species-level taxonomy can provide an initial guide for prioritizing candidate valerate-producing strains. To complement these phylogeny-based patterns, we examined genotype-phenotype associations for the major SCFA pathways (acetate, propionate, butyrate, and branched-chain fatty acids) and found strong concordance between pathway presence and metabolite production, as detailed in **Supplementary Figures 5-8 and Supplementary Table 6-9**. This robust mapping suggests that pathway-based screening holds strong potential for identifying strains with desired SCFA phenotypes, including high producers of valerate and hexanoate.

To assess the contribution of *Megasphaera* to valerate and hexanoate levels in the gut, we compared fecal samples from 100 healthy human. In this cohort, samples containing *Megasphaera* exhibited significantly higher valerate levels (**Fig. 2C, and Supplementary Table 6**). In contrast, no significant differences in hexanoate levels were observed (**Supplementary Fig. 9, and Supplementary Table 10**). These findings suggest that *Megasphaera* is a major contributor of valerate in the human gut.

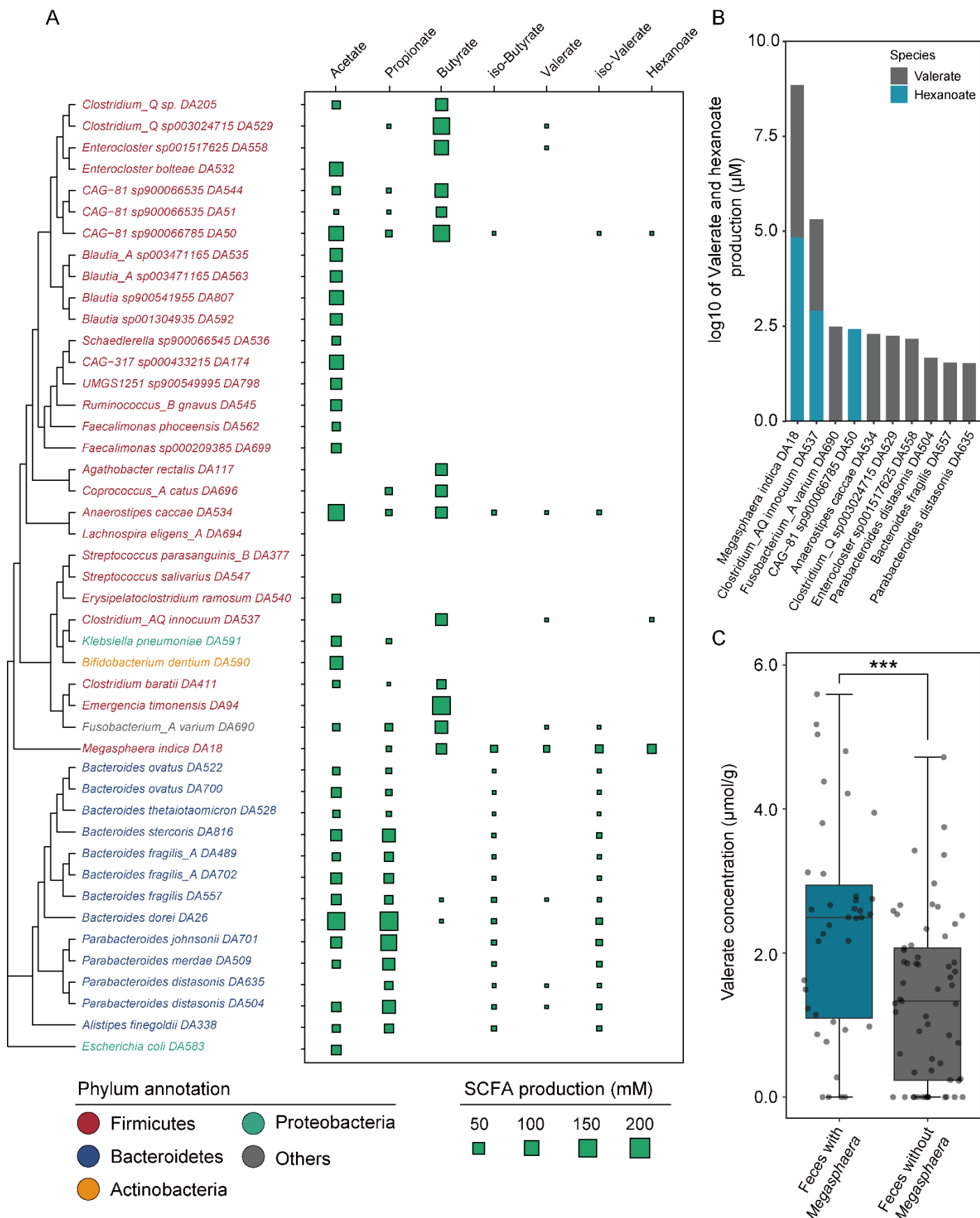


Figure 2. Systematic SCFA profiling identifies *Megasphaera indica* as a prominent producer of valerate and hexanoate. **A)** The color and size of square indicate the production of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate and hexanoate, normalized to OD600 = 1, each with n = 3 biological replicates. **B)** Stacked bar plot illustrating the total production of valerate and hexanoate, normalized to OD600

=1 and shown as log₁₀ transformed concentrations. C) Boxplots showing fecal valerate levels in 100 healthy human samples stratified by the presence or absence of the *Megasphaera* genus. Statistical significance was assessed using a two-sided Wilcoxon rank-sum test; *** indicates $p < 0.001$.

***Megasphaera indica* produces valerate and hexanoate via the reverse β -oxidation pathway**

The genetic basis of valerate production pathways in gut bacteria remains unclear. One proposed route involves proline conversion, 5-aminovalerate degradation and propionate elongation^{25,33} (**Supplementary Fig. 10A**). To determine whether *M. indica* relies on this pathway, we knocked out the key enzyme 5-aminovalerate aminotransferase (DavT) (**Methods; Supplementary Fig. 10A-B**). Gas chromatography (GC) analysis showed no significant difference in valerate production between the knockout strain (*M. indica* *DavT::Cm^r*) and the wild-type, indicating that *M. indica* does not produce valerate via the 5-aminovalerate pathway (**Supplementary Fig. 10C**).

Having excluded the 5-aminovalerate pathway, we next investigated whether *M. indica* produces valerate and hexanoate through carbon-chain elongation. In some *Clostridium* species, this process is mediated by reverse β -oxidation, in which two-carbon units are added per cycle³⁴, with propionate acting as the precursor for valerate formation²⁷. Motivated by these observations, we examined the *M. indica* genome and identified a complete set of genes encoding enzymes associated with reverse β -oxidation. In addition, genome annotations showed that propionate production in *M. indica* proceeds through the acrylate pathway using lactate as a substrate, whereas butyrate production occurs via reverse β -oxidation using acetate as a substrate. Together, these genomic features suggested that *M. indica* may extend carbon chains through reverse β -oxidation, leading to the hypothesis that valerate and hexanoate is produced via this pathway using propionate and butyrate as precursors, respectively (**Fig. 3A**).

To test this hypothesis, we performed substrate-supplementation experiments using glucose as the reference carbon source. Lactate supplementation increased propionate and valerate production, consistent

with lactate-derived propionate serving as a precursor for valerate biosynthesis. Direct supplementation with propionate further enhanced valerate production but reduced hexanoate levels. Conversely, butyrate supplementation increased hexanoate production and decreased valerate production (**Fig. 3B**). These findings support the idea that propionate and butyrate may compete for the same metabolic pathway. In addition, we performed transcriptomic analysis under four different culture conditions. The results showed that genes predicted to encode enzymes involved in the reverse β -oxidation pathway were highly expressed in *M. indica*, with expression levels ranking among the highest in the transcriptome (**Fig. 3C, Supplementary Fig. 11, and Supplementary Table 11**).

