

Intestinal permeability of N-acetylcysteine is driven by gut microbiota-dependent cysteine palmitoylation

Corresponding Author: Professor Yimin Cui

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Employing the single-pass intestinal perfusion system, the researchers identified drugs of which the accessibility was largely affected by the gut microbiota, including NAC. The negative correlation between NAC permeability and the sphingolipid synthesis by gut commensal bacteria species was revealed, being proposed as a result of NAC palmitoylation by SPT. Various machine learning models were then tested and compared on predicting NAC permeability in patients. This topic is of novelty and value in clinical applications. However, I have some major comments on experimental design and result interpretation.

Major comments:

1. Writing needs improvements. The method part should contain more details. The experimental procedures and the conclusions obtained from the results need to be better explained. Please improve the clarity and accuracy.
2. In the SPIP system you developed, the liquid collected from the proximal side of the jejunum was circulated back to perfuse the intestine for 2 hours. Was the Peff calculated based on the accumulated absorption of NAC in the perfusion solution? Considering in the case of normal oral drug administration, the drug will not be circulated back for further absorption, this design does not look like a good simulation of oral drug administration. Please explain your design if there are any specific considerations.
3. The research studied how gut microbiota affect the permeation of NAC. However, results related to Fig 2d-h investigated the effects of NAC treatment on the gut microbiota. Please explain the rationale for going in this direction. I don't see the relevance and necessity of this part to the rest of the paper.
4. The result part starting line 170:
 - a. The researchers proposed the reaction between NAC-derived cysteine and palmitoyl-CoA, catalyzed by SPT. Is there any evidence or reports on NAC being converted to cysteine in the intestine? The reaction proposed between NAC and palmitoyl-CoA should be experimentally confirmed. For example, through heterologous expression of SPT in *E. coli* which lacks sphingolipid synthesis, supplying with NAC and palmitoyl-CoA and detecting the production of cysteine-3-KS.
 - b. In Fig 2j, there was no difference between the perfused and unperfused groups which have different abundance of SL-producing bacteria species as you showed before. How should this result be explained?
5. For the result part starting line 191:
 - a. Why did you choose not to use differentiated caco-2 cells which is a better representation of small intestinal epithelial cell compositions?
 - b. The design and interpretation of this in vitro experiment is not very straightforward. Please explain more about how you inferred the permeability of NAC from the fluorescent intensity of the caco-2 cells in Fig 3e. Please also explain why this experiment was necessary. The perfusion experiment in GF rats mono-colonized with different strains is enough to prove your conclusions.
 - c. Line 205: this conclusion is not valid. The differences between NAC+ and NAC- groups could be resulted from the addition of substrate of SLs, which increased the total SL production. The control group should be done with another known substrate of SPT, for example, serine.
 - d. have you compared the lipidomes and the metabolomes between the knockout and wide type? Did the knockout changes the production of other metabolites? If so, the impact of these changes on the permeation of NAC needs to be excluded.
 - e. Please include how the monocolonization of *Bacteroides* was performed and confirmed.
6. This research illustrated how sphingolipid synthesis of the gut microbiota could affect the permeation of NAC in the gut and predictions were made employing various models for clinical guidance. However, the experimental results were

collected using rat models and there are compositional differences between rat and human gut microbiome. For example, the human gut commensal bacteria, *Prevotella copri*, was also found to produce sphingolipids. It is one of most prevalent and dominant species of human gut microbiome however missing in the rat microbiomes. How could the lack of consideration of these species affect your conclusion and the accuracy of prediction? Please refer to this paper and talk about it in the discussion: <https://doi.org/10.1128/mbio.02409-23>

Minor comments:

1. For the method related to Fig 1A, please provide more information on how this network was calculated and constructed. In the legend, you mentioned that this network was validated by a follow-up assay. What assay was it and how was the result?
2. Line 142: for the comparison between any perfused and unperfused groups, please explain the experimental procedures in the method. For example, how the samples were collected, what time point for the changes in microbiome composition, how the sequencing library was prepared, etc.
3. Line 733: in SPF rats not mice
4. Please add statistical analysis results on the plots, including but not limited to: Fig 2j, Fig 3a
5. Line 162: how was the bacteria network constructed
6. Line 163: please explain how you got this conclusion from Fig 1e
7. Supplementary Fig 2 was mentioned in the manuscript before supplementary Fig 1f-g, please reorder the figures.
8. The rats were anesthetized for up to 2 hours, will the anesthesia affect the absorption of NAC?
9. Fig 2d: please order the color key in accordance with the order in the bar plot.
10. Line 201: how was the immunofluorescent intensity being quantified?
11. Fig 3f: why the peaks of cysteine-3-KS have different retention times in different groups?
12. Line 228: to avoid confusion, should be changed to "increased alkyne-labeled SLs by copper-catalyzed cyclo-addition in rats' intestines colonized with WT strains".
13. Fig 4c: please explain how the intestine samples were prepared for imaging. were the bacteria removed?
14. Line 522: should be apical chamber not the basal side
15. For the illustration of the system in Fig 3b, the liquid level in the inner well is higher than the middle well. Is this a mistake or maintained? The colors of the medium in the middle and lower chamber should be the same to avoid confusion. Please keep the naming of the wells/chambers consistent across the manuscript.
16. Line 420: please clarify the definition and determination of steady state.

Reviewer #2

(Remarks to the Author)

This is an innovative paper, assessing a very important topic: variable permeability of oral drugs through Intestinal microbiota composition analysis and model production.

Overall quality of the data are good, technically sound, obtained with appropriate techniques, analysed and interpreted carefully and presented in detail.

Sufficiently strong evidence is provided for the authors' claims, and appropriate controls have been included.

These results are important to the field and will advance understanding.

however the paper in the present formulation has several limitations that reduce the comprehension and the clarity of the message.

- 1 - it is very important to better specify, perhaps with a dedicated paragraph, the 13-feature light gradient boosting 55 machine (LGBM) model and how it has been generated (it seems it is generated starting from mice data).
- 2 -- it is not clear the role of patients: in the methods 240 participants, while in results not clear to which categories of patients are referred. in some results you refer to n=192, in others 48 or in other 79. Consider to do a diagram if this cohort served for differential subaims.
- 3 -- results refer to duodenal/jejunal permeability and not intestinal permeability too: please specify and better clarify into the discussion section
- 4 -- for what I can understand microbiota profile influencing permeability of oral drugs is referring to mucosa composition of microbiota and not to fecal or other microbiota. do you have also data correlating with other microbiota? please speculate

minor points

- 1 - specify why and how you selected the 32 oral drugs
- 2 - describe better how and where you do expect this new proposed model can be utilized: I do not believe, at this stage, in clinical practice, perhaps for drug design or to develop further drug/class of drug models? please discuss and speculate.
- 3 - please specify better the condition where this model is working and potential limitation (absence of disease, "normal transit time", one and the same drug, standard diet etc..)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Upon revision, the method section of the manuscript includes more details and improved clarity which facilitate the understanding of research. The authors did a good job of answering previous questions and responding to the suggestions. Considering that the effects of NAC on the gut microbiota are investigated in their research and the importance of the bi-directional relationships between drugs and microbes as mentioned by the authors in their rebuttal, I suggest adding the corresponding background and significance to the introduction section as well. Other than that, I suggested this manuscript to be accepted by the journal based on the impacts and quality of the study.

Reviewer #3

(Remarks to the Author)

I was asked to comment on the machine learning approach of the manuscript. Overall, I think more clarification is needed on the overall modeling strategy. Please see my comments below.

Line 255: What does "SLs" refer to? I was unable to find its definition in the text.

Line 255: What is the "risk-based model"? Was it described elsewhere in the manuscript?

Line 256: While the cohort background is detailed in the Methods section, it should also be briefly summarized in the main text. Are these participants healthy?

Lines 257-259: What is the total number of features analyzed? Are the features categorical or continuous? Regarding the intestinal ceramide metabolome and microbiota, did you include all microbial taxa and metabolites? If so, this would result in thousands of features. What feature selection strategies were used to avoid overfitting?

Lines 261-262: "The feature values of the participants in the Validation Cohort were divided into $F_{sys} \geq 85\%$ (Fig. 5b, left panel) and $F_{sys} < 85\%$ (Fig. 5b, right panel)." I do not understand what this means. Is F_{sys} the target variable (the variable to be predicted)? If so, why was it used to divide feature values (inputs)?

Line 264: It is unclear how the machine learning model improves the stabilization and precision of the risk-based model. Again, what is the risk-based model?

Lines 268-275: Did you use validation data during model development? Do you have three datasets (train, validation, test), or only two (train, test)? If there were three, how was the validation dataset used for? Also, what criteria were used to divide all participants into discovery, validation, and test cohorts?

Line 280: What does "the classifier algorithm" refer to? Did you mean the classification counterpart of the regression algorithms?

Fig. S7b: What does the y-axis score represent? Why is the score equal to 1.0 even with the lowest number of training samples?

Lines 287-288: What insights does the agglomerative hierarchical clustering provide?

Lines 288-289: From a methodological perspective, any feature selection process, including the recursive feature elimination approach mentioned in the manuscript, should be embedded within the training procedure. Applying feature elimination after model fitting and hyperparameter selection is less rigorous.

Lines 293-295: "Removing any of these features leads to a reduction in model accuracy." How significant were these reductions when each highly correlated feature was removed? Could you investigate the underlying cause? This may indicate overfitting.

Line 296: What is meant by "suitability"?

Lines 297-298: Once again, the feature selection algorithm should be incorporated into the training process.

Line 55: The manuscript lacks clarity on how commercial models (ACAT, GITA, ADAM) make predictions. What are the inputs and outputs of these models? Are they mechanism-based and data-free? Providing some background information on these models would improve understanding.

Lines 686-688: The code availability statement is unacceptable. Without access to the code, it is impossible to fully evaluate the machine learning approach.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

I appreciate the efforts in the manuscript revision. All my questions have been addressed. Great job!

(Remarks on code availability)

The authors deposited their code to <https://codeocean.com/capsule/8060667/tree>. I created an account but my login was denied. Here is the error message

Access to create Capsules in the OSL is available for publishers and collaborating institutions. If you need to publish a Capsule in an article, please reach out to support@codeocean.com information about your publication for assistance.

Therefore, I was unable to review the codes.

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Dear Editors and Reviewers,

I am writing to submit our revised manuscript, titled “Intestinal microbiota-mediated permeability screening reveals the variable N-Acetylcysteine bioavailability by cysteine palmitoylation of *Bacteroides*” (NCOMMS-24-32817). We are grateful for the opportunity to revise our work and insightful comments provided. We have taken the feedback to heart and meticulously modified our manuscript by expanding the Methods section, refining data analysis, and incorporating additional discussions to address each reviewer's point comprehensively.

