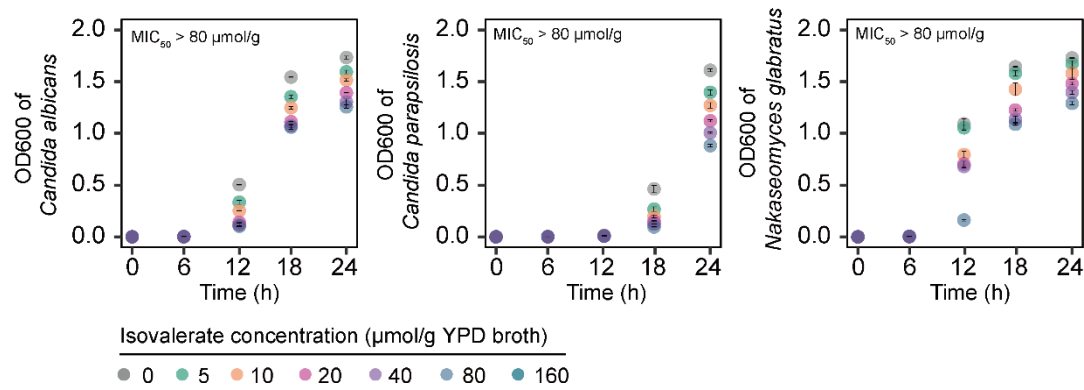


- 1 **Supplementary Materials for**
- 2 **The reverse β -oxidation pathway in *Megasphaera indica* links valerate**
- 3 **and hexanoate biosynthesis to the suppression of gut-associated fungi**
- 4 Zepeng Qu, Xinyi Gu, Hongbin Liu, *et al.*
- 5



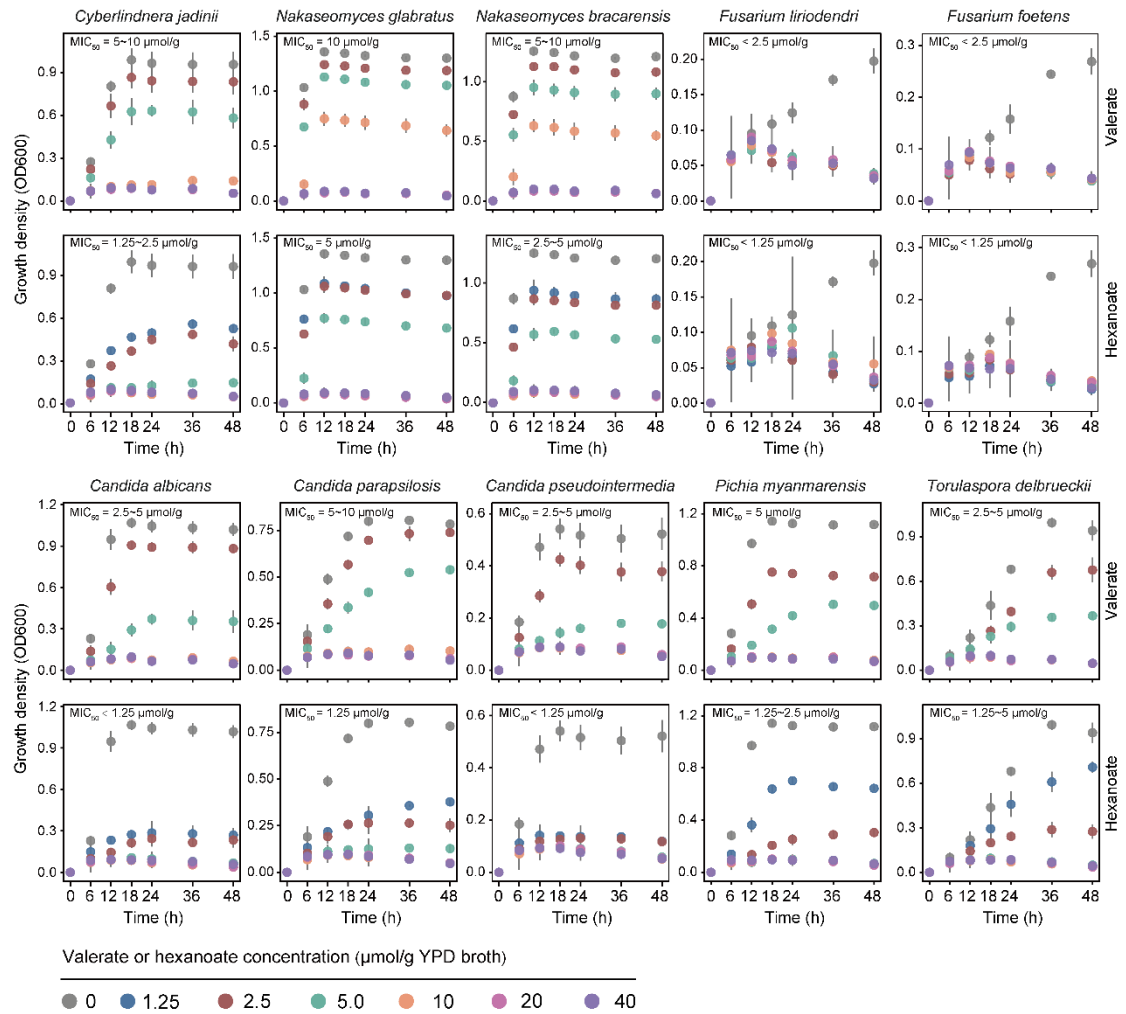
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7 **Supplementary Figure 1 The effects of acetate, propionate, and isovalerate on**
 8 **fungal growth under gradient concentrations.** The growth curve shows the changes
 9 in optical density at 600 nm (OD600) over time for three common opportunistic
 10 pathogens, *Candida albicans*, *Candida parapsilosis*, and *Nakaseomyces glabratus*, in
 11 response to a two-fold gradient of short-chain fatty acid concentrations. The
 12 concentration ranges tested were 5-80 μmol/g for isovalerate. MIC₅₀ values, defined as
 13 the minimum concentration that reduces fungal growth by 50% relative to the untreated
 14 control, are indicated on each curve. Data are presented as mean values ± SD. Assays
 15 were performed in technical triplicates (n = 3).