To determine whether *M. indica* synthesizes valerate and hexanoate via the reverse β -oxidation pathway, we knocked out the first and second enzymes in this pathway. The first enzyme, thiolase (acetyl-CoA acyltransferase), catalyzes the condensation of acetyl-CoA with an acyl-CoA to extend the carbon chain by two carbons; in *M. indica*, this enzyme is encoded by two adjacent gene copies. The second enzyme, β -hydroxyacyl-CoA dehydrogenase, uses NADH or NADPH as a reducing agent to convert the β -keto group into a hydroxyl group. The results showed that the knockout strains no longer produced butyrate, valerate, or hexanoate (**Fig. 3D-E**). These results further confirmed that *M. indica* produces valerate and hexanoate via the reverse β -oxidation pathway. Interestingly, a significant correlation was found between propionate and valerate levels in fecal samples ($R = 0.42$, $p = 1.8 \times 10^{-5}$), suggesting that the carbon chain elongation pathway from propionate is the primary route for valerate synthesis in the human gut (**Supplementary Fig. 12A-C**). In contrast, valerate showed little to no positive correlation with acetate ($R = 0.052$, $p = 0.61$) or butyrate ($R = 0.13$, $p = 0.21$), further supporting the specificity of propionate as the dominant precursor (**Supplementary Fig. 12A-C**).

To assess the prevalence and distribution of *Megasphaera* in the human gut, we analyzed an expanded dataset comprising the original 100 healthy donors and an additional 192 generally healthy individuals, yielding a total of 292 individuals. Across the combined cohort, *Megasphaera* was detected in 32.8% of

samples, with a mean relative abundance of 0.45%. Among *Megasphaera* species, *M. indica* showed a prevalence of 18.2% and a mean relative abundance of 0.66%, whereas *M. elsdenii* exhibited a prevalence of 12.3% and an abundance of 0.14% (**Supplementary Fig. 13, and Supplementary Table 12**). To further examine the geographic distribution of *Megasphaera*, we analyzed publicly available datasets from the GMrepo database³⁵(**Methods**). Notably, *M. indica* was more prevalent in the Chinese and Canadian cohorts, whereas *M. elsdenii* predominated in the Danish and French cohorts, highlighting pronounced geographic variation in *Megasphaera* species distribution (**Supplementary Fig. 14**).

To investigate the role of reverse β -oxidation in valerate and hexanoate biosynthesis *in vivo*, we colonized mice with either wild-type *M. indica* or the *thlA1-thlA2::Cm^r* mutant (**Fig. 3F**). At day 32 post-colonization, both strains colonized the intestine at comparable levels, reaching approximately 2×10^6 CFU/g feces, with no significant difference between the two groups (**Fig. 3G, and Supplementary Table 13**). Fecal valerate and hexanoate were detected in mice colonized with the wild-type strain, reaching approximately 9 $\mu\text{mol/g}$ feces and 0.8 $\mu\text{mol/g}$ feces, respectively (**Fig. 3H-I, and Supplementary Table 13**). In contrast, neither valerate nor hexanoate was detectable in fecal samples from mice colonized with the *thlA1-thlA2::Cm^r* mutant. Notably, butyrate concentrations were similar between the two groups, remaining at approximately 8 $\mu\text{mol/g}$ feces (**Supplementary Fig. 15, and Supplementary Table 13**). These results demonstrate that the disruption of *thlA1-thlA2* abolishes valerate and hexanoate production *in vivo* without affecting intestinal colonization or butyrate production, indicating that the reverse β -oxidation pathway is essential for the biosynthesis of valerate and hexanoate by *M. indica* in the gut.