The corresponding changes of the text are highlighted in red for easy identification. We have also updated the following figures to strengthen our findings and arguments:

- Figure 1a
- Figures 2c, d, g, j
- Figures 3a, b
- Figure 4a
- Figure 5a
- Supplement Figures S2a, c, d
- Supplement Figures S3b, h
- Supplement Figures S7a, b

The following are our point-to-point responses to the comments and concerns raised by the reviewers:

Reviewer comments

Reviewer #1: *Expertise: Microbiota-derived sphingolipids / metabolism / mouse.*

Employing the single-pass intestinal perfusion system, the researchers identified drugs of which the accessibility was largely affected by the gut microbiota, including NAC. The negative correlation between NAC permeability and the sphingolipid synthesis by gut commensal bacteria species was revealed, being proposed as a result of NAC palmitoylation by SPT. Various machine learning models were then tested and compared on predicting NAC permeability in patients. This topic is of novelty and value in clinical applications. However, I have some major comments on experimental design and result interpretation.

Response: We greatly appreciate the reviewer for the constructive feedback provided. Carefully considering each point, we have made the following revisions and clarifications to address the concerns raised.

1. In response to the comment on **experimental design**, we have included *Escherichia coli* which lacks sphingolipid synthesis as additional controls and replicates to strengthen the validity of our findings. Specifically, we have enlarged the range of N-Acetylcysteine (NAC) perfusion

concentrations and perfusion time for single-pass intestinal perfusion system experiments, with the rigorous statistical analysis to ensure the robustness of our results.

2. We acknowledge the reviewer's concern regarding **the interpretation of results**. To address this, we have re-evaluated our data and provided a more detailed explanation of the experimental procedures and the conclusions obtained from the results, ensuring that they are clearly outlined and understood by readers.
3. To emphasize the **novelty and clinical relevance** of our findings, we have added a discussion on the potential implications for personalized medicine and how our work could inform future clinical trials. This is now reflected in the revised manuscript, with additional references to the latest literature on the topic.

Major comments:

1. Writing needs improvements. The method part should contain more details. The experimental procedures and the conclusions obtained from the results need to be better explained. Please improve the clarity and accuracy.

Response: We sincerely appreciate the thorough review and insightful comments provided. Your expertise in microbiota-derived sphingolipids, metabolism, and mouse models has been invaluable in enhancing the quality of this manuscript. We have taken your comments to heart and implemented substantial revisions to address the concerns raised.

Improvements in Writing and Clarity: In line with the guidance provided by *Nature Communications* on clear communication, we have revised the manuscript to ensure that our findings are communicated effectively, avoiding technical jargon where possible and providing clear explanations where necessary. We have also ensured that the background, rationale, and main conclusions of this version are clearly explained, particularly in the title and abstract, to be readily intelligible to any scientist.

Enhancements in the Methods Section: As per the reviewer's suggestion and in accordance with Nature's guidelines on manuscript formatting, we have expanded the Methods section to include all elements necessary to allow for replication of the results. We have also identified commercial suppliers of reagents or instrumentation when the source is critical to the outcome of the experiments, as recommended by Nature.

Refinements in Results Interpretation: We have carefully revised all interpretations of results to improve accuracy and clarity. This includes a more detailed explanation of the experimental steps and precise conclusions obtained from the results, to ensure that our key findings and their implications are clearly articulated.

Language Editing: Recognizing the diverse readership of Nature Communications, we have had the manuscript proofread by a native English speaker to ensure that the language used is clear and accessible to all readers. All changes have been highlighted in red for easy identification.

2. In the SPIP system you developed, the liquid collected from the proximal side of the jejunum was circulated back to perfuse the intestine for 2 hours. Was the P_{eff} calculated based on the accumulated absorption of NAC in the perfusion solution? Considering in the case of normal oral drug administration, the drug will not be circulated back for further absorption, this design does not look like a good simulation of oral drug administration. Please explain your design if there are any specific considerations.

Response: We thank Reviewer #1 for highlighting the need for clarity in our experimental design, particularly regarding the Single-Pass Intestinal Perfusion (SPIP) system. We appreciate the opportunity to provide further explanation for addressing any concerns.

Clarification on SPIP System Design: The SPIP system, as originally described by *Jacobs et al.* in 1963 [PMID: 13964307], is a well-established method for assessing intestinal drug absorption. Contrary to the initial interpretation, the perfusion solution in our study does not recirculate through the intestine; instead, it is collected directly from the effluent and transferred to pre-weighted collection vials. This approach is consistent with the standard procedure to simulate a single-pass perfusion, which more accurately reflects the conditions of oral drug administration where the drug does not recirculate for further absorption.

Permeability Calculation (P_{eff}): In response to the reviewer's query about the calculation of the effective permeability (P_{eff}), we have ensured that our calculations are based on the permeation rates rather than the accumulated amounts of N-Acetylcysteine (NAC) during intestinal perfusion. This is in line with the standard practice for determining P_{eff} , which is derived from the rate of drug transport across the intestinal wall. We have updated the Methods & Materials section to include the detailed formula of calculating P_{eff} , as follows:

"The effective permeability (P_{eff} , cm/s) is calculated using the following equation:

$$P_{\text{eff}} = - \frac{Q_{\text{in}}}{A} \times \ln \frac{C_{\text{out}} \times Q_{\text{out}}}{C_{\text{in}} \times Q_{\text{in}}}$$

wherein, Q_{in} = inlet perfusate flux (cm^3/s), A ($2\pi r l$) = absorbable intestinal area considering radius and segment length (cm^2), C_{out} = outlet drug concentration (mg/mL), Q_{out} = outlet perfusate flux (cm^3/s), C_{in} = inlet drug concentration (mg/mL). " (Lines 449-454)

3. The research studied how gut microbiota affect the permeation of NAC. However, results related to Fig 2d-h investigated the effects of NAC treatment on the gut microbiota. Please explain the rationale for going in this direction. I don't see the relevance and necessity of this part to the rest of the paper.

Response: We appreciate this insightful question from Reviewer #1 regarding the rationale behind investigating the effects of N-Acetylcysteine (NAC) treatment on the gut microbiota, as presented in Figs. 2d-h. The decision to include this part was driven by the mounting evidence that highlights the complex bidirectional relationship between drugs and gut microbes.

Our study aims to explore how gut microbiota affect the permeation of NAC. However, understanding the impact of NAC on the gut microbiota is also crucial for the following reasons: First, it provides a comprehensive view of the interaction between the drug and the microbiome, which is essential for understanding the full scope of NAC's effects within the gastrointestinal environment. Second, by identifying specific microbial strains such as *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* that are prominently altered by NAC perfusion, we can target these strains for further *in vitro* and *in vivo* studies. This targeted approach is vital for verifying the colonization effects of *Bacteroides* and for elucidating the mechanisms by which NAC influences gut microbiota composition and function.

In the context of *Nature Communications'* focus on rigorous and innovative research, we believe that this part of our study adds more depth to our understanding of the drug-microbiota interaction and its implications for NAC bioavailability. It also aligns with the journal's interest in research that contributes to the understanding of host-microbiome interactions and their impact on health and disease.

4. The result part starting line 170:

a. The researchers proposed the reaction between NAC-derived cysteine and palmitoyl-CoA, catalyzed by SPT. Is there any evidence or reports on NAC being converted to cysteine in the intestine? The reaction proposed between NAC and palmitoyl-CoA should be experimentally confirmed. For example, through heterologous expression of SPT in *E. coli* which lacks sphingolipid synthesis, supplying with NAC and palmitoyl-CoA and detecting the production of cysteine-3-KS.

Response: We sincerely appreciate the valuable comments from Reviewer #1. The concerns raised about the conversion of NAC to cysteine in the intestine and the experimental confirmation of the reaction between NAC and palmitoyl coenzyme A (palmitoyl-CoA) are both pertinent to our study.

Evidence of NAC Conversion to Cysteine: It has been well-documented in the literature that NAC is readily transported by enterocytes and colonocytes and converted into L-cysteine within the intestinal cells [PMID: 24080471]. This conversion is a crucial step in understanding NAC bioavailability and metabolic fate of NAC within the gastrointestinal tract.

Experimental Confirmation of the Reaction: To address the proposed reaction between NAC-derived cysteine and palmitoyl-CoA catalyzed by serine palmitoyltransferase (SPT), a series of experiments were conducted as suggested by the reviewer. We have designed isotope labeling

experiments using isotopically-labeled ^{15}N -NAC to monitor the synthesis of cysteine-containing sphingolipids (SLs) (Figs. S3d-g). The results showed a marked decline in ^{15}N -cysteine-3-ketosphinganine, -sphinganine, -sphingosine, and -sphingosine-1-phosphate levels after the knockdown of SPT in both *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*, indicating the importance of SPT in the metabolism of NAC-derived cysteine.

To further validate the reaction as suggested by the reviewer, we performed heterologous expression of SPT in *E. coli*, which naturally lacks sphingolipid synthesis. By supplying NAC and palmitoyl-CoA to these SPT-overexpressing *E. coli* and detecting the production of cysteine-3-ketosphinganine (Figs. S2c, d), we observed a significant increase in cysteine-3-ketosphinganine levels and a decrease in palmitoyl-CoA, implying the emergence of sphingolipid synthesis reaction in SPT-overexpressing *E. coli*.

The following sentences and Figures are now added as follows:

"To further elucidate the effects of bacterial SPT on NAC permeability, we added isotopically-labeled ^{15}N -NAC in the medium to monitor newly synthesized SLs (Supplementary Fig. 3c). At the end of the three-hour incubation, ^{15}N -cysteine-3-ketosphinganine, cysteine-sphinganine, cysteine-sphingosine and cysteine-sphingosine-1-phosphate in SL pools were significantly down-regulated by SPT deficiency in co-culturing with both *B. thetaiotaomicron* and *B. fragilis* (Supplementary Fig. 3d-g)." (Lines 209-215)

Fig. S2c

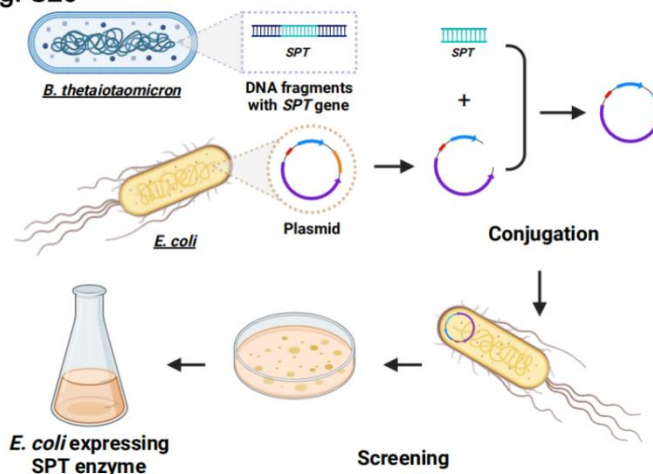


Fig. S2d

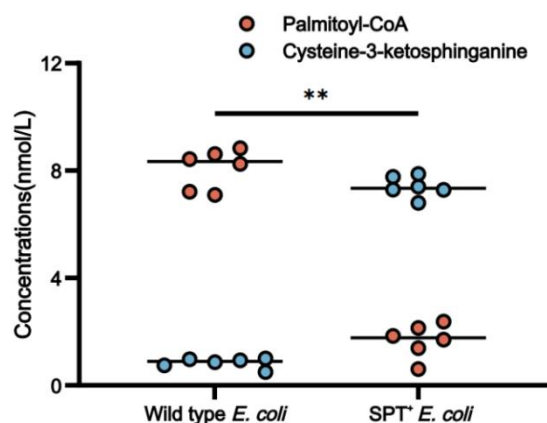


Fig. S2 (c) Schematic representation of the SPT gene transfer workflow into *E. coli*, created using BioRender. **(d)** Quantitative analysis of palmitoyl-CoA and cysteine-3-ketosphinganine levels in wild-type *E. coli* and SPT-overexpressing *E. coli*, respectively. Data are presented as mean $\pm$ standard deviation. Statistical significance was determined using one-way ANOVA; * $P < 0.05$, ** $P < 0.01$.