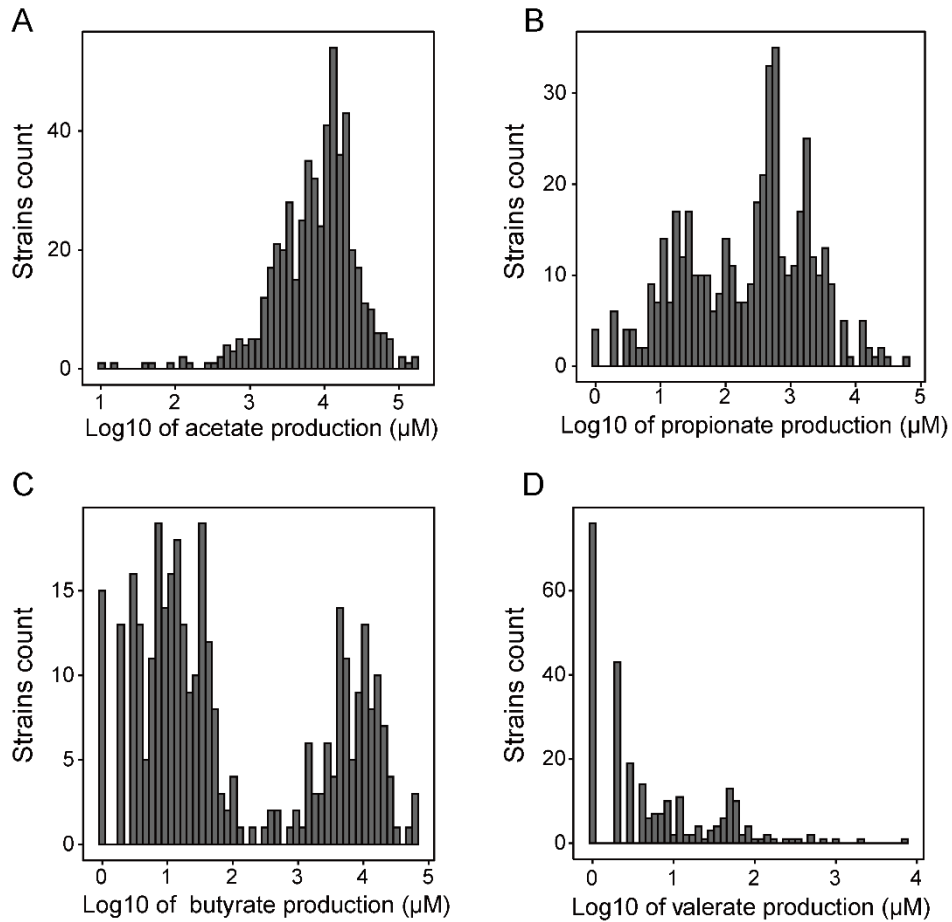
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	Species	CGMCC Num.	37°C	Microaerophilic	Anaerobic	Prevalence
	<i>Meyerozyma caribbica</i>	CGMCC 3.35096	+	+	+	56.52%
	<i>Meyerozyma guilliermondii</i>	CGMCC 3.35118	+	+	+	45.65%
	<i>Candida parapsilosis</i>	CGMCC 3.35042	+	+	+	97.83%
	<i>Wickerhamomyces anomalus</i>	CGMCC 3.35009	+	+	+	42.39%
	<i>Pichia myanmarensis</i>	CGMCC 3.35120	+	+	+	91.3%
	<i>Torulaspora delbrueckii</i>	CGMCC 3.35095	+	+	+	75%
	<i>Kodamaea ohmeri</i>	CGMCC 3.35114	+	+	+	95.65%
	<i>Cyberlindnera jadinii</i>	CGMCC 3.35015	+	+	+	28.26%
	<i>Fusarium liriodendri</i>	CGMCC 3.35320	+	+	+	64.13%
	<i>Fusarium foetens</i>	CGMCC 3.35499	+	+	+	100%
	<i>Candida lusitanae</i>	CGMCC 3.35041	+	+	+	98.91%
	<i>Candida pseudointermedia</i>	CGMCC 3.35054	+	+	+	89.13%
	<i>Candida albicans</i>	CGMCC 3.35058	+	+	+	96.74%
	<i>Pichia kudriavzevii</i>	CGMCC 3.35097	+	+	+	18.48%
	<i>Nakaseomyces brachycephalus</i>	CGMCC 3.35040	+	+	+	10.84%
	<i>Nakaseomyces glabratus</i>	CGMCC 3.35039	+	+	+	57.61%

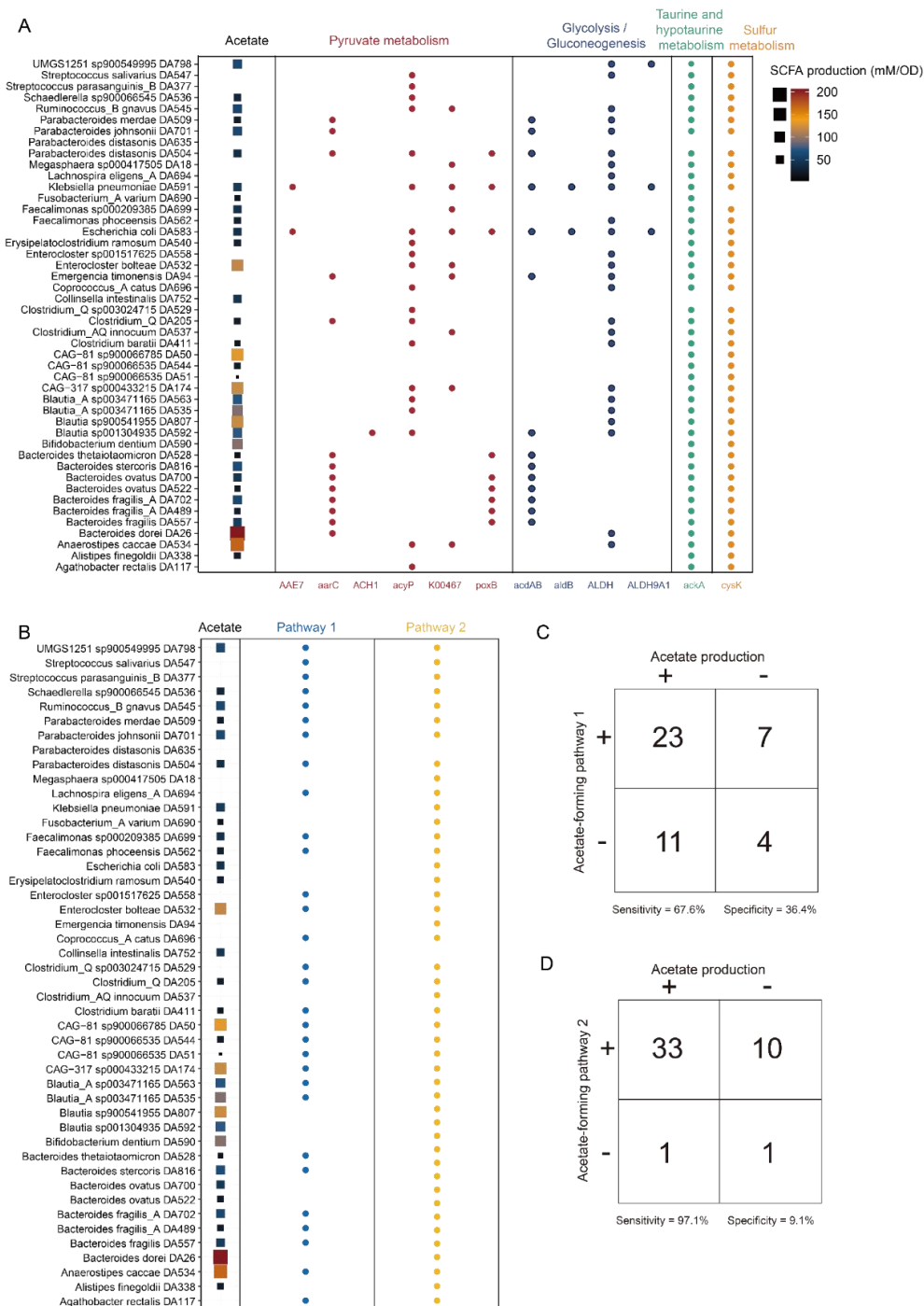
Supplementary Figure 2 Cultivable anaerobic gut fungi commonly found in the human gut. For filamentous fungi, colonies with a diameter greater than 0.5 cm were considered as positive growth “+”. For yeasts, an OD₆₃₀ above 0.2 in liquid medium was considered as positive growth “+”. All fungal strains were detected after 7 days of incubation. Prevalence: Data from 100 healthy volunteers from four different geographical regions in China¹.