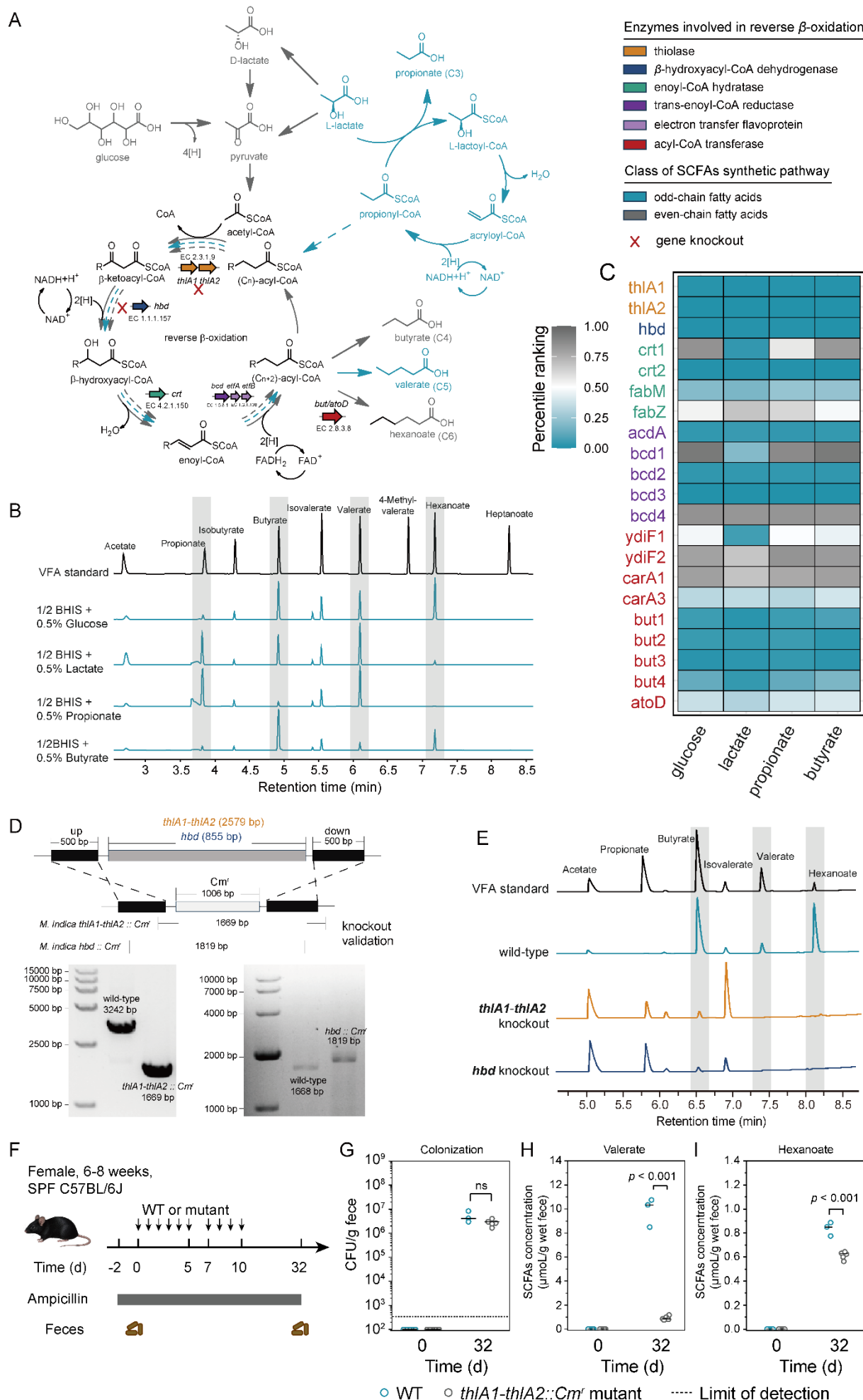


Figure 3. The reverse β -oxidation pathway is required for valerate and hexanoate biosynthesis by *M. indica*. **A)** The predicted SCFAs production pathway in *Megasphaera*. Two molecules of acetyl-CoA are converted into butyrate via the reverse β -oxidation pathway, and two additional carbon atoms are added to form hexanoate. Lactic acid is metabolized through the acrylate pathway to propionate, which then enters the reverse β -oxidation pathway, adding two carbon atoms to produce pentanoate. **B)** Gas chromatogram showing the changes in SCFAs product distribution of *M. indica* when fed different carbon sources, including glucose, lactate, propionate, and butyrate. **C)** Heatmap displaying the expression rankings of reverse β -oxidation-related genes under four different carbon source conditions. Colors range from deep cyan (higher expression) to white to dark gray (lower expression). **D).** Schematic diagram of gene knockout and validation, demonstrating gene knockouts using homologous recombination. The nucleic acid gel image shows successful knockout of the target gene. **E)** Gas chromatogram displaying the changes in SCFAs product distribution between the gene knockout mutant strain (*M. indica thlA1-thlA2::Cm^r*, and *M. indica hbd::Cm^r*) and the wild-type strain. **F)** Experimental design of the mouse colonization model. Female SPF C57BL/6J mice (6–8 weeks old) were treated with an antibiotic cocktail followed by ampicillin-containing drinking water and orally gavaged with either wild-type (WT) *M. indica* XA511 or the *thlA1-thlA2::Cm^r* mutant. **G)** Fecal colonization levels of WT and mutant strains at the indicated time points. **H)** Valerate and **I)** Hexanoate concentrations in fecal samples of two groups (wild-type (WT) *M. indica* XA511 or the *thlA1-thlA2::Cm^r* mutant). Black and blue open circles represent mice colonized with the WT and *thlA1-thlA2::Cm^r* mutant strains, respectively. Each symbol represents an individual mouse, and horizontal bars indicate the mean. The dotted line indicates the limit of detection of the plate-counting assay. Statistical significance was determined using an unpaired two-tailed Student's *t*-test. ns, not significant.

***M. indica* inhibits gut opportunistic pathogenic fungi through valerate and hexanoate**

Having established that reverse β -oxidation is required for valerate and hexanoate biosynthesis by *M. indica*, we next investigated whether these metabolites contribute to its antifungal activity. Culture supernatants from wild-type *M. indica* XA-511 inhibited the growth of *Candida albicans*, *Candida parapsilosis*, and *Nakaseomyces glabratus*, whereas supernatants from the *thlA1-thlA2::Cm^r* mutant showed markedly reduced inhibition (**Fig. 4A, and Supplementary Table 14**). To determine whether this reduction resulted from the loss of valerate and hexanoate, we supplemented the *thlA1-thlA2::Cm^r* mutant culture supernatant with

valerate and hexanoate to match their respective concentrations measured in the wild-type *M. indica* XA-511 culture supernatant. Supplementation restored fungal growth inhibition to levels comparable to, or greater than, those observed with the wild-type *M. indica* XA-511 supernatant (**Fig. 4A, and Supplementary Table 14**). These metabolite rescue results indicate that valerate and hexanoate substantially contribute to the antifungal activity of *M. indica*.

Following this direct metabolite rescue, we next investigated whether altering precursor availability could modulate the endogenous production of valerate and hexanoate by *M. indica* and the resulting antifungal activity. Wild-type *M. indica* XA-511 and the *thlA1-thlA2::Cm^r* mutant were cultured with different precursor substrates that bias reverse β -oxidation toward valerate or hexanoate production. Propionate supplementation led to robust valerate accumulation in the wild-type culture, whereas butyrate supplementation selectively increased hexanoate production. In contrast, the *thlA1-thlA2::Cm^r* mutant produced neither valerate nor hexanoate under either condition (**Fig. 4B–C**). These precursor supplementation experiments generated valerate-enriched and hexanoate-enriched wild-type supernatants, together with metabolite-deficient mutant controls, allowing us to further evaluate how pathway-dependent production of valerate and hexanoate affects fungal growth.

We next compared the antifungal activity of supernatants from wild-type and reverse β -oxidation–deficient knockout strains across ten representative fungal species isolated from the human gut³⁶ (including *Candida albicans*, *Candida parapsilosis*, *Nakaseomyces glabratus*, *Wickerhamomyces anomalus*, *Candida lusitanae*, *Candida pseudointermedia*, *Meyerozyma caribbica*, *Pichia kudriavzevii*, *Kodamaea ohmeri*, *Meyerozyma guilliermondii*). Supernatants were collected from cultures grown in unsupplemented 1/2 BHIS, propionate-supplemented medium, or butyrate-supplemented medium, which generated baseline, valerate-enriched, and hexanoate-enriched supernatants, respectively. Across most conditions, wild-type supernatants inhibited fungal growth, whereas knockout supernatants, which lack the ability to produce valerate and hexanoate, showed markedly reduced inhibition (**Fig. 4D, Supplementary Fig. 16, and Supplementary**

Table 15). This pattern was robust across the panel: all eight fungi showed diminished inhibition in the baseline medium, seven in the propionate-supplemented condition, and five in the butyrate-supplemented condition.

We next compared the inhibitory effects of *M. indica* culture supernatants generated under different substrate conditions. Several fungi were not significantly inhibited in the hexanoate-enriching group, including *M. caribbica*, *C. pseudointermedia*, and *M. guilliermondii* (**Supplementary Fig. 16, and Supplementary Table 15**). This outcome is likely attributable to the relatively high butyrate background generated under this culture condition, which may mask the antifungal effect of hexanoate. In contrast, some fungi were more sensitive to hexanoate; for example, *P. kudriavzevii* displayed the lowest absolute growth OD in the hexanoate-enriching group compared with both the baseline and valerate-enriching conditions (**Supplementary Fig. 16, and Supplementary Table 15**). We also identified cases in which valerate and hexanoate acted synergistically. *W. anomalus* showed the lowest absolute growth OD in the baseline 1/2 BHIS condition (**Supplementary Fig. 16, and Supplementary Table 15**), where both SCFAs were present at moderate concentrations, suggesting a combinatorial inhibitory effect that exceeded the activity of either SCFA alone. These results indicated that fungal responses varied with both fungal species and the *M. indica* culture conditions, highlighting that different growth environments or cross-feeding interactions among gut microbes may lead to distinct antifungal outcomes.

To investigate whether propionate-producing bacteria could promote valerate production by *M. indica* and inhibit fungal growth in the gut environment, we selected two propionate-producing strains, *Phocaeicola dorei* DA26 and *Parabacteroides johnsonii* DA701. We found that co-culturing *M. indica* with *P. dorei* DA26 and *P. johnsonii* DA701 enhanced valerate production while inhibiting hexanoate production (**Fig. 4E, and Supplementary Table 16**). Furthermore, this co-cultivation significantly suppressed the growth of opportunistic pathogenic fungi. In contrast, when the reverse β -oxidation mutant strain *M. indica* mutant was co-cultured with *P. dorei* DA26 and *P. johnsonii* DA701, the inhibitory effect on the fungi was greatly

diminished (**Fig. 4E, and Supplementary Table 16**). This indicates that *M. indica* can utilize propionate precursors from gut microbes through cross-feeding, converting them into valerate via reverse β -oxidation, thereby exerting antifungal effects.

To further investigate whether other valerate- and hexanoate-producing gut bacteria could also inhibit fungal growth, we tested multiple *Megasphaera* spp. strains from different sources. *M. indica* DA18 was isolated in this study, whereas the XA-series strains, including *M. indica* XA-511, *M. elsdenii* XA-5208, *M. hominis* XA-4277, and *M. stantonii* XA-5244, were obtained from Xbiome Co., Ltd. (China) and were originally isolated from fecal samples of healthy adult donors. In addition, *M. indica* XA-511 *thlA1-thlA2::Cm^r*, *M. stantonii* XA-5244, *Ligilactobacillus salivarius* DA858, and *Acidaminococcus provencensis* DA778 were included as negative controls because they do not produce valerate or hexanoate. Interestingly, the valerate- and hexanoate-producing strains *M. indica* XA-511, *M. indica* DA18, *M. elsdenii* XA-5208, and *M. hominis* XA-4277 exhibited potent antifungal activity (**Fig. 4F, and Supplementary Table 16**). In contrast, the strains that do not produce these metabolites, including *M. indica* XA-511 *thlA1-thlA2::Cm^r*, *M. stantonii* XA-5244, *L. salivarius* DA858, and *A. provencensis* DA778, showed significantly weaker inhibition of fungal growth (**Fig. 4F, and Supplementary Table 16**). These findings suggest that valerate- and hexanoate-producing gut bacteria may contribute to the suppression of opportunistic fungi in the gut ecosystem.