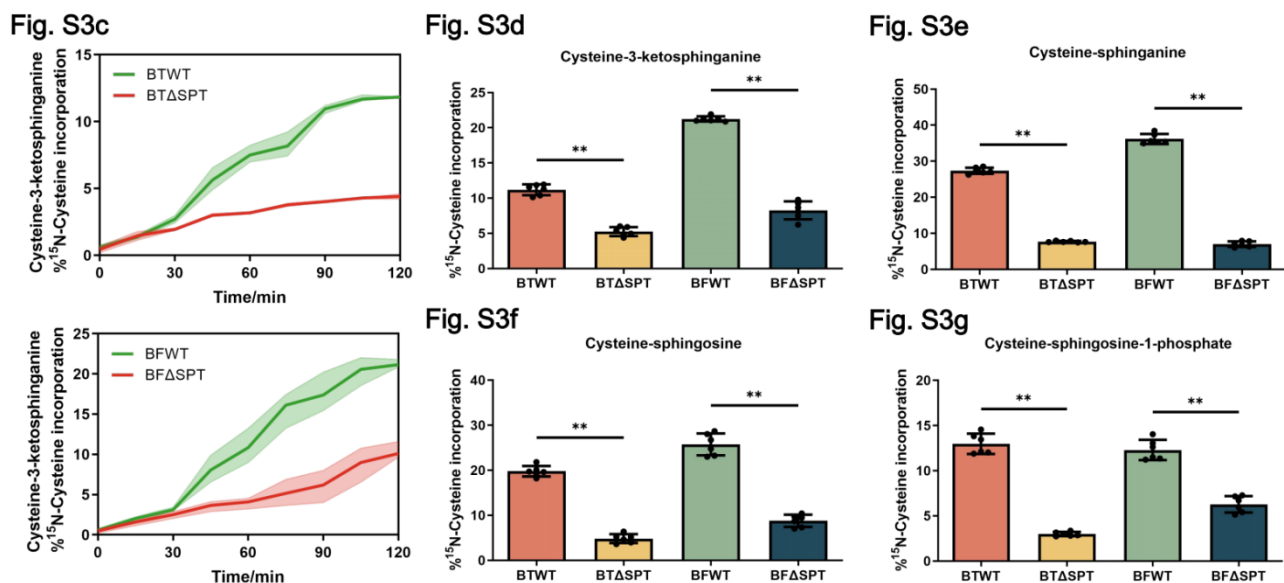


Fig. S3 (c) Time course of effective permeability (P_{eff}) values over 2 hours for the Single-Pass Intestinal Perfusion (SPIP) model in Germ-Free rats (GF^{BTWT}) and $\text{GF}^{\text{BT}\Delta\text{SPT}}$ (top panel) and GF^{BFWT} , $\text{GF}^{\text{BF}\Delta\text{SPT}}$ rats (bottom panel), monitored at 30-min intervals. **(d-g)** Schematic diagram illustrating the incorporation of ¹⁵N-NAC to trace newly synthesized cysteine-3-ketosphinganine (d), -sphinganine (e), -sphingosine (f), -sphingosine-1-phosphate (g). ¹⁵N-NAC was added to culture medium and the [M + 1] isotopes due to ¹⁵N-cysteine incorporation into newly synthesized SLs were monitored by liquid chromatography-mass spectrometry (LC-MS). Bars represent the mean $\pm$ standard deviation of three biological replicates. Statistical significance was determined by two-sided t-test, * $P < 0.05$, ** $P < 0.01$.

b. In Fig 2j, there was no difference between the perfused and unperfused groups which have different abundance of SL-producing bacteria species as you showed before. How should this result be explained?

Response: We thank the reviewer for this query regarding the observed lack of difference between the perfused and unperfused groups in Fig. 2j, despite the differences in the abundance of sphingolipid (SL)-producing bacteria species as previously shown. This is indeed a critical point that requires clarification.

To address this apparent contradiction, we repeated the experiments by broadening the range of NAC perfusion concentrations. We sought to determine whether an insufficient NAC perfusion concentration (20 mg/L) could account for the lack of statistical difference in palmitoyl-CoA and cysteine-3-ketosphinganine between the NAC-perfused and NAC-unperfused intestinal segments, which originally contained endogenous cysteine. In this revision, we introduced a higher concentration of 30 mg/L NAC and re-examined the levels of palmitoyl-CoA and cysteine-3-ketosphinganine. The results of this revision demonstrated a significant increase in cysteine-3-ketosphinganine and a decrease in palmitoyl-CoA in the SPF-perfused segment compared with the

SPF-unperfused segment (Fig. 2j). This finding aligns with the expected outcomes based on the presence of SL-producing bacteria species. The original records of two rounds of Single-Pass Intestinal Perfusion (SPIP) experiments have been uploaded as data attachments to ensure transparency and reproducibility.

The following sentences and figures have been revised to reflect these changes:

"Then, a perfusion solution containing 30 mg/L NAC was administered at 37 °C through the intestinal lumen at a constant flow rate of 0.2 mL/min." (Lines 441-443)

"By employing ultra-performance liquid chromatography-coupled time-of-flight mass spectrometry (UPLC-ESI-QTOF/MS) metabolite profiling, we further quantified a significant increase in palmitoyl-CoA but a decrease in cysteine-3-ketosphinganine in GF rats compared with SPF rats (Fig. 2j)." (Lines 183-187)

Fig. 2j

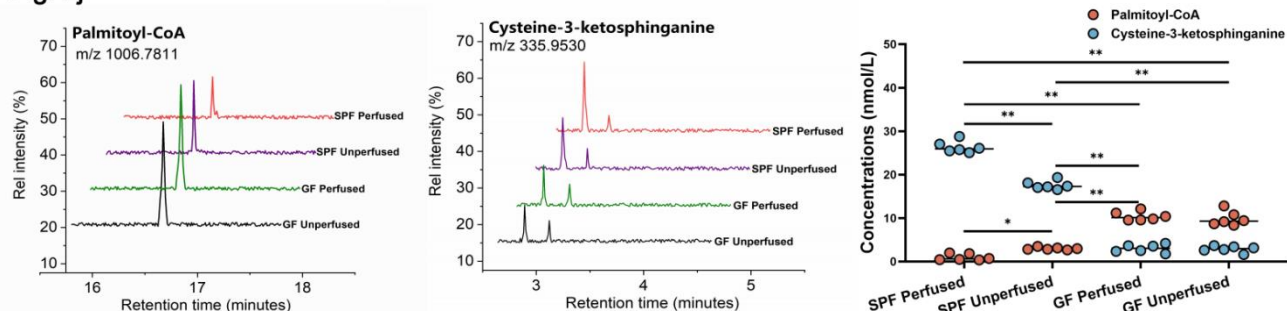


Fig. 2 (j) Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was utilized to identify and quantify palmitoyl-CoA (left panel) and cysteine-3-ketosphinganine (middle panel). Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was assessed using one-way ANOVA, with $*P < 0.05$, $**P < 0.01$ indicating significant differences.

5. For the result part starting line 191:

a. Why did you choose not to use differentiated caco-2 cells which is a better representation of small intestinal epithelial cell compositions?

Response: We thank the reviewer for their valuable feedback to highlight the importance of using differentiated Caco-2 cells in our study. It is indeed true that differentiated Caco-2 cells provide a more accurate representation of the small intestinal epithelial cell composition, which are crucial for studying intestinal absorption.

Inclusion of Differentiated Caco-2 Cells: In response to the reviewer's comment, we acknowledge that the use of differentiated Caco-2 cells was not explicitly mentioned in the initial submission. We have now included this information to clarify our experimental approach. Differentiated Caco-2 cells were indeed utilized in this study, and we have added a detailed description of their use and the establishment of the differentiated Caco-2 cell model to the revised manuscript.

Revisions and Additions: The following sentences and figures have been added to the manuscript to address the reviewer's concern:

"Differentiated Caco-2 cells were co-cultured with wild-type *Bacteroides thetaiotaomicron* (BTWT), *Bacteroides fragilis* (BFWT), *Bacteroides thetaiotaomicron* SPT-knockout (BTΔSPT), or *Bacteroides fragilis* SPT-knockout (BFΔSPT) cells in a three-well transfer system." (Lines 197-199)

"To establish the differentiated Caco-2 cell model, the cell line was routinely subcultured using the low-density protocol⁴³. Caco-2 cells were initially seeded at 6.2×10^3 cells/cm² and subcultured for at least 10 passages at 50% confluence (5.4×10^4 cells/cm²). Differentiation was achieved by seeding cells on polyethylene-terephthalate (PET) membrane inserts at a density of 3×10^5 cells/cm² and maintained for 21 days in complete medium, which was changed three times a week." (Lines 582-588)

Fig. 3b

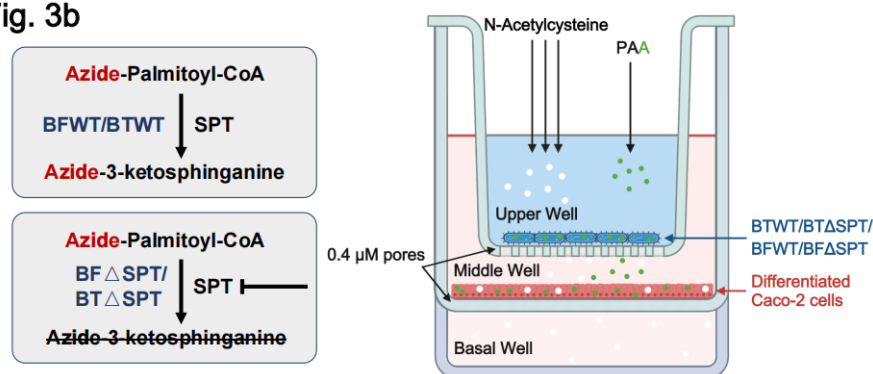


Fig. 3 (b) Schematic representation of the three-well co-culture system. NAC is transferred through BTWT/BFWT or BTΔSPT/BFΔSPT cells in the upper well to differentiated Caco-2 cells in the middle well, followed by infiltration into the basal well for concentration measurement.

b. The design and interpretation of this in vitro experiment is not very straightforward. Please explain more about how you inferred the permeability of NAC from the fluorescent intensity of the caco-2 cells in Fig 3e. Please also explain why this experiment was necessary. The perfusion experiment in GF rats mono-colonized with different strains is enough to prove your conclusions.