Supplementary Figure 3 Valerate and hexanoate inhibits the growth of a broad range of gut fungi. The growth curve shows changes in optical density at 600 nm over time for 10 common gut fungi at 37°C. Except for two *Fusarium* strains grown in PDB medium, all other 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 $\mu\text{mol/g}$). Data are presented as mean values $\pm$ SD. Assays were performed in technical triplicates ($n = 3$).



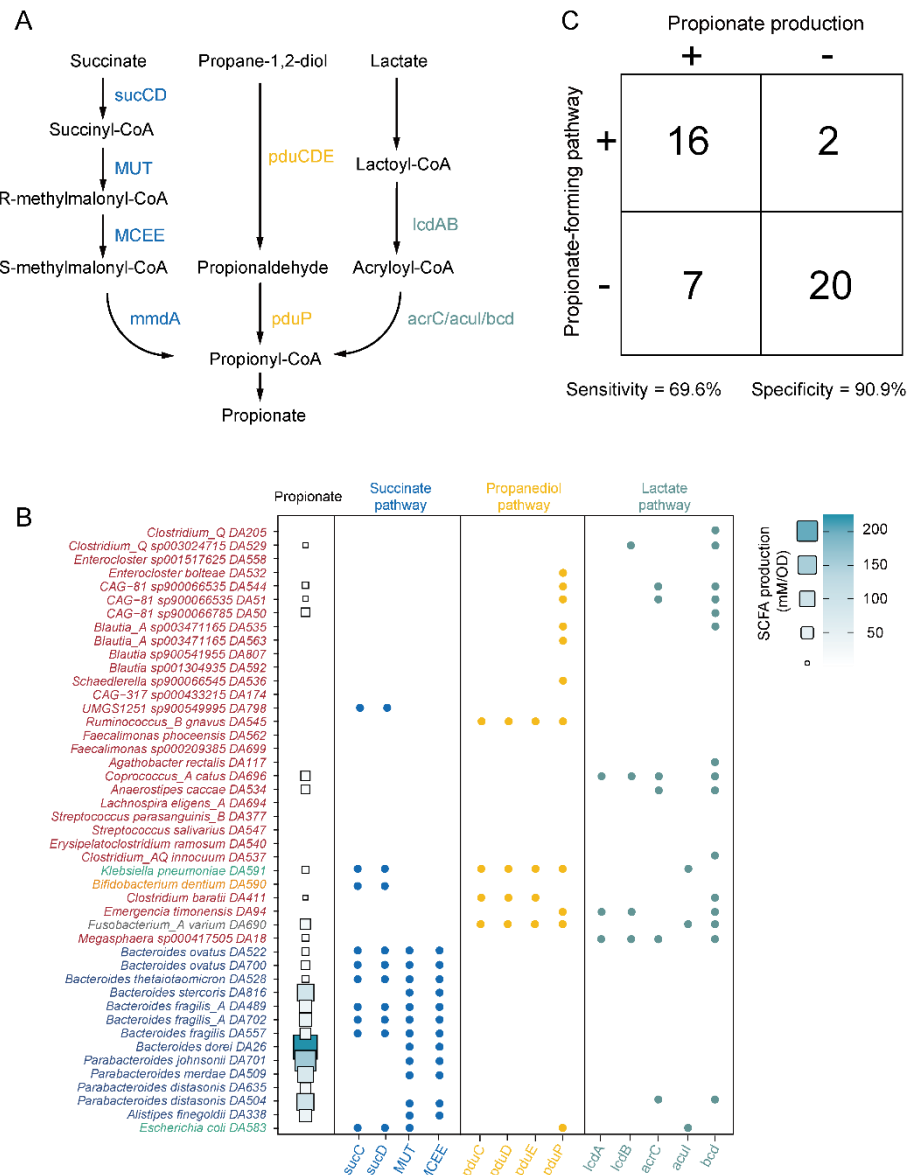
Supplementary Figure 4 Profiling the SCFA metabolism of gut bacterial strains with diverse phylogeny. Histogram showing distribution of A) acetate production, B) propionate production, C) butyrate production, D) valerate production, at log10 scale, each with no biological replicates.



Supplementary Figure 5 Association between genotype and phenotype for acetate production. A) The diagram shows the distribution of acetate-production and enzyme of acetate-forming pathway final step. B) The diagram shows the distribution of acetate-production and acetate-forming pathways predicted by DRAM. Confusion matrix shows the number of bacteria positive or negative for acetate-forming pathway 1 C) and acetate-forming pathway 2 D) versus positive or negative for acetate production.

47 For acetate production, the KEGG database contains 20 relevant pathways. The
48 distribution of the acetate-forming genes were predicted by aligning the reviewed gene
49 sequences of enzymes catalyzing final step from KEGG² (**Supplementary Table 6A-**
50 **B**). We found that, for pyruvate metabolism, 12/13 (92.3%) aarC, and 9/9 (100%) poxB
51 coding strains produced acetate; for glycolysis/ gluconeogenesis, 13/14 (92.9%) acdAB
52 coding strains produced acetate (**Supplementary Fig. 5A, C**). Additionally, even when
53 using the integrated functional annotation tool DRAM (version 1.4.0)³, the mapping
54 between the acetate-forming pathway and acetate production was not ideal
55 (**Supplementary Fig. 5B, D, and Supplementary Table 6C**). These data with poor
56 genotype-phenotype mapping may serve as valuable resources for bioinformaticians to
57 further optimize algorithms.