To evaluate whether these metabolite–fungus relationships extend to the human gut environment, we analyzed fecal samples from a cohort of 100 healthy individuals. Concentrations of acetate ($R = -0.095$, $p = 0.35$), propionate ($R = -0.098$, $p = 0.33$), and butyrate ($R = -0.041$, $p = 0.69$) exhibited weak and non-significant negative correlations with *Candida* abundance (**Supplementary Fig. 17A-C, and Supplementary Table 16**). In comparison, valerate showed a stronger, albeit still non-significant, negative association with *Candida* abundance ($R = -0.11$, $p = 0.28$; **Supplementary Fig. 17D, and Supplementary Table 16**). Notably, hexanoate displayed a significant negative correlation with *Candida* abundance ($R = -0.22$, $p = 0.028$; **Supplementary Fig. 17E, and Supplementary Table 16**). Together with the genetic and *in vitro* functional

evidence, these human fecal association data support a potential role for *M. indica* and its chain-elongated fatty acids in constraining the expansion of opportunistic pathogenic fungi in the human gut.

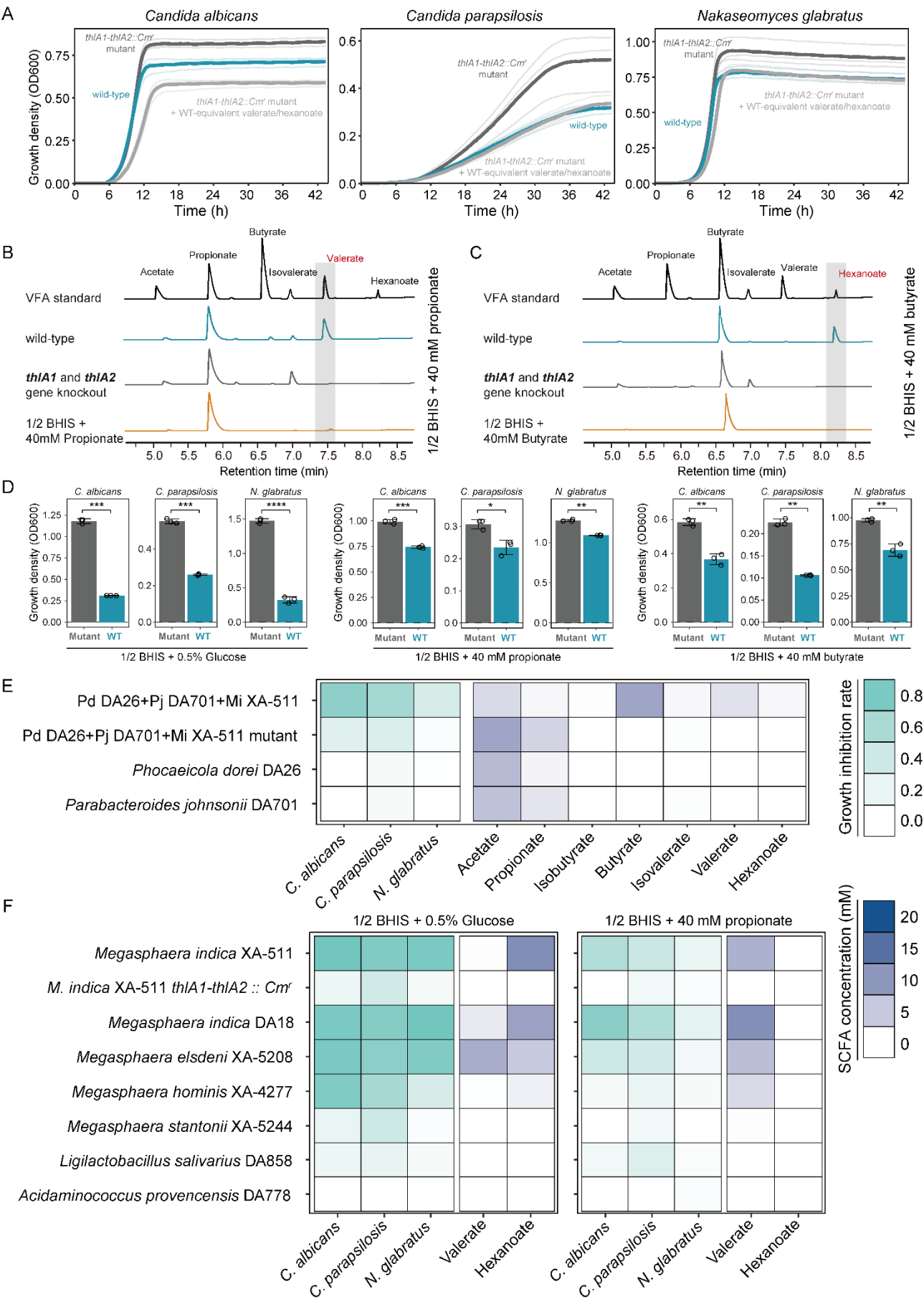


Figure 4. Reverse β -oxidation-dependent production of valerate and hexanoate contributes to fungal suppression by *M. indica*. **A)** Growth curves of *Candida albicans*, *Candida parapsilosis*, and *Nakaseomyces glabratus* incubated with culture supernatants from wild-type *M. indica* XA-511, the *thlA1-thlA2::Cm^r* mutant, or the mutant supplemented with valerate and hexanoate to assess restoration of antifungal activity. Thick lines represent mean values, and thin lines represent individual technical replicates (n = 3). **B)** Gas chromatographic analysis of SCFA product composition in wild-type and mutant strains under 40 mM propionate supplementation in 1/2 BHIS medium. **C)** Gas chromatographic analysis of SCFA product composition in wild-type and mutant strains under 40 mM butyrate supplementation in 1/2 BHIS medium. **D)** Growth differences of eight gut fungal strains incubated with supernatants from wild-type and mutant *M. indica* cultures under three conditions: 0.5% glucose, 40 mM propionate supplementation, and 40 mM butyrate supplementation in 1/2 BHIS medium. Data are presented as mean values $\pm$ SD. Assays were performed in technical triplicates (n = 3). Heatmaps showing the inhibitory effects of supernatants from **E)** synthetic communities and **F)** individual gut bacterial isolates on three opportunistic gut fungal species, together with the corresponding SCFA concentrations in the supernatants. Experiments were performed in triplicate (n = 3). Mi XA-511 mutant denotes *M. indica* XA-511 *thlA1-thlA2::Cm^r*.

Discussion

Our findings establish reverse β -oxidation in *Megasphaera indica* as the metabolic pathway responsible for valerate and hexanoate biosynthesis and link pathway-dependent production of these metabolites to the suppression of diverse gut-associated fungi. This mechanistic connection expands the known antifungal functions of microbiota-derived SCFAs beyond acetate, propionate, and butyrate. Valerate and hexanoate inhibited multiple opportunistic fungi at concentrations relevant to the human gut, whereas disruption of reverse β -oxidation abolished their production and markedly weakened the antifungal activity of *M. indica*. Importantly, supplementation of the *thlA1-thlA2::Cm^r* mutant culture supernatant with valerate and hexanoate at concentrations matching those measured in the wild-type *M. indica* XA-511 supernatant reinstated fungal growth inhibition. This functional rescue provides direct evidence that valerate and hexanoate substantially mediate the antifungal activity of *M. indica*. Together, these results identify a gut bacterial source and

genetically validated biosynthetic pathway for C5/C6 SCFAs and establish their functional contribution to the suppression of opportunistic fungi.