Response: We thank the reviewer for their insightful comments on the *in vitro* experiment depicted in Fig. 3e. We understand the need for clarity in our experimental design and interpretation.

Clarification on the Use of Fluorescence Intensity: The fluorescence intensity of PAA-derived sphingolipids (SLs) in the Caco-2 cells (Fig. 3e) was not intended to directly measure the permeability of N-Acetylcysteine (NAC). Instead, it served as a proxy to verify the efficient knockdown of microbial serine palmitoyltransferase (SPT), which is a key enzyme in sphingolipid synthesis (Figs. 3d, e). This approach allowed us to assess the impact of SPT activity on SL production, which is central to our study's objectives.

Rationale for the *In Vitro* Experiment: This *in vitro* experiment was essential for several reasons. Firstly, it enabled us to screen *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* from

multiple strains, which were subsequently used for *in vivo* colonization in SPIP model. This screening process is critical for selecting strains that exhibit significant impact on SL production and NAC permeability. Secondly, the three-well co-culture system, which includes differentiated Caco-2 cells, has been reformed from the conventional drug permeability evaluation using sterile Caco-2 cells alone. This system is designed to more closely mimic the physiological patterns of drug permeability in the intestinal tract, providing a more accurate representation of *in vivo* conditions.

Necessity of the *In Vitro* Experiment: While the perfusion experiment in germ-free (GF) rats mono-colonized with different strains does provide valuable evaluation into *in vivo* permeability of NAC, the *in vitro* experiment complements the underlying mechanism by offering a controlled environment to dissect the specific contributions of microbial SPT to NAC permeability. This dual approach strengthens our conclusions by providing both mechanistic insights from the *in vitro* system and physiological relevance from the *in vivo* model.

c. Line 205: this conclusion is not valid. The differences between NAC+ and NAC- groups could be resulted from the addition of substrate of SLs, which increased the total SL production. The control group should be done with another known substrate of SPT, for example, serine.

Response: We appreciate the reviewer's scrutiny of our experimental design and the concern raised regarding the interpretation of the results in Fig. 3e.

Clarification on the Role of NAC in SL Synthesis: In our experiment, the fixed amount of azide-tagged PAA was the rate-limiting step, but not the availability of NAC-derived cysteine. Therefore, the addition of NAC does not alter the total SL production. The low-permeability PAA reacts with NAC-derived cysteine in the presence of SPT, leading to the generation of high-permeability azide-tagged SLs. This process is illustrated in the schematic diagram provided in left panel below. The NAC+ groups with wild-type *Bacteroides* exhibited stronger fluorescence at the 3.227-hour time point, indicating a faster entry of high-permeability SLs into the Caco-2 cell layer compared to the NAC- or SPT-depleting groups (Figs. 3d, e). This observation suggests that NAC-derived cysteine is indeed involved into the sphingolipid synthesis process and is consumed in the process, as depicted in Fig. 3c.

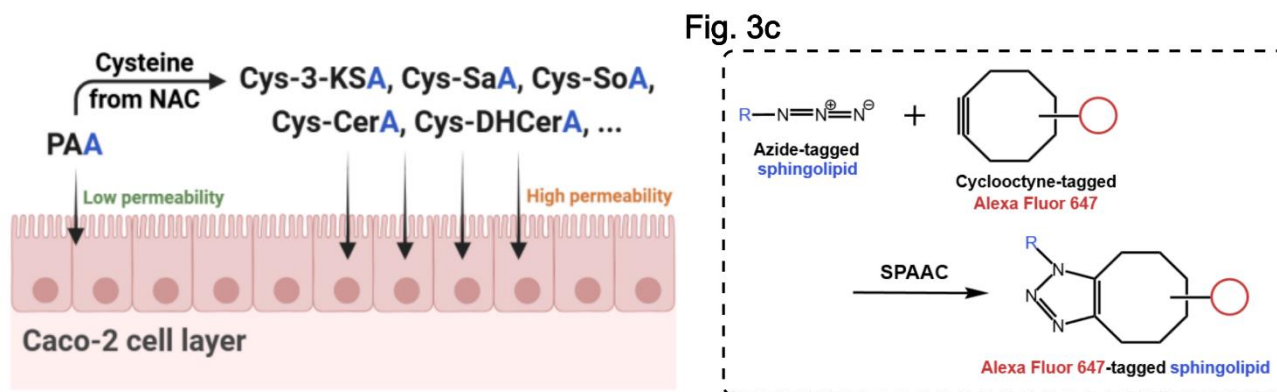


Fig. 3 (c) Analysis of sphingolipid penetrability in small intestine epithelial cells. The left panel illustrates the interaction of azide-tagged sphingolipids with cyclooctyne-tagged Alexa Fluor 647. The reaction of azide-tagged sphingolipids with cyclooctyne-tagged Alexa Fluor 647 by strain-promoted azide-alkyne cycloaddition (SPAAC) principle, which facilitates the visualization and quantification of sphingolipid uptake and transport across the intestinal epithelium.

d. Have you compared the lipidomes and the metabolomes between the knockout and wide type? Did the knockout changes the production of other metabolites? If so, the impact of these changes on the permeation of NAC needs to be excluded.

Response: We thank the reviewer for the insightful question regarding the comparison of lipidomes and metabolomes between the knockout and wild-type strains, and the potential impact of these changes on the permeation of NAC.

Lipidome and Metabolome Comparison: To address the concern raised, we have conducted a comprehensive metabolomic analysis to compare the wild-type and serine palmitoyltransferase (SPT)-knockout *Bacteroides* strains. This analysis was crucial to ensure that the observed changes in NAC permeability were not confounded by alterations of other metabolites. Our results indicate that depletion of SPT did not significantly alter the synthesis of other metabolites, but prominently affected the content of sphingolipids (Fig. S3b). This finding is consistent with the high selectivity of SPT for acyl coenzyme A (acyl-CoA) substrates, as reported in the literature [PMID: 33558761], where it is stated that "ssSPTs (small subunits of SPT) not only greatly stimulate the enzymatic activity of SPT, but also regulate its acyl-CoA substrate specificity. SPT complex containing ssSPTa is highly selective for palmitoyl-CoA..."

Exclusion of Impact from Other Metabolites: To further exclude the impact of other metabolites affected by SPT knockout, we have added the metabolomics data between wild-type and SPT-depleting *Bacteroides* in the revised manuscript. The data showed that SPT knockdown primarily influenced the metabolism of acyl-CoA classes and did not significantly affect other metabolites, thus ensuring that the observed effects on NAC permeability can be attributed to changes in sphingolipid synthesis.

Revisions and Additions: The following sentences and figures have been added to the manuscript to address the reviewer's concern:

"Excluding the intervention of SPT knockout on other metabolomes in *Bacteroides* (Supplementary Fig. 3b), we co-cultured differentiated Caco-2 cells with wild-type *Bacteroides thetaiotaomicron* (BTWT), *Bacteroides fragilis* (BFWT), *Bacteroides thetaiotaomicron* SPT-knockout BTΔSPT or *Bacteroides fragilis* SPT-knockout BFΔSPT cells into the three-well transfer system." (Lines 196-199)

Fig. S3b

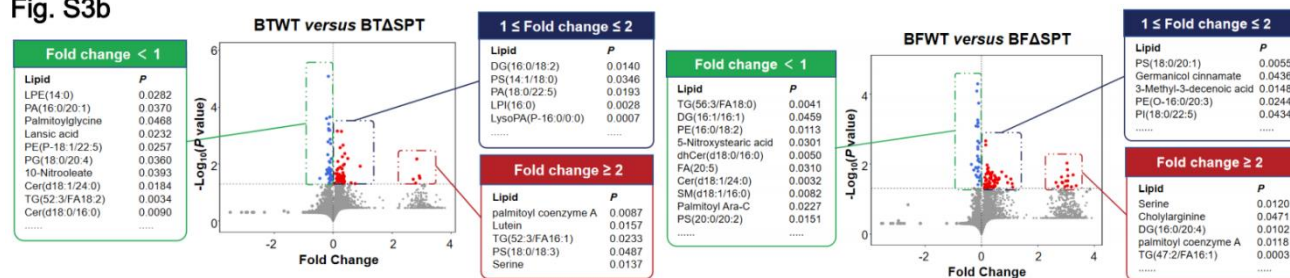


Fig. S3 (b) Volcano plots depicting the metabolomic changes following SPT knockdown in *Bacteroides thetaiotaomicron* (left panel) and *Bacteroides fragilis* (right panel). Metabolites exhibiting significant alterations ($P < 0.05$, Student's t -tests) are highlighted, with those having a fold change ≥ 2 depicted in red, sphingolipids with $1 \leq$ fold change < 2 in blue, and sphingolipids with fold change < 1 in green. These plots effectively visualize the differential metabolite profiling, facilitating the identification of key metabolic shifts associated with SPT knockdown, particularly in the context of sphingolipid metabolism.

e. Please include how the monocolonization of Bacteroides was performed and confirmed.

Response: We appreciate the reviewer's request for clarification on the monocolonization process and confirmation of *Bacteroides* in our study. Here, we provide a detailed account of how the monocolonization was performed and confirmed.

Monocolonization Process: The monocolonization of germ-free (GF) rats with specific strains of *Bacteroides* was conducted under strict sterile conditions. The bacterial culture medium was centrifuged for 10 minutes at 8,000 g and 4°C, and the pellets were resuspended in oxygen-free PBS to obtain bacteria for oral administration. This step ensures that the bacteria are properly prepared for colonization without exposure to oxygen, which could affect their viability.

Confirmation of Monocolonization: To verify the monocolonization, we isolated and identified the bacterial composition of the gut immediately after sacrificing the rats. We confirmed the exclusive presence of *Bacteroides thetaiotaomicron* or *Bacteroides fragilis*, with no other bacterial interventions (Fig. S3h). This step is crucial to ensure that the colonization is specific to the intended microbial strain and no other microorganisms to confound the results.