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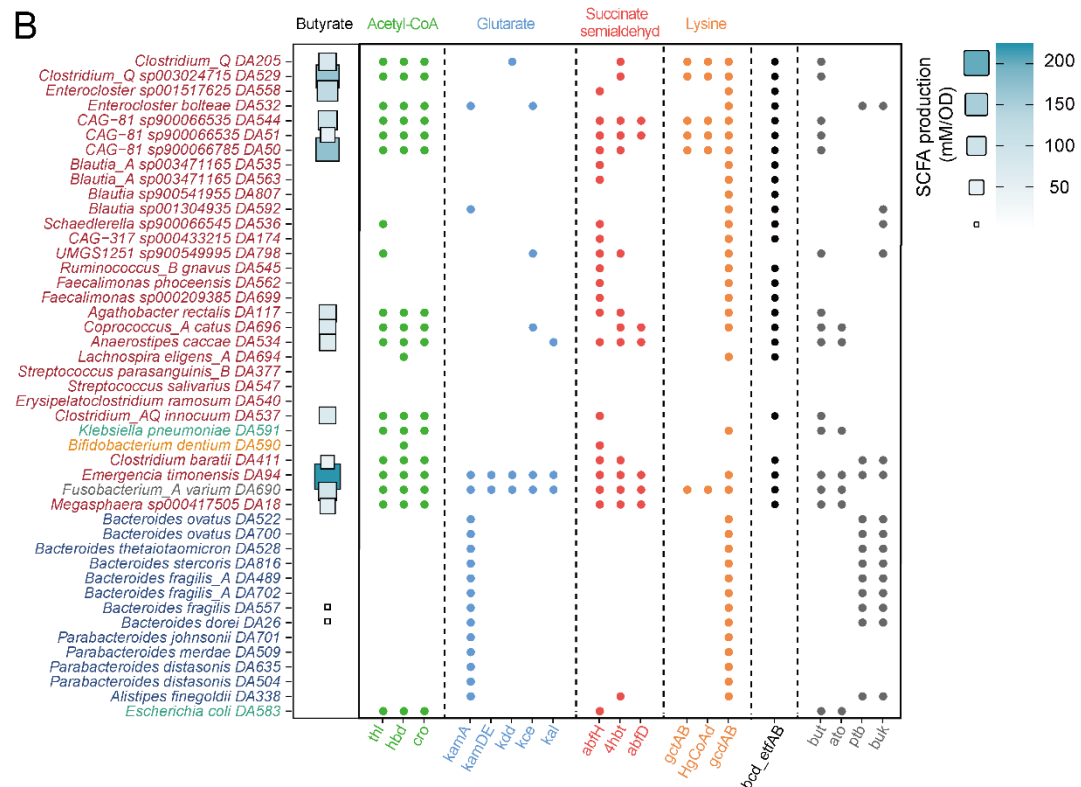
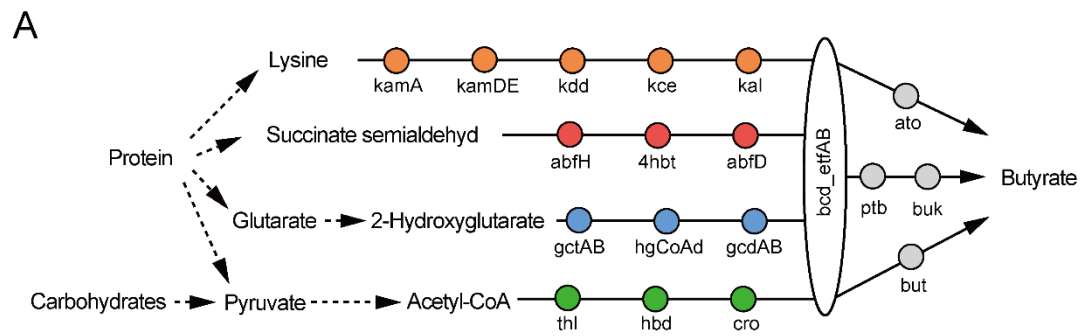


59

60 **Supplementary Figure 6 The genotype-phenotype association of propionate**
61 **production.** A) The schematic diagram shows three propionate-forming pathways,
62 including all major genes involved. B) The diagram shows the distribution of
63 propionate-production and propionate-forming pathways. C) Confusion matrix shows
64 the number of bacteria positive or negative for propionate-producing pathway (one
65 complete pathways at least) vs. positive or negative for propionate production. For
66 propionate, three major biosynthetic pathways were reported⁴, including succinate
67 pathway, lactate pathway, and propanediol pathway (Supplementary Fig. 6A, and
68 **Supplementary Table 7**). The distribution of the propionate-forming genes were
69 predicted by aligning the reviewed gene sequences from KEGG². We observed strong

concordance between the presence of biosynthetic genes (MUT and MCEE from succinate pathway produced propionate; complete propanediol pathway; complete lactate pathway) with propionate production (Sensitivity = 69.6%, Specificity = 90.9%) (Supplementary Fig. 7B-C).