Our genetic and metabolic analyses establish reverse β -oxidation as the principal pathway for valerate and hexanoate biosynthesis in *M. indica*. Valerate production has previously been linked to proline conversion and 5-aminovalerate degradation in *Clostridium aminovalericum*^{25,26}. However, disruption of the 5-aminovalerate aminotransferase gene *davT* did not affect valerate production in *M. indica*, arguing against this pathway as its major biosynthetic route. By contrast, deletion of either the adjacent thiolase genes *thlA1-thlA2* or the β -hydroxyacyl-CoA dehydrogenase gene *hbd* abolished butyrate, valerate and hexanoate production. Substrate-supplementation experiments further supported the two-carbon elongation of propionate to valerate and butyrate to hexanoate. These findings are consistent with recent work showing that the rumen bacterium *Megasphaera hexanoica* produces hexanoate through reverse β -oxidation and that its pathway enzymes support hexanoate production when heterologously expressed in *Escherichia coli*³⁷. Our study extends this metabolic principle to a human gut commensal and provides genetic evidence for both odd- and even-chain fatty acid elongation. The positive association between fecal propionate and valerate concentrations further supports the relevance of propionate elongation in the human gut.

The antifungal effects of acetate, propionate and butyrate have been well documented¹⁴, and recent studies have begun to clarify their underlying mechanisms. Microbiota-derived SCFAs suppress *Candida albicans* by disrupting carbon metabolism and inducing intracellular acidification⁴, whereas valerate was recently shown to inhibit *Candida parapsilosis* through an acidification-dependent mechanism¹⁰. Previous studies, however, have focused primarily on individual metabolites and a limited number of *Candida* species. Our study extends this scope to a broader collection of gut-associated fungi. Valerate inhibited not only *C. albicans*, *C. parapsilosis* and *Nakaseomyces glabratus*, but also emerging opportunistic yeasts, including *Meyerozyma guilliermondii*, *M. caribbica*, *Kodamaea ohmeri*, *Candida lusitanae*, *Pichia kudriavzevii* and *Wickerhamomyces anomalus*. Several of these fungi have been associated with bloodstream infections or other

host disorders³⁸. For example, *M. guilliermondii* has been reported to aggravate alcoholic hepatic steatosis through the production of prostaglandin E₂³⁹. The broader activity observed here therefore suggests that C5/C6 SCFAs may constrain a wider range of gut-associated opportunistic fungi than previously recognized.

In the human gut, much less is known about the key microbial producers of valerate compared with the well-studied SCFAs acetate, propionate, and butyrate. In addition to *M. indica*, other species of *Megasphaera*, such as *M. elsdenii*, also contribute to valerate production³⁰⁻³², indicating that valerate production is a conserved trait within the *Megasphaera* genus in the human gut. Our analysis of fecal samples from two independent cohorts in China revealed a significant correlation between the presence of *Megasphaera* species and higher levels of valerate. These results suggest that *Megasphaera* is a major contributor to valerate production in the human gut. Consistent with this observation, a recent Danish cohort study reported *M. elsdenii* as a principal contributor to human gut valerate⁴⁰, highlighting geographical variation in dominant *Megasphaera* species across populations.

As the gut fungal resource database expands and our understanding of fungal-host interactions deepens, human-associated fungi will evolve from being merely studied for infections to presenting new therapeutic targets. For example, *Aspergillus tubingensis*, a gut fungus, has been implicated in promoting polycystic ovary syndrome through secondary metabolites⁴¹. Characterizing interactions between gut bacteria and a wider range of gut fungi will be crucial for developing strategies to control emerging pathogenic fungi. Additionally, metabolic engineering⁴², such as enhancing the production of valerate via the reverse β -oxidation pathway, holds promise for developing live biotherapeutic products that can be used to treat fungal infections. Looking ahead, the discovery of high valerate-producing *M. indica*, the elucidation of its valerate and hexanoate synthesis pathways, and its antifungal activity offer new possibilities for treating drug-resistant fungal infections with potential benefits for both human and animal health.

Methods

Collection of human fecal samples

All human participants from the Shenzhen Institutes of Advanced Technology cohort (hereafter referred to as the “SIAT cohort”) provided written informed consent. This cohort has been previously described and reported by previously published study⁴³. The study protocol was approved by the Institutional Review Board of the Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (SIAT-IRB-200315-H0438). Fecal samples (n = 119) were collected from healthy volunteers who had not used antibiotics within two months prior to sampling. As these samples were used for subsequent bacterial isolation and cultivation, they were freshly collected and transferred into an anaerobic chamber (Vinyl Anaerobic Chambers, Coy Laboratory Products, USA) with an atmosphere of 85% N₂, 5% CO₂, and 10% H₂ within 30 min of collection. Samples were then stored at −80 °C in sterile phosphate-buffered saline supplemented with 0.1% L-cysteine and 20% glycerol.

An independent cohort was obtained from a previously published study⁴⁴ conducted at Westlake University (hereafter referred to as the “Westlake cohort”), comprising fecal samples from 173 individuals. The study was approved by the Westlake University Ethics Committee (WePrecision No. 20210802ZJS001) and registered at ClinicalTrials.gov (NCT05054153; registration date: September 23, 2021). In the present study, samples from this cohort were used exclusively for short-chain fatty acid profiling and metagenomic analyses. Fecal specimens were immediately frozen at −80 °C after collection, without the addition of glycerol or other cryoprotectants, as previously described.

Isolation and cultivation of human gut bacterial strains

Megasphaera indica XA-511, *M. elsdeni* XA-5208, *M. hominis* XA-4277, and *M. stantonii* XA-5244 were obtained from Xbiome Co., Ltd. (China) and were originally isolated from fecal samples of healthy adult donors. All other bacterial strains used in this study were isolated from fecal samples collected from the SIAT

cohort. Briefly, fecal samples stored at -80°C were thawed, serially diluted, and plated onto modified yeast casitone fatty acids (mYCFA) agar. Plates were incubated anaerobically at 37°C for 2–3 days under an atmosphere of 85% N_2 , 5% CO_2 , and 10% H_2 . Single colonies were picked, restreaked on fresh plates, and incubated anaerobically for an additional 2–3 days; this purification step was repeated once to obtain pure isolates. Pure strains were subsequently cultured in liquid medium at 37°C for 2 days. Full-length 16S rRNA genes were amplified and sequenced using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Strains showing mixed sequencing peaks were either discarded or subjected to an additional round of purification. All purified isolates were stored at -80°C in 20% (v/v) glycerol supplemented with 0.1% L-cysteine. Taxonomic assignment was performed by comparing full-length 16S rRNA gene sequences against the Genome Taxonomy Database⁴⁵ (GTDB, release 226).

Metagenomic sequencing and taxonomic profiling

Fecal metagenomic sequencing and taxonomic profiling were performed as previously described⁴³. Briefly, genomic DNA was extracted from 200 mg of stool using the QIAamp Power Fecal Pro DNA Kit (Qiagen). Libraries were prepared with the Hieff NGS® OnePot II DNA Library Prep Kit for Illumina® (Yeasten), purified and size-selected using Hieff NGS® DNA Selection Beads, and quantified with the Qubit dsDNA High Sensitivity Assay (Thermo Fisher). Library quality and fragment size distribution were assessed using the Bioanalyzer High Sensitivity DNA Kit (Agilent). Equimolar pooled libraries were sequenced on the Illumina HiSeq X Ten platform (150 bp paired-end; Annoroad Gene Technology), targeting ~0.3 Gbp raw data per sample⁴⁶.