Additional Methodological Details: We have added the following details into the "Microbial Strains and Gene-Editing Strategies" section of *Materials & Methods*:

"The bacterial culture medium was centrifuged for 10 min at 8,000 g and 4°C, and the pellets were resuspended with oxygen-free PBS to obtain bacteria for oral administration. Colonization fitness of the four strains in rats was analyzed by 2% agarose gel electrophoresis and 3500 ultraviolet (UV) images (Supplementary Fig. 3h)." (Lines 575-579)

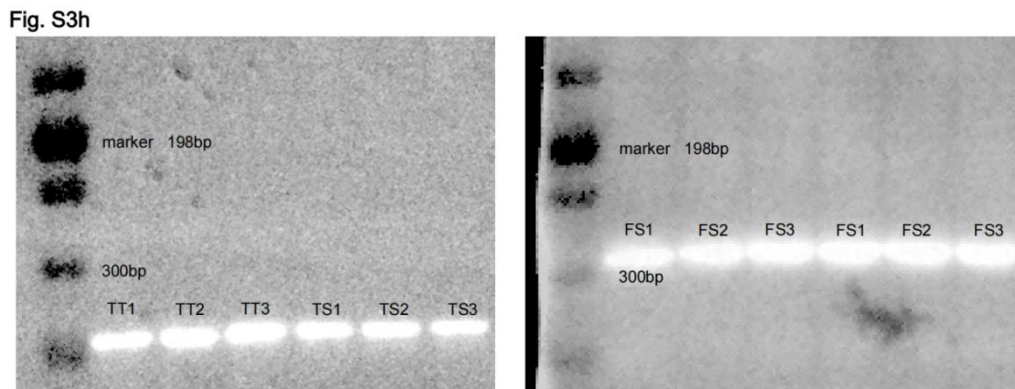


Fig. S3 (h) Assessment of colonization fitness of *Bacteroides thetaiotaomicron* wild-type (BTWT), *Bacteroides thetaiotaomicron* SPT-knockout (BTΔSPT), *Bacteroides fragilis* wild-type (BFWT), and *Bacteroides fragilis* SPT-knockout (BFΔSPT) in rats.

6. This research illustrated how sphingolipid synthesis of the gut microbiota could affect the permeation of NAC in the gut and predictions were made employing various models for clinical guidance. However, the experimental results were collected using rat models and there are compositional differences between rat and human gut microbiome. For example, the human gut commensal bacteria, *Prevotella copri*, was also found to produce sphingolipids. It is one of most prevalent and dominant species of human gut microbiome however missing in the rat microbiomes. How could the lack of consideration of these species affect your conclusion and the accuracy of prediction? Please refer to this paper and talk about it in the discussion: <https://doi.org/10.1128/mbio.02409-23>

Response: We thank the reviewer for highlighting the important consideration of species differences between the rat and human gut microbiota, and for the recommendation to the reference of sphingolipids synthesis in *Prevotella copri*. This is a critical point that needs to be addressed to ensure the translatability of our findings from rat models to human clinical settings.

Addressing Species Differences: We acknowledge that the rat models used in our study may not fully represent the complexity of the human gut microbiome, particularly with respect to species like *Prevotella copri*, which is prevalent in humans but absent in rats. To bridge this gap, we have considered the approach of human fecal microbiota transplantation (FMT) in germ-free rats. However, as noted, the FMT model presents variability based on individual human donors, which underscores the need for further in-depth investigation. This is indeed a limitation of our current study.

Incorporating the Reference: We have now included this reference to the paper provided by the reviewer, which discusses the sphingolipid metabolism of *Prevotella copri*, in our revised discussion. This reference will help contextualize our findings within the broader literature on gut microbiota and its impact on drug metabolism.

Revisions to the Discussion: The following sentences have been added to the discussion section to address the reviewer's comments:

"While our study provides valuable insights into the role of gut microbiota in NAC permeability using rat models, it is important to recognize the compositional differences between rat and human gut microbiomes. Notably, *Prevotella copri*, a prominent human gut commensal bacterium known to produce sphingolipids, is absent in rat microbiomes³³. The inclusion of such species in future studies could offer additional insights into the mechanisms of NAC permeability in humans. Our screening model, therefore, serves as a foundation that can be further refined through systematic research involving fecal microbiota transplantation (FMT) from diverse human donors, which would help to more accurately model the human gut environment and refine our predictions for clinical applications." (Lines 375-385)

Minor comments:

1. For the method related to Fig 1A, please provide more information on how this network was calculated and constructed. In the legend, you mentioned that this network was validated by a follow-up assay. What assay was it and how was the result?

Response: We appreciate the reviewer's interest in the methodology behind Fig. 1a and the follow-up assay used to validate the network. In response to the request for more details, we have expanded the description of the assay and its results in the revised manuscript as follows:

Network Calculation and Construction: "The network was constructed by correlating the abundance of symbiotic bacteria (or microbial metabolites) in the intestine with the effective permeability (P_{eff}) during the drug perfusion steady state. For each bacterium-drug interaction, triplicates were analyzed, along with single bacteria-free controls for each drug. Subsequently, Single-Pass Intestinal Perfusion (SPIP) experiments were conducted for each drug in bacterially colonized germ-free (GF) rats (see below for colonization method). Quantitative PCR (qPCR) was used to validate bacterial abundances, and liquid chromatography-mass spectrometry (LC-MS) was employed to quantify metabolites, thereby verifying the initial screening results. Statistical significance was assessed using Wilcoxon's rank sum test with a P -value cut-off of 0.05 after false discovery rate (FDR) correction." (Lines 462-471)

Results of the Follow-Up Assay: "In our screening process, we systematically profiled *in vitro* interactions between 29 oral drugs and 18 representative microbial strains. The resulting 60 potential bacteria-drug pairs were subjected to two independent screenings (Fig. 1b). A false discovery rate (FDR)-corrected *P* value cut-off of 0.05 and a threshold of 30% depletion were defined as indicative of a potential interaction. These targeted interactions were further investigated in bacterially colonized GF rats, revealing an *in vivo* network that spanned all tested strains and 91% of the tested drugs (29 of 32). Notably, multiple drugs, including NAC, showed typical interactions with *Bacteroides* species." (Lines 110-119)

2. Line 142: *for the comparison between any perfused and unperfused groups, please explain the experimental procedures in the method. For example, how the samples were collected, what time point for the changes in microbiome composition, how the sequencing library was prepared, etc.*

Response: We appreciate the reviewer's request for additional details on the experimental procedures for the comparison between perfused and unperfused groups. We have expanded the Methods section to include the following detailed procedures:

Sample Collection: "Upon sacrifice, the perfused and unperfused intestinal segments were removed to assess microbiomic changes. Each intestinal segment was perfused with 37°C saline at a rate of 0.2 mL/min for 10 minutes. The intestinal fluids collected from these segments were then used for subsequent microbiological analysis (refer to 'Metabolome Analysis of Intestinal Microbiota' and 'Taxonomic Profiles of Intestinal Microbiota' sections)." (Lines 455-460)

Time Point for Microbiome Composition Changes: "We uniformly sampled intestinal fluid at the endpoint of perfusion, which was 120 minutes, to capture the changes in microbiome composition." (Lines 460-462)

Sequencing Library Preparation: "For the generation of paired-end clusters, the V4 kit was utilized across 8 lanes on an Illumina cBot and subsequently applied for sequencing on an Illumina HiSeq 2500 platform. The median cluster density was determined to be 908.5 for Nextera XT. The open-source software package DADA2 (version 1.10) was employed for amplicon data processing, enabling single-nucleotide resolution of amplicons. The forward and reverse reads were truncated to 200 and 150 bases in length, respectively. Read pairs were excluded if they contained ambiguous bases with expected errors higher than two or originated from PhiX spike-in controls. One million reads from each sequencing run were used to infer error profiles. Subsequent steps included replication, error correction, and merging of forward and reverse reads." (Lines 521-531)

3. Line 733: *in SPF rats not mice*

Response: Thank you for pointing out the species discrepancy in the manuscript. We have made the necessary correction to ensure accuracy in our description of the animal models used in the study.

Revision Made: The phrase "in SPF mice" has been revised to "in SPF rats" to correctly reflect the animal model used in the experiments. This change has been implemented in the manuscript to maintain consistency and precision in our reporting.

4. Please add statistical analysis results on the plots, including but not limited to: Fig 2j, Fig 3a

Response: We appreciate the reviewer's guidance on enhancing the presentation of our results. In compliance with the standards of transparency and clarity set by Nature communication, we have carefully reviewed all statistical analyses and have included the results directly on the plots as well as in the figure captions for Figs. 2j and 3a.

Statistical Analysis Results Added: For Fig. 2j, we have added the results of the one-way ANOVA test, indicating the level of significance with $*P < 0.05$, $**P < 0.01$, to the figure caption. For Fig. 3a, we have included the results of the statistical tests used, with details on the significance levels, directly in the figure caption.

These additions ensure that readers can quickly assess the statistical significance of the data presented in the figures, aligning with the best practices for scientific communication. We hope that these revisions meet the expectations for detailed statistical reporting as per the journal's requirements.

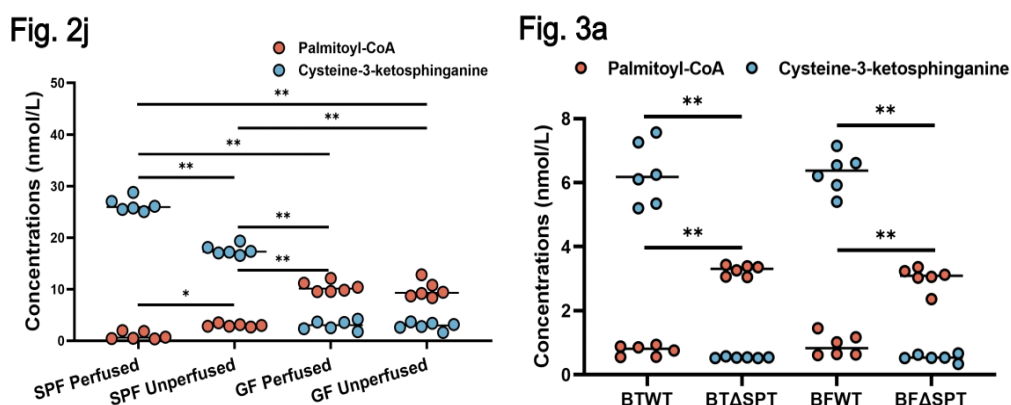


Fig. 2 (j) Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was utilized to identify and quantify palmitoyl coenzyme A (palmitoyl-CoA, left panel) and cysteine-3-ketosphinganine (middle panel). Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was assessed using one-way ANOVA, with $*P < 0.05$, $**P < 0.01$ indicating significant differences.

Fig. 3 (a) LC-MS/MS to quantify palmitoyl-CoA and cysteine-3-ketosphinganine in the separated BTWT/BFWT or BTΔSPT/BFΔSPT treated by 25 μ M palmitoyl-CoA and 10 μ M NAC. Data are the means $\pm$ SD. One-way ANOVA: $*P < 0.05$, $**P < 0.01$.