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C

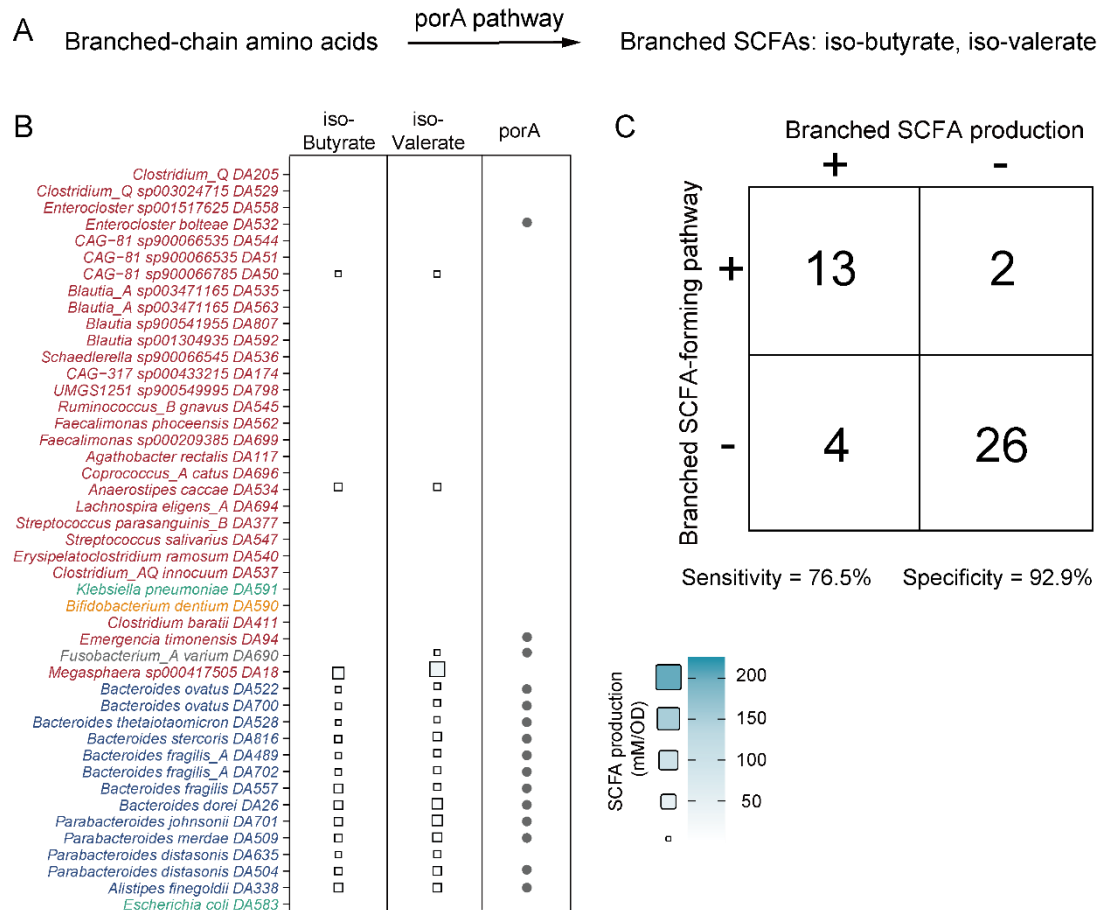
Butyrate production

		+	-
Butyrate-forming pathway	+	13	1
	-	3	28

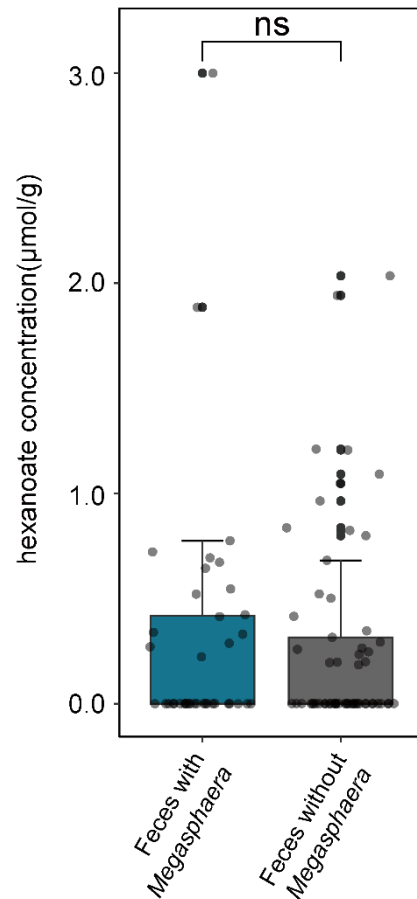
Sensitivity = 81.3% Specificity = 96.6%

Supplementary Figure 7 The genotype-phenotype association of butyrate

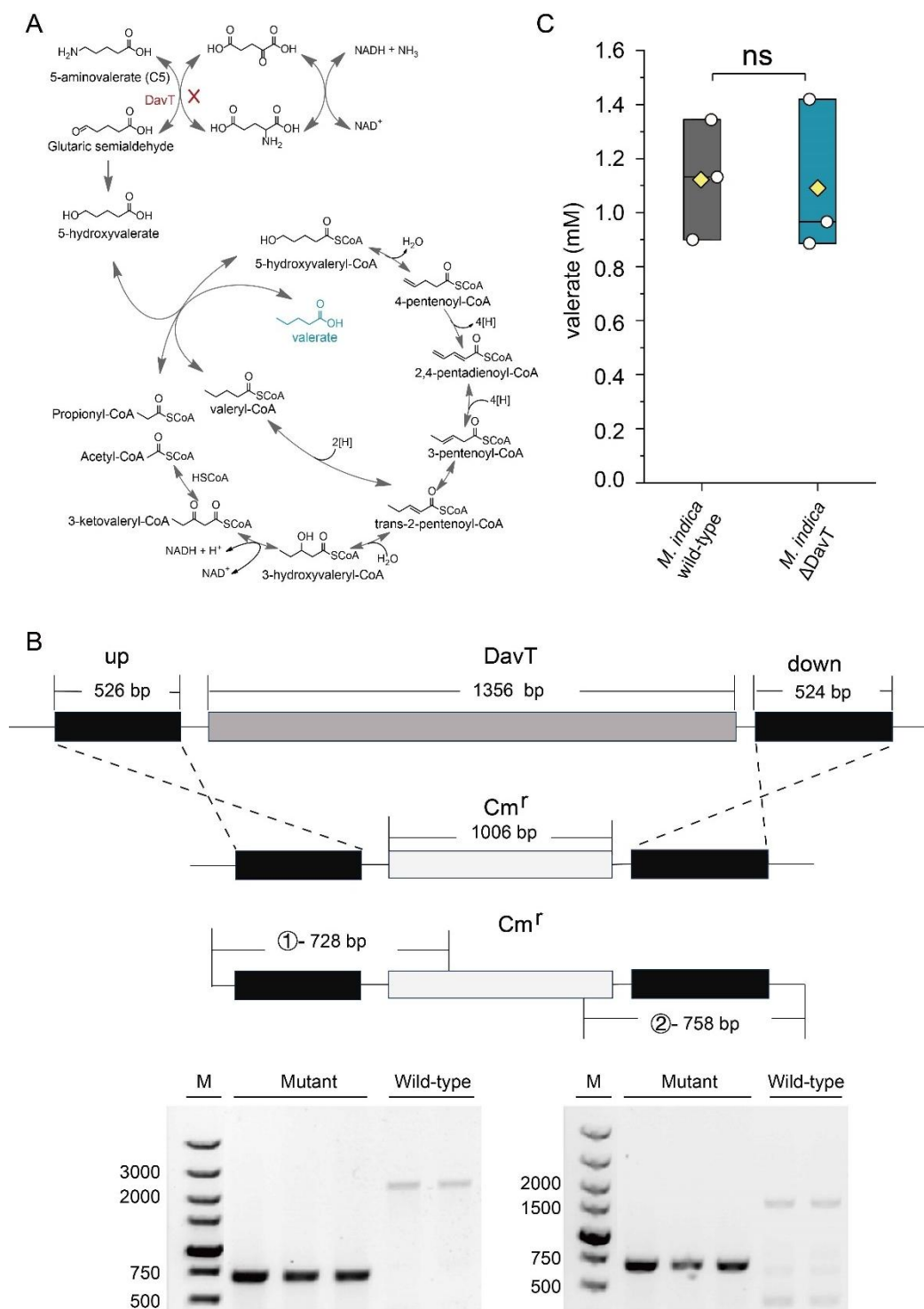
production. **A)** The schematic diagram shows four butyrate-forming pathways, including all major genes involved. **B)** The diagram shows the distribution of butyrate-production and butyrate-forming pathways. **C)** Confusion matrix shows the number of bacteria positive or negative for C) butyrate-producing pathway (one complete pathways at least) vs. positive or negative for butyrate production vs. positive or negative for butyrate production. Four major pathways, including one carbohydrates fueled pathway (acetyl coenzyme A pathway) and three proteins fed pathway (glutarate, 4-aminobutyrate, and lysine pathways) were reported for butyrate biosynthesis⁵⁻⁷ (**Supplementary Fig. 7A, and Supplementary Table 8**). We found that the genomes of butyrate producers encoded one or more complete butyrate-forming pathways, which indicated a good genotype-phenotype correlation (Sensitivity = 81.3%, Specificity = 96.6%) (**Supplementary Fig. 7B-C**).