Samples with fewer than 10^5 clean reads were excluded. Reads were quality-filtered by removing reads with >50% bases below Q19, >5% ambiguous bases (N), or paired-end reads in which either mate failed these criteria. Taxonomic profiling was performed using the SHOGUN pipeline with the RefSeq database (v82), following previously published methods⁴⁶.

ITS amplicon sequencing and data processing

Total fecal DNA was extracted and purified as described previously². Briefly, 200 μ L of liquid stool or 200 μ g formed stool was mixed with 500 μ L of lyticase buffer (50 mM Tris, pH 7.6, 10 mM ethylenediaminetetraacetic acid, 28 mM β -mercaptoethanol), 100 U lyticase (Sigma L2524), 0.2 ml of 0.1-mm diameter silicone beads and 0.3 ml of 0.5-mm diameter silicone beads. Samples were vortexed and then incubated at 32 °C for 30 min. Samples were supplemented with 30 μ L of 4 M NaCl, 47 μ L of 2 M Tris-HCl, pH 8.0, 13 μ L of 0.5 M ethylenediaminetetraacetic acid, 200 μ L of 20% sodium dodecylsulfate and 500 μ L of phenol chloroform and then homogenized by bead-beating for 2 min. The debris was spun at 13,300 g for 5 min at 4 °C. The upper aqueous layer was mixed with 100 μ L of DEB buffer (0.2 M NaCl, 0.2 M Tris, pH 8.0, 0.5 M EDTA) and 500 μ L of phenol chloroform. After spinning down, the upper aqueous layer was recovered and mixed with phenol chloroform for two additional rounds. 200 μ L of the upper aqueous layer was mixed with 200 μ L buffer AL (QIAamp DNA Mini Kit, catalog no. 51306) and 200 μ L of 100% ethanol. Further extraction processing was according to the instruction of QIAamp DNA Mini Kit. DNA was final eluted with 80 μ L ultrapure water (Invitrogen, catalog no. 10977).

The ITS2 amplicon sequencing strategy was adapted from ITS1 rDNA sequencing as described previously²: forward primer ITS2-F (5'-GCATCGATGAAGAACGCAGC-3') and reverse primer ITS2-R (5'-TCCTCCGCTTATTGATATGC-3')⁴⁷ were modified with additional barcode nucleotides for fungal ITS2 region amplification. Purified fecal DNA was amplified with Phusion High-Fidelity DNA Polymerase (Thermo Fisher, F530L) for 35 cycles (98 °C for 30 s, 55 °C for 30 s and 72 °C for 5 s). Fragment size of PCR amplicons was checked by agarose gel electrophoresis (required from 500 bp to 700 bp). PCR amplicons were purified using VAHTS DNA Clean Beads (Vazyme, Cat. No. N411-03) and then grouped to sequencing on the Illumina Nova platform with PE250 setting. Raw sequencing reads were demultiplexed according to the barcodes, and then removed primer and barcode. DADA2 was used to quality-filter and generate amplicon sequence variants (ASVs)⁴⁸. Taxonomy was assigned to ASVs using National Center for Biotechnology

Information (NCBI) RefSeq⁴⁹ as a reference database. The ASV tables, taxonomic assignment and sample metadata were assembled using the phyloseq package⁵⁰.

Isolation and cultivation of fungal strains

Candida albicans SC5314 was the reference strain originally obtained from a bloodstream infected sample⁵¹. Fungal isolation, identification and preservation were performed as described previously². Briefly, 50 μ L of liquid stool or 50 mg of formed stool was homogenized in 150 μ L of sterile phosphate-buffered saline (PBS) and serially diluted on yeast extract peptone dextrose (YPD) agar with 0.1 mg mL⁻¹ of vancomycin and 0.5 mg mL⁻¹ of gentamicin. The samples were cultured at 37 °C for 48 h under aerobic conditions. Single yeast colonies were picked and stored in 20% glycerol stocks at -80 °C. Species identification was performed by Sanger sequencing of ITS rDNA using forward primer ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and reverse primer ITS4-R (5'-GCTGCGTTCTTCATCGATGC-3').

Sixteen high-prevalence human gut fungal isolates were obtained from fecal samples of healthy adult donors in China³⁶. *Kodamaea ohmeri* CGMCC 3.35114, *Pichia myanmarensis* CGMCC 3.35120, *Cyberlindnera jadinii* CGMCC 3.35015, *Wickerhamomyces anomalus* CGMCC 3.35009, *Pichia kudriavzevii* CGMCC 3.35097, *Nakaseomyces glabratus* CGMCC 3.35039, *Nakaseomyces bracarensis* CGMCC 3.35040, *Torulaspora delbrueckii* CGMCC 3.35095, *Meyerozyma guilliermondii* CGMCC 3.35118, *Meyerozyma caribbica* CGMCC 3.35096, *Candida parapsilosis* CGMCC 3.35042, *Candida albicans* CGMCC 3.35058, *Candida pseudointermedia* CGMCC 3.35054, and *Candida lusitanae* CGMCC 3.35041 were cultured in yeast extract-peptone-dextrose (YPD) medium at 30 °C with shaking at 220 rpm for 24 h to obtain seed cultures for subsequent antifungal assays. *Fusarium foetens* CGMCC 3.35499 and *Fusarium liriodendri* CGMCC 3.35320 were cultured in potato dextrose broth (PDB) at 30 °C with shaking at 220 rpm for 24 h prior to use.

Quantification of SCFA concentration in fecal samples and bacterial culture

Fecal SCFAs were quantified by gas chromatography (GC). For the sample extraction, 100 mg of frozen human fecal sample was transferred into a 1.5 mL microcentrifuge tube containing 50 mg of 1.0 mm zirconia/silica beads (BioSpec, Bartlesville, OK, USA). Samples were homogenized three times for 20 s at 6,500 rpm, incubated at 4 °C with shaking for 30 min, and subsequently centrifuged at 13,000 × g for 30 min. A 300 µL aliquot of the supernatant was mixed with 60 µL of 20% (w/v) metaphosphoric acid, vortexed, and incubated at −20 °C overnight to precipitate proteins. After thawing, samples were centrifuged at 12,000 rpm for 10 min, and 300 µL of the clarified supernatant was transferred to a new tube and extracted with an equal volume of ethyl acetate by vigorous vortexing for 1–2 min. Following centrifugation at 12,000 rpm, the organic phase was carefully collected, filtered through a 0.22 µm organic membrane, and analyzed using an Agilent 8890 gas chromatograph equipped with a capillary column (30 m × 0.53 mm × 1.00 µm).

The SCFAs of bacterial culture supernatant samples were analyzed by GC-MS. For the sample extraction, 200 µL of frozen supernatant were mixed with 100 µL of pure water containing caproic acid-6,6,6-d₃ (CDN Isotopes, Quebec, Canada) as internal standard (final concentration 10 µg/mL). Following extraction with anhydrous diethyl ether, the SCFA extract was accurately transferred into a glass insert in a GC vial and capped tightly after adding 5 µL of N, O-bis(trimethyl-silyl)-trifluoroacetamide and vortexed for 5 s. The mixture was kept in the GC vial and incubated at room temperature (22 °C) overnight (or over 8 h) before loading to GC/MS. The analysis of acetate, propionate, iso-butyrate, butyrate, iso-valerate, valerate, and hexanoate was performed by Agilent 8890/7000D triple quadrupole GC/MS equipped with a capillary HP-5 ms capillary column (30 m × 0.25 mm × 0.25 µm film thickness) (Agilent Technologies). The analytes were quantified in the selected ion monitoring (SIM) mode using the target ion and confirmed by confirmative ions. The integrated areas for all SCFAs were normalized with the internal standard and quantified with the standard curve, as previously described.