5. Line 162: how was the bacteria network constructed.

Response: Thank you for your inquiry regarding the construction of the bacterial network. We have expanded upon the methodology in the Materials & Methods section to provide a clear description of how the network was constructed, as follows: "Amplicon sequence variants (ASVs) with an average relative abundance greater than 0.1% within the microbiome were selected for correlation analysis of their occurrence patterns. The SparCC algorithm was employed to estimate the correlations between intestinal microbes. Pseudo *P*-values were calculated using 1,000 bootstrap replicates. Correlations with an absolute value of the correlation coefficient (*r*) greater than 0.2 and a *P*-value less than 0.01 were considered statistically significant. For each genus with a significant SparCC correlation, its degree was calculated as an indicator of its weight within the network by summing up its edges." (Lines 535-543)

6. Line 163: please explain how you got this conclusion from Fig S1e

Response: We appreciate the reviewer's question regarding the conclusion drawn from Supplementary Fig. 1e. To clarify, the microbial co-occurrence network depicted in the figure represents the correlations between different bacterial species. The size and thickness of the lines connecting the dots (representing bacterial species) indicate the strength and number of correlations among bacteria. A larger and thicker network of correlations suggests a more stable bacterial community structure. Herein, the strong correlations observed between high-abundance species such as *Bacteroides thetaiotaomicron* and *Bacteroides fragilis* with other bacteria underscore their pivotal roles in maintaining the stability of the microbiota. To enhance understanding, we have revised the description of Fig. S1e as follows:

"The microbial co-occurrence network illustrating all intestinal microbiota correlated with *Bacteroides* in the NAC-perfused group is presented in Supplementary Fig. 1e. This network suggests that commensal intestinal bacteria, such as *Bacteroides thetaiotaomicron* and *Bacteroides fragilis*, play significant roles in stabilizing the composition of intestinal microbiota under the influence of NAC perfusion." (Lines 161-165)

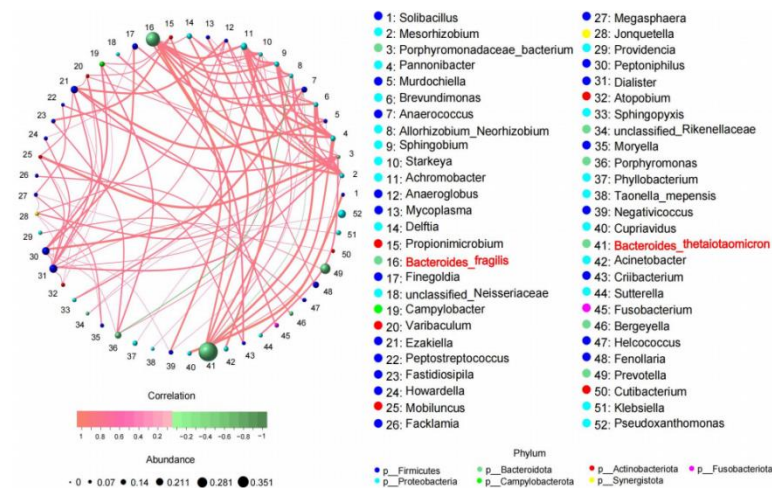


Fig. S1 (e) Analysis of the intestinal microbial co-occurrence network focused on core microbial species with an average relative abundance greater than 0.1%. The size of the nodes represents the abundance of the respective bacteria. The color of the lines connecting the nodes indicates the nature of the correlation—pink for positive and green for negative—and the thickness of the lines corresponds to the strength of the correlation. Spearman’s rank correlation coefficient was employed for the statistical analysis, providing a measure of the relationships between microbial species within the network.

7. *Supplementary Fig 2 was mentioned in the manuscript before supplementary Fig 1f-g, please reorder the figures.*

Response: We appreciate the reviewer's attention to the organization of the supplementary figures in the manuscript. To ensure a logical sequence and to avoid any confusion, we have revised the order of the supplementary figures as follows:

Revision Made: The figures have been renumbered to maintain a consistent order with their mention in the manuscript. The previous Supplementary Figs. 1-2 are now renumbered as Supplementary Figs. 1-3, respectively. We have also updated the references to these figures within the text to match the new numbering, ensuring that the figures are referenced in order. This change enhances the clarity and readability of the manuscript, aligning with the high standards of presentation expected by Nature journal.

8. *The rats were anesthetized for up to 2 hours, will the anesthesia affect the absorption of NAC?*

Response: We appreciate the reviewer's concern regarding the potential impact of anesthesia on the absorption of N-Acetylcysteine (NAC) in the rat model. To address this question, we have conducted additional experiments to investigate the effects of prolonged anesthesia on intestinal absorption.

Extended Perfusion Time: In our revised study, we extended the perfusion time to 180 minutes to observe any potential effects of prolonged anesthesia on the intestinal absorption capacity of

the rats. Our findings indicate that the extended anesthesia did not significantly influence the NAC absorption, as the intestinal absorption reached a steady state within 120 minutes and remained constant thereafter. For example, Fig. 4b below illustrates *in situ* effective permeability (P_{eff}) values of NAC across different groups, which remained stable after reaching the steady state at 120 minutes. This suggests that the anesthesia used in our study does not significantly affect the absorption of NAC, at least within the time-frame of our experiments. This finding is crucial for ensuring the validity of our results and their translational potential.

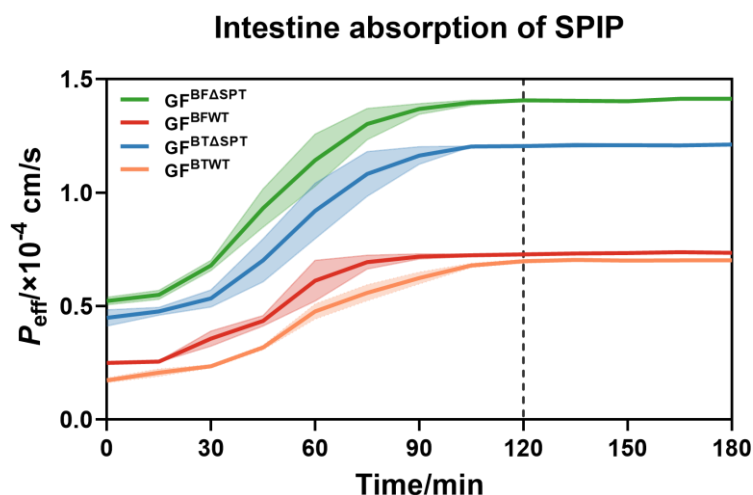


Fig. 4 (b) Monitoring of intestinal P_{eff} -values over a 3-hour period for each group, with measurements taken at 15-minute intervals.

9. Fig 2d: please order the color key in accordance with the order in the bar plot.

Response: Thank you for your attention to the details in Fig. 2d. We have made the necessary adjustments to ensure that the color key is ordered consistently with the bars in the plot.

Revision Made: The color key in Fig. 2d has been reordered to match the sequence of the bar plot. We appreciate the reviewer's guidance in improving the presentation of our data.

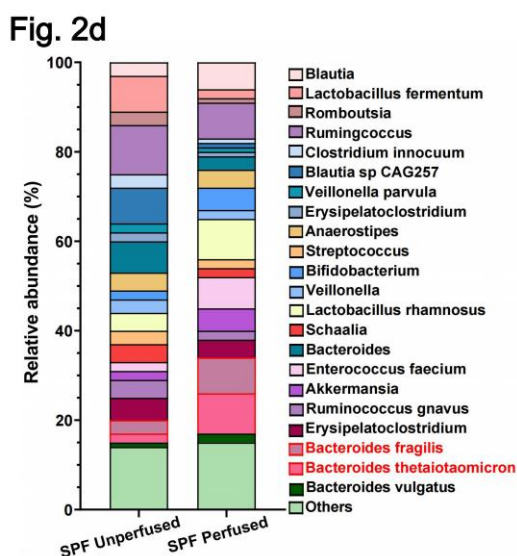


Fig. 2 (d) Summary of genus and species taxonomic changes in the gut microbiome of specific pathogen-free (SPF) rats following perfusion with 30 mg/L N-Acetylcysteine (NAC).

10. Line 201: how was the immunofluorescent intensity being quantified?

Response: We appreciate the reviewer's inquiry regarding the quantification of immunofluorescent intensity. To address this, we have detailed the quantification method in the Materials & Methods section as follows:

Quantification Method for Immunofluorescence Intensity: "The PM2000/AQUA immunofluorescent platform was utilized to capture images of Caco-2 cells within the middle well of each core tissue microarray. The slide was automatically scanned using the PM2000 hardware (HistoRx/Genoptix), and fluorescent images were captured at ×20 magnification in two channels: DAPI (for cell nuclei) and Alexa Fluor 647 (for azide-tagged sphingolipids) at predefined locations. The ScanScopeFL/TissueStudio fluorescence immunohistochemistry platform was employed to digitally capture images of each channel (DAPI, Alexa Fluor 647) across the entire slide at ×20 magnification using the ScanScopeFL (Aperio/Leica Biosystems). The two-color immunofluorescence images were then separated into single-channel fluorescence images to allow for the quantification of each fluorescent channel individually. The immunofluorescence intensity of Caco-2 cells was quantified using the following formula:

$$\text{Mean Gray Value} = \text{Integrated Density/Area}$$

To account for noise interference, the relative fluorescence intensity was calculated, with the initial immunofluorescent intensity set as the reference value of 1." (Lines 606-620)

We believe that these revisions provide a clear and detailed explanation of the immunofluorescence intensity quantification process, aligning with the rigorous standards of *Nature communications*.

11. Fig 3f: why the peaks of cysteine-3-KS have different retention times in different groups?

Response: We thank the reviewer for their observation regarding the variation in retention times of cysteine-3-ketosphinganine (cysteine-3-KS) peaks across different groups in Fig. 3f. We have provided an explanation for this phenomenon as follows:

Explanation of Retention Time Variation: In Fig. 3f, the observed retention times for azide-tagged cysteine-3-ketosphinganine across the various groups varied from 16.63 to 16.81 minutes. It is important to note that retention time deviations of less than 20 seconds are commonly attributed to the mechanical tolerance of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system used in our analyses. This minor variability is within the acceptable range for LC-MS/MS and does not significantly impact the identification or quantification of the analytes.

We hope that this explanation clarifies the reason for the observed differences in retention times and satisfies the reviewer's query.

12. Line 228: to avoid confusion, should be changed to "increased alkyne-labeled SLs by copper-catalyzed cyclo-addition in rats' intestines colonized with WT strains".

Response: We appreciate the reviewer's suggestion to clarify the sentence at line 228 to avoid any potential confusion. We have revised the sentence to more accurately describe the process, as follows:

Revision Made: "We then determined the Alexa Fluor-647 azide (AF647-Az) intensity resulting from the increased alkyne-labeled SLs through copper-catalyzed cyclo-addition in the intestines of rats colonized with wild-type strains." (Lines 229-231)

13. Fig 4c: please explain how the intestine samples were prepared for imaging. Were the bacteria removed?

Response: We appreciate the reviewer's question regarding the preparation of intestinal samples for imaging and whether bacteria were removed. To address this, we have provided a detailed description of the sample preparation process in the revised manuscript:

Preparation of Intestinal Samples for Imaging: "Following the *in vivo* click-chemistry experiments, rats were euthanized, and both perfused and unperfused intestinal segments were directly embedded in O.C.T compound (Sakura Finetek, Tokyo, Japan) and placed on ice. After one hour of stabilization on ice, the samples embedded in O.C.T compound were rapidly frozen in 2-methylbutane chilled with liquid nitrogen. These frozen blocks were then wrapped in labeled foil and stored at -80°C until further use." (Lines 477-482)

It is important to note that the bacteria were not removed from the intestinal samples for imaging. This approach was intentional, as the *in vivo* click-chemistry experiments were designed to validate the impact of SPT knockdown on the synthesis of downstream cysteine-sphingolipids (SLs) metabolites within the context of intact microbiome.