Supplementary Figure 8 The genotype-phenotype association of isobutyrate and isovalerate production. **A)** The schematic diagram shows *porA* metabolic pathway is involved in the conversion of branched-chain amino acids to branched SCFA. **B)** The diagram shows the distribution of branched SCFA-production and branched SCFA-forming pathway. **C)** Confusion matrix shows the number of bacteria positive or negative for branched SCFA-producing pathway vs. positive or negative for branched SCFA production. For the branched SCFA production, *porA* pathway was responsible for the branched SCFA producing⁸ (**Supplementary Fig. 8A, and Supplementary Table 9**). The distribution of the branched SCFA forming pathways were predicted by gutSMASH. We found that almost strains coding *porA* produced branched SCFA (Sensitivity = 80.0 %, Specificity = 92.0%) (**Supplementary Fig. 8B-C**). In conclusion, we have established a robust genotype-phenotype mapping for butyrate, propionate, and branched SCFAs production of cultivated gut bacteria.

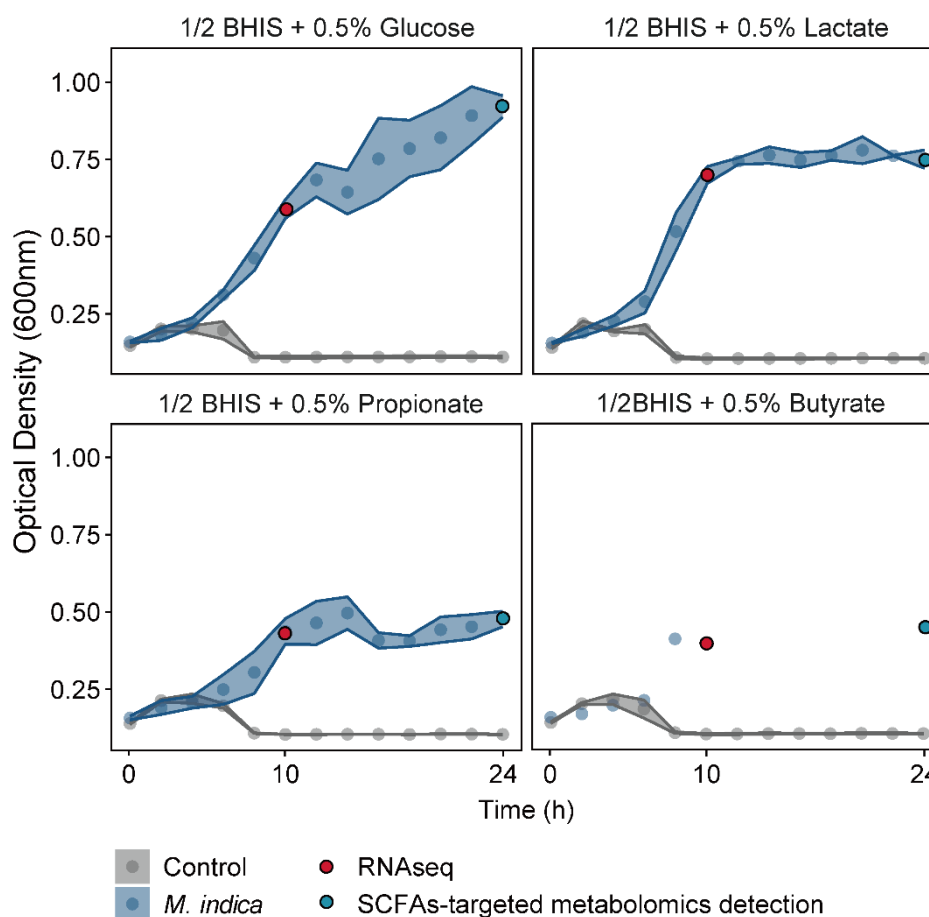


Supplementary Figure 9 The presence of *Megasphaera* does not have a significant effect on the level of hexanoate in human feces. Boxplots showing the differences in hexanoate levels in feces with or without *Megasphaera* genus across 100 healthy human feces samples. Statistical significance was assessed using a two-sided Wilcoxon rank-sum test; ns indicates $p > 0.05$.

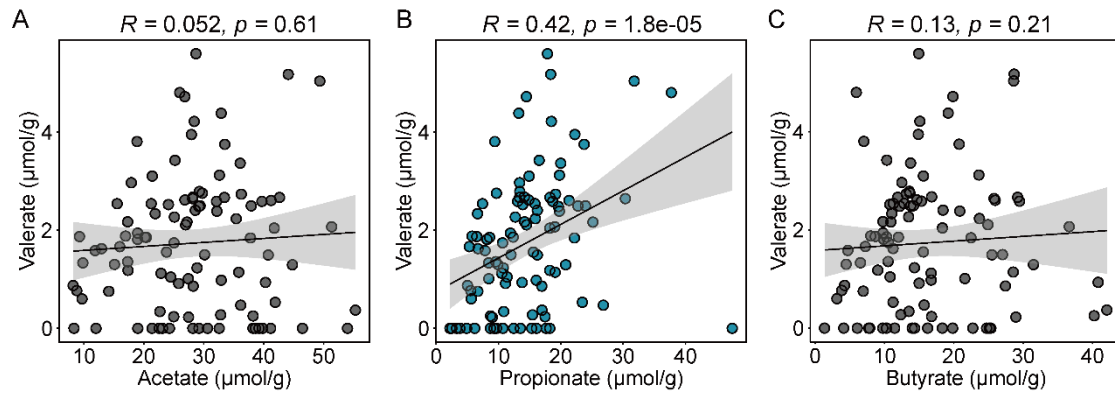


Supplementary Figure 10 *M. indica* produces valerate and hexanoate independently of the 5-aminovaleate pathway. **A)** The anaerobic degradation of 5-aminovaleate by gut anaerobic bacteria leads to valerate, acetate, propionate, and ammonia through intermediates including glutaric semialdehyde, 5-hydroxyvalerate, 5-hydroxyvaleryl-CoA, 4-pentenoyl-CoA, 2,4-pentadienoyl-CoA, trans-2-pentenoyl-