Antifungal activity measurement

To evaluate the antifungal activity of acetate, propionate, butyrate, valerate, hexanoate and isovalerate *in vitro*,

sterilized YPD broth was supplemented with each compound at a series of final concentrations ranging from 2.5 to 160 mM. Each treated YPD broth was adjusted to pH 7.0 using 10 mM NaOH and then sterilized by filtration. *Candida albicans*, *C. parapsilosis* and *C. glabrata* were cultured in YPD broth with shaking at 37 °C to the stationary phase. Fungal cells were pelleted, washed with PBS and inoculated into 96-well plates at 1000 CFU/well in treated YPD broth. Untreated YPD broth was set as the control. The growth curves were measured at 37 °C for 24 h with shaking in a BioTek LogPhase 600 Microbiology Reader (Agilent) by optical density at 600 nm (OD₆₀₀).

To evaluate the antifungal activity of valerate on prevalent human gut fungi, valerate (2.5–40 mM) was prepared by two-fold serial dilution in YPD or PDB. Sixteen representative gut fungal isolates were pre-cultured at 30 °C with shaking for 24 h, adjusted to OD₆₀₀ = 1.0, and inoculated at a 1:100 dilution. Antifungal assays were conducted under both anaerobic conditions at 37 °C and aerobic conditions at 30 °C to mimic gut and environmental growth, respectively. Fungal growth was measured at 0, 6, 12, 18, 24, 36, and 48 h by OD₆₀₀.

For culture supernatant-based antifungal assays, bacteria were grown anaerobically in 1/2 BHIS medium supplemented (1:1, v/v) with pre-reduced carbon source solutions (0.5% glucose, 40 mM sodium propionate, or 40 mM sodium butyrate). Bacterial strains, including *Megasphaera* spp., *Ligilactobacillus salivarius* DA858, *Acidaminococcus provencensis* DA778, and *M. indica* mutant strains, were inoculated at OD₆₀₀ = 0.1 (1:10 dilution) and cultured for 24 h. Culture supernatants were collected by centrifugation, filter-sterilized (0.22 µm), and mixed with 4× YPD at a 3:1 ratio. Pre-cultured fungi (OD₆₀₀ = 1.0) were inoculated at 1:100, and growth was monitored for 48 h by OD₆₀₀.

For synthetic community assays, *Phocaeicola dorei* DA26, *Parabacteroides johnsonii* DA701, and *Megasphaera indica* XA-511 (or *Megasphaera indica* XA-511 *thlA1-thlA2* :: *Cm^r*) were mixed at equal OD, inoculated at a final OD₆₀₀ of 0.01, and cultured anaerobically for 24 h. Supernatants were harvested and evaluated for antifungal activity following the same procedure as for single-strain supernatants.

RNA extraction, transcriptome sequencing and analysis

RNA extraction, library preparation, sequencing, and downstream analyses were performed largely as described previously⁵². Briefly, total RNA was extracted using the TRIzol method with DNase treatment to remove genomic DNA. RNA quality was assessed using a NanoDrop One spectrophotometer and an Agilent 4200 TapeStation (Agilent, G2991AA). Ribosomal RNA was depleted using the Ribo-Zero rRNA Removal Kit (Epicenter, 15066012) for bacteria. Strand-specific RNA-seq libraries were constructed using the NEBNext Ultra II Directional RNA Library Prep Kit (NEB, e7760) and sequenced on a DNBSEQ-G400 platform (MGI Tech). Raw reads were quality-filtered using fastp⁵³, and rRNA-derived reads were removed by alignment to the Rfam database. Clean reads were mapped to the reference genome using Bowtie2⁵⁴, and gene-level read counts were generated with RSEM⁵⁵.

Construction of *M. indica* mutants

The $\Delta thlA1-thlA2::Cm^r$ and $\Delta hbd::Cm^r$ mutants were constructed by electroporating the wild type *M. indica* DA18 with the methylated integration vector pXA511-double-crossover⁵⁶.

To construct plasmids for deletion mutagenesis, two 500 bp PCR fragments were generated corresponding to upstream or downstream of the target gene from the genome of *M. indica* DA18. The backbone plasmid and the chloramphenicol resistance cassette were amplified from the plasmid pXA511-mini. Phusion High-Fidelity DNA Polymerase (Thermo Fisher, F530L) was used for PCR cloning steps. PCR fragment of the backbone, upstream, the chloramphenicol resistance cassette and downstream were ligated in order using ClonExpress Ultra One Step Cloning Kit (Vazyme, Cat. No. C115-02) and then introduced into *E. coli* Stbl3 competent cells. Assembled plasmids were sequenced following cloning to ensure insertions were without mutations. *E. coli* DB24 (Methyls⁺) is an engineered strain for overexpressing two methyltransferases from *M. indica* XA511⁵⁶. Assembled plasmids were transformed into *E. coli* DB24 (Methyls⁺) competent cells to obtain the methylated integration vector pXA511-double-crossover.

To construct *M. indica* mutants, *M. indica* DA18 was picked into fresh BHIs medium supplemented with 2%

fructose (BHI-Fructose) at 37 °C in anaerobic chamber. Overnight cultures were diluted into fresh BHI-Fructose medium to an OD₆₀₀ of 0.6. After ice-cold incubation for 20 min, cells were pelleted at 4 °C, washed three times with pre-chilled sterile water, and resuspended in pre-chilled 20% glycerol to obtain the competent cells. A total 1 µg of the constructed plasmid DNA was mixed with competent cells and electroporated using a 2 mm cuvette at 2.5 kV, 200 Ω, and 25 µF. The electroporated mixture was immediately resuspended in BHI-Fructose to incubate for 3 h. The cultures were plated on the BHIs agar with 5 µg/mL of thiamphenicol. Colonies were inoculated and subcultured three times on fresh BHI medium to obtain the mutants. All strains were confirmed by PCR amplification using the primer pairs hbd_F (5'-CGTTCCAGCAGCTGTCCTGTG-3') and hbd_R (5'-GGATTAGCACCTGCGCCGG-3') for *Δhbd::Cmr* mutant, and thlA1_F (5'-AGTTCTTCATTTCAGCGTAACCCCC-3') and THLA_R (5'-CATGGCCAGGGCTAAAGAGCC-3') for *ΔthlA1-thlA2::Cmr* mutant.

For the animal experiments, wild-type XA511 and the *ΔthlA1-thlA2::Cmr* mutant were transformed with the ampicillin-resistant plasmid pXA02, yielding the WT and mutant strains, respectively.

Analysis of *Megasphaera* species across cohorts

Publicly available metagenomic datasets PRJEB8094⁵⁷, PRJEB1220⁵⁸, PRJEB6070⁵⁹, PRJNA388263⁶⁰, together with the SIAT cohort, were used to assess the relative abundance of *Megasphaera* species across multiple healthy human populations. Metagenomic analysis was performed as previously described⁶¹. Briefly, raw sequencing reads were downloaded and subjected to quality control and host contamination removal using KneadData (v0.12.3). Adapter trimming and quality filtering were performed with Trimmomatic⁶² using a 4-base sliding window, requiring an average Phred quality score >20 per base and a minimum read length of 90 bp. Reads mapping to the human reference genome were removed. Only samples retaining more than 1,000,000 high-quality, non-host reads were included in downstream analyses. Taxonomic classification was performed using Kraken2⁶³ with default parameters against the Genome Taxonomy Database⁶⁴ (GTDB, release r226). Following initial classification, Bracken⁶⁵ was applied to re-estimate bacterial relative

abundances from species to phylum levels, using a read length of 150 bp. The prevalence of each taxon was calculated as the proportion of samples in which the taxon was detected at a relative abundance greater than 0.01%.