14. Line 522: should be apical chamber not the basal side.

Response: We appreciate the reviewer's correction regarding the terminology used at line 522. To ensure accuracy and consistency with standard scientific nomenclature, we have made the necessary revision:

Revision Made: The term "basal side" has been updated to "apical chamber" to correctly describe the compartment in the experimental setup.

15. For the illustration of the system in Fig 3b, the liquid level in the inner well is higher than the middle well. Is this a mistake or maintained? The colors of the medium in the middle and lower chamber should be the same to avoid confusion. Please keep the naming of the wells/chambers consistent across the manuscript.

Response: We apologize for the oversight in the illustration of the system in Fig. 3b. We have taken the reviewer's comments into account and made the following corrections:

Revisions Made:

- The liquid levels in the upper and middle wells have been adjusted to be at the same level to accurately represent the experimental setup.
- The colors of the medium in the middle and lower wells have been made consistent to avoid any confusion.
- The terminology for "wells/chambers" has been standardized to "wells" throughout the manuscript to maintain consistency.

Figure Revision: Fig. 3b has been revised accordingly to reflect these changes, ensuring that the illustration accurately represents the experimental system and avoids any misinterpretation.

Fig. 3b

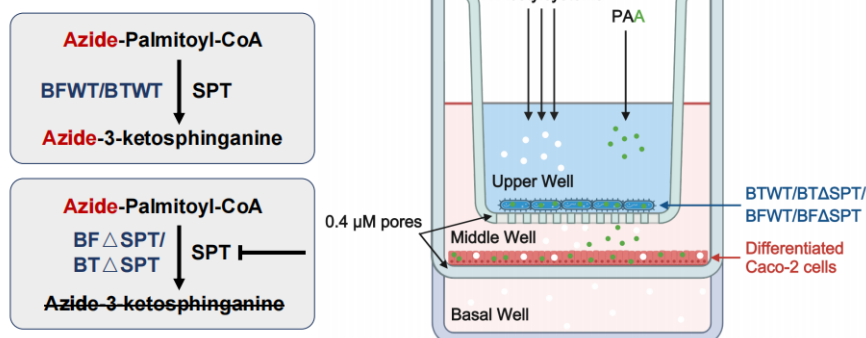


Fig. 3 (b) Schematic representation of the three-well co-culture system. N-Acetylcysteine (NAC) is transferred through BTWT/BFWT or BTΔSPT/BFΔSPT cells in the upper well to differentiated Caco-2 cells in the middle well, followed by infiltration into the basal well for concentration measurement.

16. Line 420: please clarify the definition and determination of steady state.

Response: We appreciate the reviewer's request for clarification on the definition and determination of the steady state in our study. To address this, we have provided a detailed explanation in the revised manuscript:

Definition and Determination of Steady State: "To ascertain the steady state during the perfusion process, perfusate samples were collected from the distal portion of the jejunum at 15-minute intervals (at 15, 30, 45, 60, 75, 90, 105, and 120 minutes). These samples were collected in pre-weighted vials to monitor **when the effective permeability (P_{eff}) values at each point of**

the perfused segment remained constant, indicating that the system had reached a steady state. The samples were immediately frozen at -20°C until subsequent analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS)." (Lines 443-449)

Reviewer #2: Expertise: Microbiome / Intestinal permeability / Drugs.

This is an innovative paper, assessing a very important topic: variable permeability of oral drugs through Intestinal microbiota composition analysis and model production. Overall quality of the data is good, technically sound, obtained with appropriate techniques, analyzed and interpreted carefully and presented in detail. Sufficiently strong evidence is provided for the authors' claims, and appropriate controls have been included. These results are important to the field and will advance understanding. However, the paper in the present formulation has several limitations that reduce the comprehension and the clarity of the message.

Response: We are grateful for the reviewer's positive evaluation of our manuscript and for recognizing the innovation and importance of our work on the variable permeability of oral drugs through intestinal microbiota and model production. We appreciate the acknowledgment of the technical soundness of our data and the careful analysis provided in our study.

We also take to heart the reviewer's note about the limitations affecting the comprehension and clarity of our message. In response to this feedback, we have taken steps to address these issues in our revised manuscript:

Clarity of Message: We have revised the manuscript to ensure that our key findings and their implications are clearly articulated, with a focus on making our discussion more accessible to readers.

Comprehension: We have worked to simplify complex sections and added clarifying figures and tables where necessary to enhance understanding.

Limitations Addressed: We have explicitly addressed potential limitations in the discussion, providing a balanced view of our study's scope and the context of our findings.

1. It is very important to better specify, perhaps with a dedicated paragraph, the 13-feature light gradient boosting machine (LGBM) model and how it has been generated (it seems it is generated starting from mice data).

Response: We appreciate the reviewer's suggestion to provide a clearer description of the 13-feature light gradient boosting machine (LGBM) model and its generation process. In this version, we have re-segmented the section of *multi-parameter training models for clinically predicting N-Acetylcysteine (NAC) permeability*, dividing the 13-feature LGBM model generation into a dedicated paragraph. Inspired by previous screening and verified results of rats' experiments, the first paragraph of clinical data aimed to describe the process of encompassing descriptors for nine machine learning (ML) models. Then the second separated paragraph revealed how LGBM algorithm was selected and trained from the 17-feature model into the final 13-feature model through recursive feature elimination.

In response, we have expanded our manuscript to include a dedicated section that details the development of the LGBM model and we have described the section of LGBM construction in ***ML model development and evaluation*** of **Materials & Methods** section as follows:

Process of encompassing descriptors for ML models: "For developing the machine learning (ML) model of incorporating intestinal microbial features, we initially included intestinal common SLs characteristics into the risk-based model (Supplementary Fig. 6). Discovery Cohort ($n = 192$ participants; Fig. 5a) was established with input features to describe NAC systemic bioavailability (F_{sys}), including available age, prescription, NAC dose, intestinal blood flow, intestinal content viscosity, intestinal pH, intestinal transit rate, intestinal ceramide metabolome and microbiota, followed by the validation of an independent Validation Cohort ($n = 48$ participants; Fig. 5a). The feature values of the participants in Validation Cohort were divided into $F_{sys} \geq 85\%$ (Fig. 5b, left panel) and $F_{sys} < 85\%$ (Fig. 5b, right panel)." (Lines 253-262)

LGBM algorithm selection and training: "Given that utilizing too many, potentially irrelevant features might increase the risk of spurious correlations between individual features and observed outcomes, thus degrading the generality of model, the features redundancy of the LGBM model was then evaluated by agglomerative hierarchical clustering (Fig. 5e). Following the removal of least important features by recursive feature elimination, the optimal version of the 13-feature LGBM model has reached the lowest MAE by excluding prescription, NAC dose, intestinal transit rate and age (Table 1). For the categorical variable *prescription*, we have simultaneously evaluated it as an exclusion feature based on the SHAP visualization (Supplementary Fig. 7c-e). Despite the strong correlation between cysteine-sphingolipid metabolites, removing any of these features leads to a reduction in model accuracy (Supplementary Table 4)." (Lines 284-295)

2. It is not clear the role of patients: in the methods 240 participants, while in results not clear to which categories of patients are referred. in some results you refer to $n=192$, in others 48 or in other 79. Consider to do a diagram if this court served for differential sub-aims.

Response: We thank the reviewer for highlighting the need for clarity regarding the participant cohorts in our study. To address this, we have revised the manuscript to include a clear diagram that outlines the distribution and categorization of the participants.

Revision Made: We have updated Fig. 5a to include a comprehensive diagram that details the participant cohort information. This diagram clarifies the breakdown of the 319 participants included in the study. Specifically, it shows that out of the total participants, 240 were collected for machine learning (ML) model building and screening. Within this group, feature information from 192 participants was utilized for model construction, while the remaining 48 participants' data were used for model validation. The diagram also indicates that the last 79 participants were used to compare the performance of the 13-feature light gradient boosting machine (LGBM) model

developed in this study with three other commercially available models, independently of the ML model construction process and the comparative analysis between the models.

Explanation in the Results Section: To further clarify, we have added the following explanation in the Figure legends:

Fig. 5a

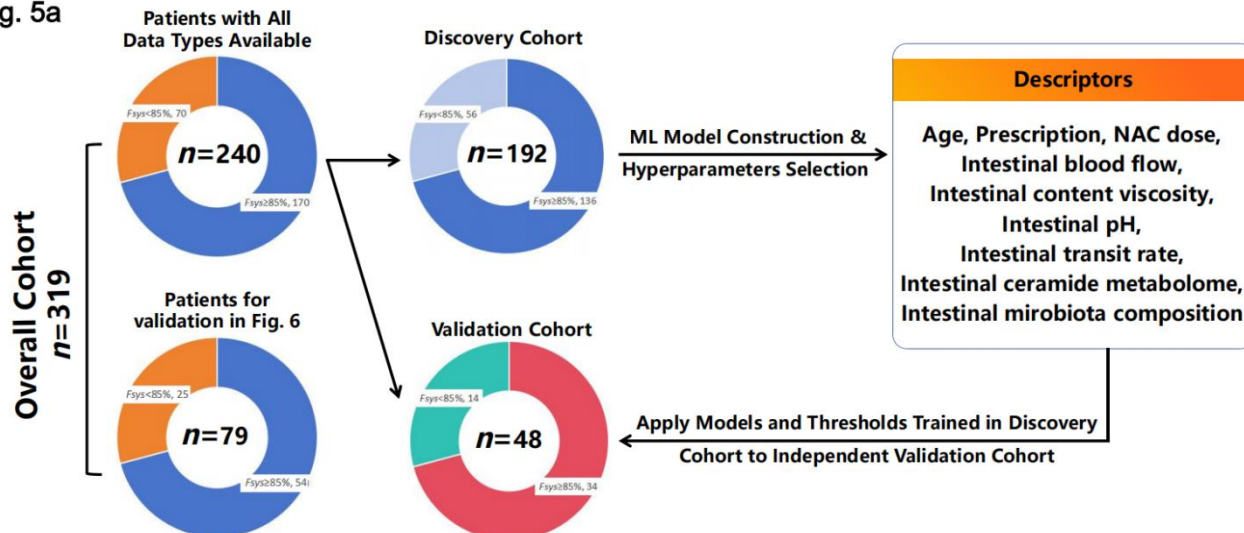


Fig. 5 (a) A total of 319 participants were enrolled. For the development and screening of the ML models, 240 participants were selected, with 192 contributing to the model building phase and 48 to the model selection phase. An additional 79 participants were independently used to assess the performance of the 13-feature LGBM model against three other commercial models, providing a robust evaluation of predictive capabilities.