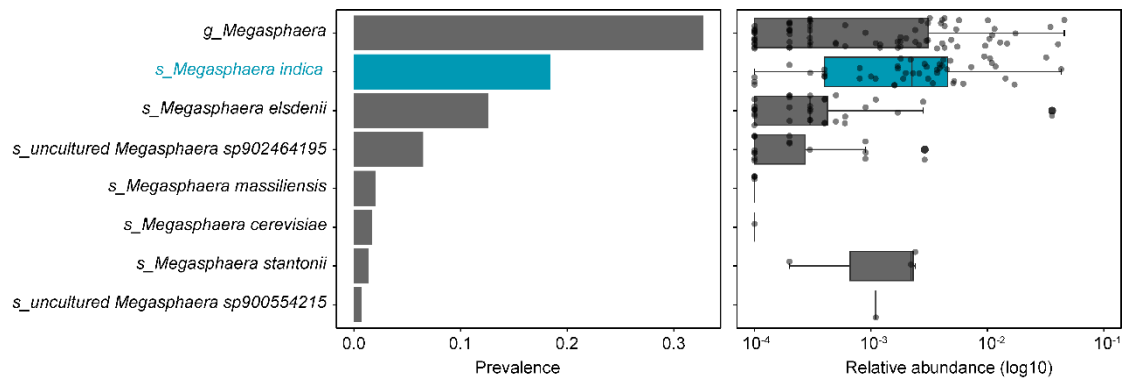
CoA, L-3-hydroxyvaleryl-CoA, 3-ketovaleryl-CoA, acetyl-CoA, and propionyl-CoA. Trans-2-pentenoyl-CoA is the direct precursor of valeryl-CoA. **B)** Schematic diagram of gene knockout and validation, demonstrating gene knockouts using homologous recombination. The nucleic acid gel image shows successful knockout of the target gene. **C)** Gas chromatogram displaying the changes in SCFAs product distribution between the gene knockout mutant strain (*M. indica* *DavT::Cm^r*) and the wild-type strain. Differences in valerate production between wild-type and mutant strains were assessed using a two-sided Wilcoxon rank-sum test.



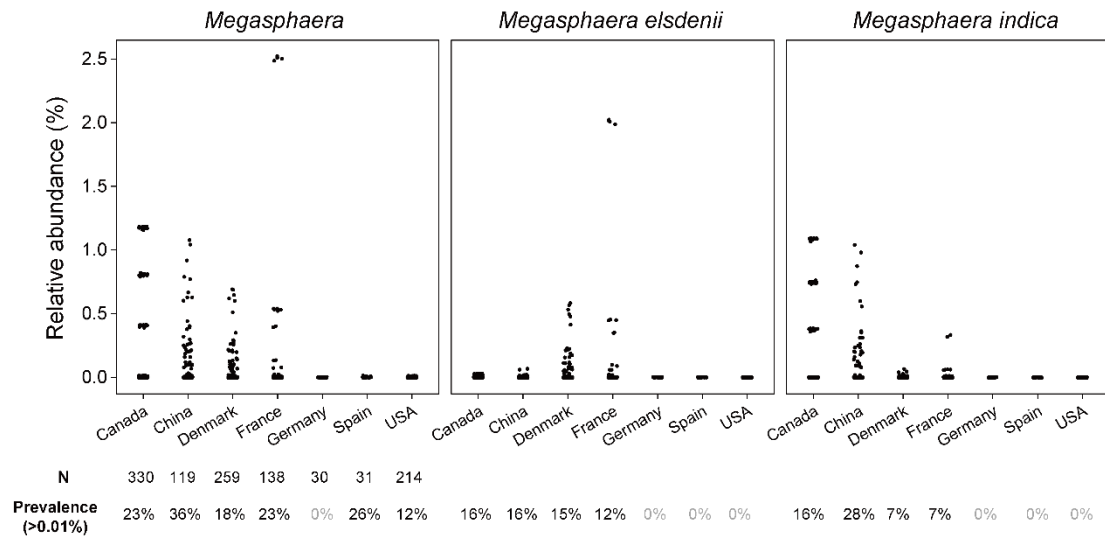
Supplementary Figure 11 Growth dynamics of *M. indica* under different carbon source conditions for transcriptomic and SCFA profiling. The growth curve shows changes in optical density at 600 nm (OD600) over time for *M. indica* under various carbon source conditions. Red points represent the time points for RNA sequencing, while dark cyan points correspond to those for targeted SCFAs metabolomics analysis. Data are presented as mean $\pm$ SD, with assays performed in technical triplicates ($n = 3$), except for the butyrate-fed group.



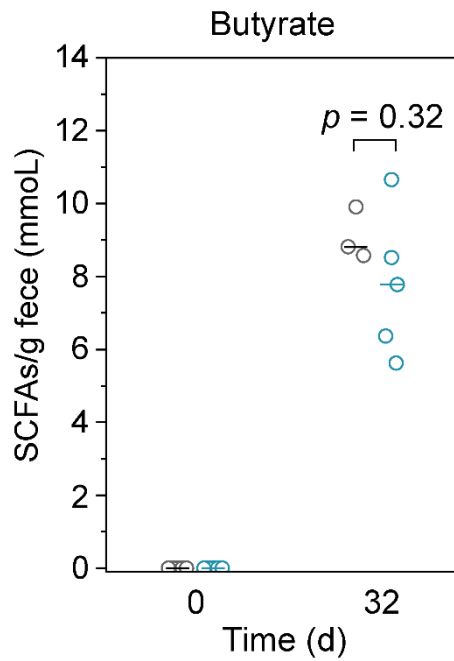
Supplementary Figure 12 Valerate levels are significantly positively correlated with propionate levels in human feces. A-C) Correlation scatter plot showing the relationship between valerate levels and A) acetate, B) propionate, and C) butyrate levels in 100 healthy human feces. Correlations were assessed using Spearman's rank correlation, with p values calculated by a two-sided test. The solid line indicates a linear regression fit for visualization, with the shaded area representing the 95% confidence interval.