Mice studies

Female C57BL/6J mice (6-8 weeks old) were obtained from Charles River Laboratories Co., Ltd (Guangdong, China) and maintained under specific pathogen-free (SPF) conditions. Mice were housed under a 12-h light /dark cycle, at 20-22°C with 40-60% relative humidity. All animal procedures complied with federal and institutional guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences (Approval No. SIAT-BSI-IRB-250704-HCS-ZB-A0189).

To establish intestinal colonization, mice were treated with ampicillin (1 g/L) in drinking water beginning 2 days prior to bacterial administration and throughout the experiment. Mice were orally gavaged daily on day 0-5 and 7-10 with either WT *M. indica* XA511 or the $\Delta thlA1-thlA2::Cm^r$ mutant carrying plasmid pXA02 (inoculum: 1×10^9 CFU per mouse per day). Fecal samples were collected at day 0 and 32 for bacterial enumeration and SCFAs quantification. Bacterial loads were determined by plate counting, and SCFA concentrations were measured by GC-FID.

Substrate-supplementation experiments for SCFA production by *Megasphaera indica*

Carbon sources (1% w/v glucose, sodium lactate, sodium propionate, or sodium butyrate) were dissolved in pre-reduced PBS, sterile-filtered through 0.22 μ m membranes, and prepared anaerobically. Each carbon source solution was mixed 1:1 (v/v) with BHIS medium to generate feeding media. *Megasphaera indica* was revived from -80 °C stocks and cultured anaerobically for 24 h. Cells were washed three times with anaerobic PBS, adjusted to an OD₆₀₀ of 0.1, and inoculated at a 1:10 ratio into 96-well plates. Cultures were incubated at 37 °C under anaerobic conditions, with OD₆₀₀ measured every 2 h. Samples were collected at 10 h during

the late logarithmic phase for transcriptomic analysis. Cells were harvested by centrifugation at $5,000 \times g$ for 2 min and stored at -80°C . For SCFA profiling, culture supernatants were collected at 24 h during the stationary phase (**Supplementary Fig. 11**), clarified by centrifugation, and stored at -80°C prior to analysis.

Valerate and hexanoate rescue assay

To determine whether valerate and hexanoate mediate the antifungal activity of *M. indica*, wild-type *M. indica* XA-511 and the *thlA1-thlA2::Cmr* mutant were cultured anaerobically in BHIS medium at 37°C for 24 h. Culture supernatants were collected by centrifugation and sterilized by filtration through a $0.22\ \mu\text{m}$ membrane. Valerate and hexanoate concentrations in the wild-type and mutant culture supernatants were quantified by GC-MS as described above. Pure valeric acid and hexanoic acid were added directly to the mutant culture supernatant to match their respective concentrations measured in the wild-type *M. indica* XA-511 culture supernatant. Wild-type, mutant, and valerate- and hexanoate-supplemented mutant culture supernatants were mixed with $2 \times$ YPD medium at a ratio of 1:1 (v/v). *Candida albicans*, *Candida parapsilosis*, and *Nakaseomyces glabratus* were precultured in YPD medium, adjusted to an OD600 of 1.0, and inoculated into the supernatant-containing medium at a 1:100 dilution. Fungal growth was continuously monitored at 37°C by measuring OD600. For each replicate, the OD600 value at 0 h was subtracted from subsequent measurements, and negative values after baseline correction were set to zero. Experiments were performed in technical triplicates ($n = 3$).

Synthetic community cultivation and antifungal assay

To determine whether propionate-producing gut bacteria promote valerate production by *M. indica* and enhance fungal suppression, *Phocaeicola dorei* DA26, *Parabacteroides johnsonii* DA701, and either wild-type *M. indica* XA-511 or the *thlA1-thlA2::Cmr* mutant were used to construct defined synthetic communities. Overnight bacterial cultures were adjusted to the same OD600 and mixed at equal proportions. The mixtures were inoculated into BHIS medium at a final total OD600 of 0.01 and cultured anaerobically at 37°C for 24

h. Culture supernatants were collected by centrifugation and sterilized by filtration through a 0.22 μm membrane. SCFA concentrations in the supernatants were quantified by GC-MS as described above. For antifungal assays, the sterile bacterial culture supernatants were mixed with 4 $\times$ YPD medium at a ratio of 3:1 (v/v). *Candida albicans*, *Candida parapsilosis*, and *Nakaseomyces glabratus* were precultured in YPD medium, adjusted to an OD₆₀₀ of 1.0, and inoculated into the supernatant-containing medium at a 1:100 dilution. Fungal growth was monitored at 37 °C by measuring OD₆₀₀. Sterile uninoculated bacterial medium processed using the same procedure was used as the control. Fungal growth inhibition was calculated as $(OD_{\text{control}} - OD_{\text{supernatant}})/OD_{\text{control}}$. Experiments were performed in triplicate (n = 3).

Antifungal activity of individual gut bacterial isolates

To examine whether antifungal activity is associated with valerate and hexanoate production across gut bacterial isolates, *M. indica* XA-511, *M. indica* DA18, *Megasphaera elsdenii* XA-5208, *Megasphaera hominis* XA-4277, and *Megasphaera stantonii* XA-5244 were tested individually. The *M. indica* XA-511 *thlA1-thlA2::Cm^r* mutant, *Ligilactobacillus salivarius* DA858, and *Acidaminococcus provencensis* DA778 were included as nonproducing controls. Each bacterial strain was inoculated into 1/2 BHIS medium supplemented with 0.5% glucose at an initial OD₆₀₀ of 0.1 and cultured anaerobically at 37 °C for 24 h. Culture supernatants were collected by centrifugation, sterilized through a 0.22 μm membrane, and analyzed for SCFA concentrations by GC-MS. Sterile bacterial culture supernatants were mixed with 4 $\times$ YPD medium at a ratio of 3:1 (v/v). Precultured *C. albicans*, *C. parapsilosis*, and *N. glabratus* cells were adjusted to an OD₆₀₀ of 1.0 and inoculated at a 1:100 dilution. Fungal growth was monitored at 37 °C by measuring OD₆₀₀. Sterile uninoculated bacterial medium processed using the same procedure was used as the control, and fungal growth inhibition was calculated relative to this control. Experiments were performed in triplicate (n = 3).

Availability of data and materials

Whole-genome sequencing data generated in this study have been deposited in the European Nucleotide Archive (ENA) under the study accession number PRJEB67733. RNA sequencing data generated in this study have also been deposited in ENA under accession number PRJEB106487. Human fecal metagenomic data from the SIAT cohort were obtained from a previously published study and are available under accession number PRJEB60398⁴³. Fecal metagenomic data from the Westlake cohort were retrieved from a previously published work⁴⁴. Additional publicly available human fecal metagenomic datasets from different geographic regions were obtained from ENA and NCBI under accession numbers PRJEB8094⁵⁷, PRJEB1220⁵⁸, PRJEB6070⁵⁹, and PRJNA388263⁶⁰.

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Author contributions

L.D. and B.Z. designed the project, supervised all experiments, and revised the manuscript. Z.Q., X.G., and H.L. performed most of the experiments and contributed to experimental design. Y.L., M.R., F.X., Y.Z., and X.L. assisted with the *M. indica* gene-knockout experiments. K.Z. and J.Z. contributed to healthy human fecal sample collection for the Westlake cohort, and L.W. contributed to sample collection for the SIAT cohort. M.R. assisted with the valerate inhibition assays on gut commensal fungi. Y.L., M.R., and J.Y. assisted with animal assays. W.Z. assisted with transcriptomic data analysis. X.X. assisted with quantification of fecal SCFAs. Z.Q.

wrote the draft manuscript, L.D., B.Z., X.G., Y.T., and N.Z. revised the manuscript.

Declaration of interests

L.D. is the cofounder of SynBiome Biotech. Y.T. is the cofounder of XBiome Biotech. The authors have filed patent applications on *Megasphaera indica* and fungi-inhibiting bacterial strains reported in this paper.

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