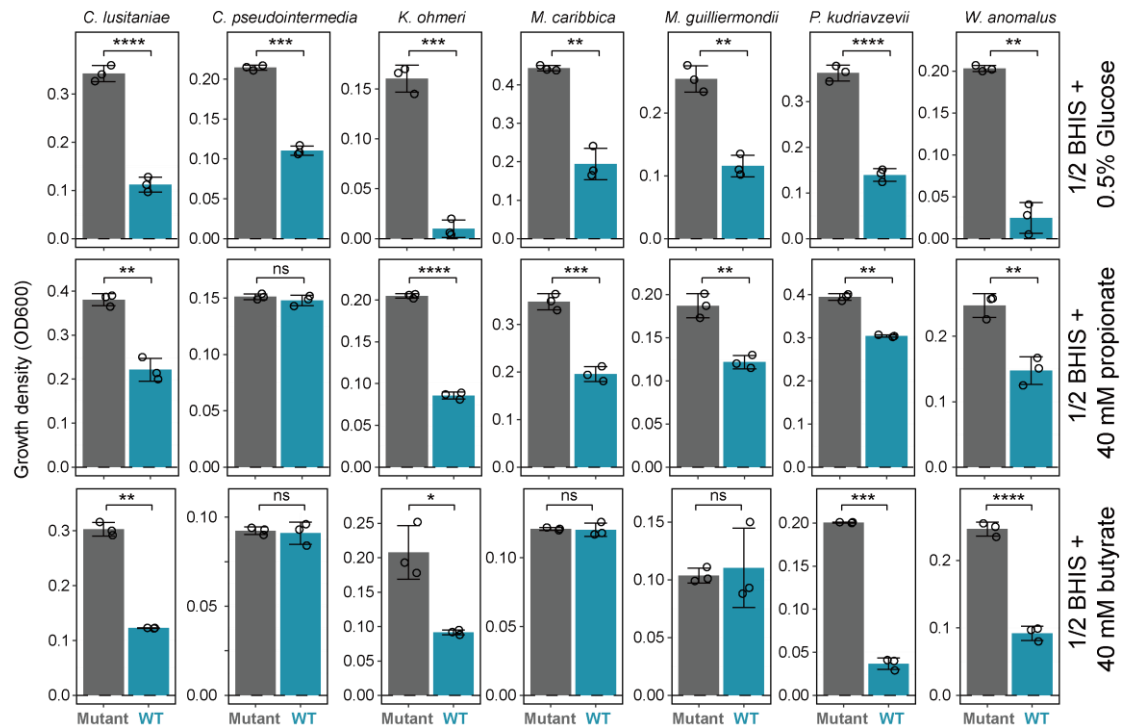
Supplementary Figure 13 *Megasphaera indica* is widely distributed in the human gut. The prevalence (bar chart) and the relative abundance (boxplot) of *Megasphaera* spp. in 292 healthy human fecal samples.



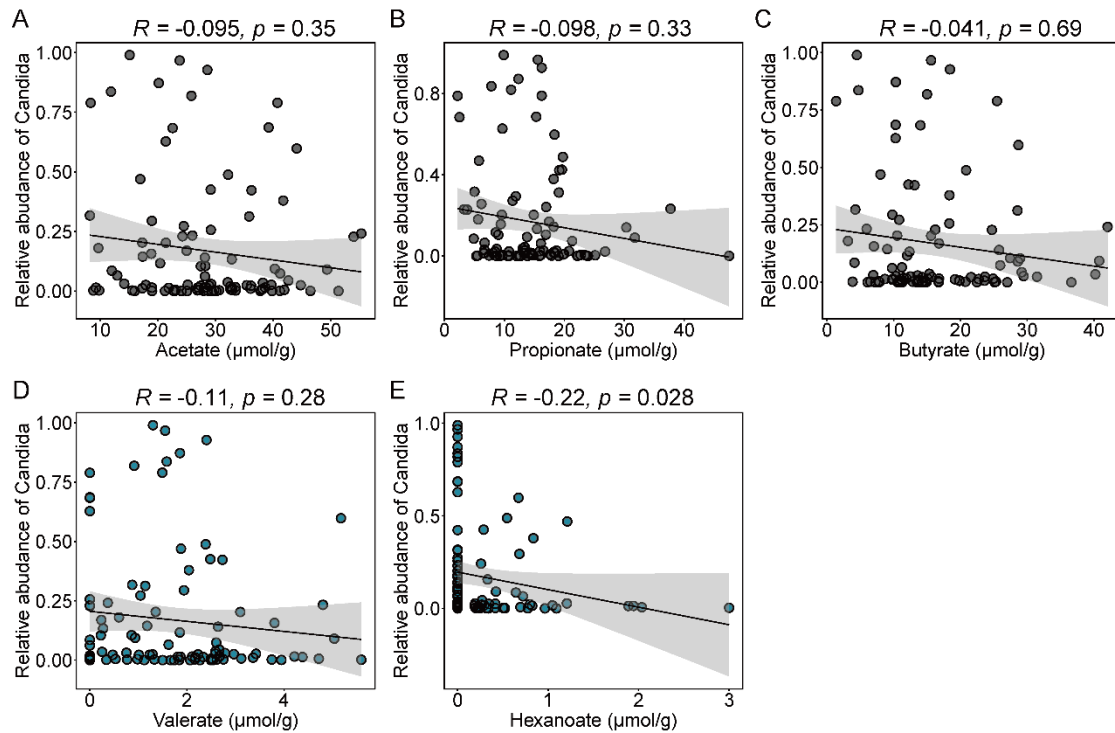
Supplementary Figure 14 The distribution of dominant *Megasphaera* species varies geographically. We analyzed the prevalence and relative abundance of *Megasphaera* genus, as well as *Megasphaera elsdenii* and *Megasphaera indica*. The Chinese cohort was from the SIAT cohort, while the other cohorts were from GMrepo⁹.



Supplementary Figure 15 The production of butyrate by *M. indica* in vivo. Butyrate concentrations in fecal samples of two groups (wild-type (WT) *M. indica* XA511 or the *thlA1-thlA2::Cmr* mutant). Black and blue open circles represent mice colonized with the WT and *thlA1-thlA2::Cmr* mutant strains, respectively. Each symbol represents an individual mouse, and horizontal bars indicate the mean. Statistical significance was determined using an unpaired two-tailed Student's *t*-test. ns, not significant.



Supplementary Figure 16 Antifungal activity of wild-type and mutant *M. indica* culture supernatants under different substrate conditions. Growth differences of 7 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).



Supplementary Figure 17 The relative abundance of *Candida* negatively correlates with valerate and hexanoate levels in human feces. A-E) Correlation scatter plot showing the relationship between relative abundance of *Candida* and A) acetate, B) propionate, C) butyrate, D) valerate, and E) hexanoate levels in 100 healthy human feces. Correlations were assessed using Spearman's rank correlation, with p values calculated by a two-sided test. The solid line indicates a linear regression fit for visualization, with the shaded area representing the 95% confidence interval.

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