3. Results refer to duodenal/jejunal permeability and not intestinal permeability too court: please specify and better clarify into the discussion session.

Response: We thank the reviewer for prompting us to clarify the distinction between duodenal and jejunal permeability within our study. We have taken this feedback into account and provided the following clarification in the discussion section:

Clarification on Duodenal/Jejunal Permeability: In our study, we specifically examined the permeability of N-Acetylcysteine (NAC) in the duodenum and jejunum, rather than intestinal permeability as a whole. Preliminary experiments in rats indicated no significant difference in *in-situ* effective permeability (P_{eff}) values between these two segments, which informed our subsequent approach. However, in the clinical sample collection, the oral introduction of the catheter made it challenging to accurately distinguish between the duodenum and jejunum due to the invisibility of the catheter's distal end. This limitation led us to describe the collected intestinal fluid as originating from an unspecified segment of the small intestine, acknowledging the inability to definitively attribute it to either the duodenum or jejunum.

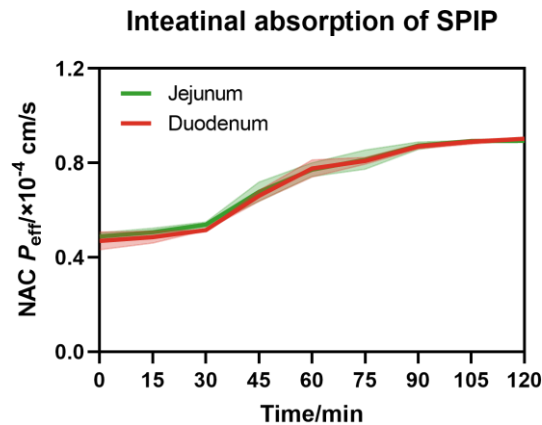


Figure of preliminary experiments. Monitoring of intestinal P_{eff} -values for N-Acetylcysteine (NAC) in the jejunum and duodenum over a 2-hour period, with measurements taken at 15-minute intervals.

Revisions in the Discussion: "In this study, we conducted a prospective cohort study involving 240 participants, integrating intestinal microbial features into the input feature profile to derive the most interpretable light gradient boosting machine (LGBM) model for predicting NAC systemic bioavailability (F_{sys}) using machine learning approaches. Given the challenges in precisely identifying the origin of the collected intestinal fluid from either the duodenum or jejunum due to the oral catheterization, we characterized the fluid based on limited intestinal features, which is a recognized limitation of our study." (Lines 406-411)

4. For what I can understand microbiota profile influencing permeability of oral drugs is referring to mucosa composition of microbiota and not to fecal of other microbiota. Do you also have data correlating with other microbiota? please speculate.

Response: We appreciate the reviewer's insightful comment regarding the specificity of our microbiota profile analysis in relation to the permeability of oral drugs. To address this, we have provided further clarification in the revised manuscript:

Clarification on Microbiota Profile Analysis: Our study focused on the mucosal composition of the microbiota rather than fecal microbiota when examining its influence on the permeability of oral drugs, as rats' intestinal fluid was directly collected from the perfused intestinal segments and human intestinal fluid was obtained by orally introducing a catheter, with the distal end positioned 90 cm below the duodenum site. Both methods have ensured that the collected samples were not influenced by fecal microbiota, as they did not pass through the colon. This attributes that N-Acetylcysteine (NAC) is known to be almost completely absorbed in the small intestine, with minimal absorption occurring in the colon (PMID: 25553484). Previous animal and clinical studies have also indicated that fecal microbiota does not significantly affect NAC permeability (PMID: 37186118). Therefore, our study concentrated on extracting the microbiome from the small intestine, which is the primary site of NAC absorption.

Minor comments

1. Specify why and how you selected the 32 oral drugs.

Response: We appreciate the reviewer's question regarding the selection of the 32 oral drugs used in our study. The selection was based on the drugs' controversial Biopharmaceutics Classification System (BCS) classification, which relates to their intestinal permeability. Specifically, we focused on drugs with BCS class I/III or II/IV classifications, which represent a spectrum of permeability characteristics that are subject to debate or variation.

To identify these drugs, we utilized online resources known for their comprehensive listings of BCS classifications. The drugs were selected from the following URLs, which provide extensive databases of drugs categorized under the BCS system:

<https://www.pharmaspecialists.com/p/available-bcs-classification-of-drugs.html>,

and <https://www.pharmaspecialists.com/p/available-bcs-classification-of-drugs-2.html>.

2. Describe better how and where you do expect this new proposed model can be utilized: I do not believe, at this stage, in clinical practice, perhaps for drug design or to develop further drug/class of drug models? please discuss and speculate.

Response: We thank the reviewer for the question regarding potential applications of our proposed 13-feature light gradient boosting machine (LGBM) model. While it is true that the model is not yet ready for direct clinical application, it serves as an important step forward in understanding the role of gut microbiota in drug absorption. Here, we discuss and speculate on the possible uses of this model:

Potential Applications of the LGBM Model: Our 13-feature LGBM model, which incorporates gut microbial composition and metabolites as independent variables, is not intended for immediate clinical practice but rather to highlight the significance of gut microbiota in the absorption of oral drugs. The model's superior predictive performance suggests that gut microbes play a crucial role in drug absorption, a factor that has been underappreciated in traditional Biopharmaceutics Classification System (BCS) assessments. We anticipate that this model could prompt a reevaluation of BCS systems to include the impact of intestinal microbiota on drug permeability. Furthermore, we envision that this model could be a valuable tool in drug design, where understanding how gut microbiota interact with potential drugs could inform the development of more effective medications. It may also serve as a foundation for developing further class-specific models that can predict drug behavior in the presence of different microbial compositions.

Revisions in the Discussion: "The enhanced predictive accuracy of the LGBM model, which includes characteristics of the gut flora, underscores the significant role of gut microbes in drug

absorption and the potential for refining BCS assessment systems. Given the multitude of factors that influence drug absorption, such as diet, drug interactions, and physiological conditions including digestive juice composition, gastrointestinal blood flow, and disease states⁴², there is a clear need for more sophisticated models. Machine learning offers a promising avenue for developing such comprehensive models in pharmaceutical research." (Lines 420-427)

3. Please specify better the condition where this model is working and potential limitation (absence of disease, "normal transit time", one ad the time drug, standard diet etc.).

Response: We appreciate the reviewer's question regarding the conditions under which our model operates and its potential limitations. We have expanded upon this discussion to provide a clearer understanding of the model's parameters and constraints.

Conditions and Limitations of the Model: Our model operates under several assumptions and conditions that are important to note. Initially, our study was conducted in the absence of disease states, which could significantly alter the gut microbiota composition and, consequently, the absorption of drugs. We also assumed 'normal transit time' for the oral drugs, as variations in gastrointestinal motility can impact drug absorption rates. Additionally, our model was developed with the consideration of a single drug at a time, which may not account for the complex interactions that can occur with multiple drugs or in the context of polypharmacy. Furthermore, our study utilized a standard diet to control for dietary variations that could influence the gut microbiota and drug interactions.

It is important to recognize that these conditions limit the generalizability of our model. More comprehensive and precise models that account for a broader range of factors, such as diet, drug combinations, physiological processes, and internal environment—including the composition of digestive juices, gastrointestinal blood flow, and the presence of diseases—require further exploration in the pharmaceutical field. Machine learning offers a promising approach for developing such models, which can better predict drug behavior under various real-world conditions.

Revisions in the Discussion: "The application of our model is contingent upon several conditions, including the absence of disease, which can significantly alter gut microbiota and drug absorption. We also assumed normal transit times for the oral drugs and a standard diet to control for dietary variations. However, these assumptions limit the model's applicability. Future models should aim to incorporate a wider range of factors that affect drug absorption, such as drug combinations, physiological processes, and internal environment conditions, to enhance their predictive accuracy in diverse clinical scenarios." (Lines 411-419)

We trust that the changes we have made in response to the reviewers' feedback have adequately addressed their concerns and that the manuscript is now suitable for publication in *Nature Communications*.

Thank you once again for your valuable input.

Yours Sincerely

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Dear Editors and Reviewers,

I am very pleased to submit our revised manuscript, titled "Intestinal microbiota-mediated permeability screening reveals the variable N-Acetylcysteine bioavailability by cysteine palmitoylation of *Bacteroides*" (NCOMMS-24-32817B). We also express our great appreciation for giving us critical and insightful comments and suggestions. Taking these feedback to heart, we have meticulously modified our manuscript by expanding the section of Introduction, clarifying the modeling strategy of machine learning, and uploading the original source code as well as its README.md file to address each reviewer's point comprehensively.

The corresponding changes of the main text are highlighted in red for easy identification. We have also updated the following figures to strengthen our findings and arguments:

- [Figures 5a, 5b, 5c, 5d, 5e, 5f, 5g](#)
- [Figures 6a, 6b, 6c, 6d](#)

The following are our point-to-point responses to the comments and concerns raised by the reviewers:

Reviewer #1:

Upon revision, the method section of the manuscript includes more details and improved clarity which facilitate the understanding of research. The authors did a good job of answering previous questions and responding to the suggestions.

Considering that the effects of NAC on the gut microbiota are investigated in their research and the importance of the bi-directional relationships between drugs and microbes as mentioned by the authors in their rebuttal, I suggest adding the corresponding background and significance to the introduction section as well. Other than that, I suggested this manuscript to be accepted by the journal based on the impacts and quality of the study.

Response: We greatly appreciate the reviewer for the positive evaluation of our manuscript and for recognizing the innovation of our work on the variable permeability of oral drugs through intestinal microbiota and model production. Carefully considering the insightful suggestion on emphasizing the importance of the bi-directional relationships between drugs and microbes, we have added the following sentences into the introduction section as follows:

Revision Made: Herein we unearthed insights into the underlying mechanism of intestinal microbiota in NAC permeability by analyzing their host-microbiome interactions, which is essential to understanding the full scope of NAC's effects within the gastrointestinal environment.

By identifying the specific microbial strains that were prominently altered by NAC perfusion, we targeted these strains to elucidate the mechanisms by which NAC influenced gut microbiota composition and function. (Lines 77-82)

Reviewer #3:

I was asked to comment on the machine learning approach of the manuscript. Overall, I think more clarification is needed on the overall modeling strategy. Please see my comments below.

Response: We are grateful for your expertise and for the constructive feedback in the field of machine learning. Carefully considering each point, we have made the following revisions and clarifications to address the concerns of overall modeling strategy. In the context of all insightful comments provided, we have taken steps to address these issues in our revised manuscript:

Clarity of Message: We revised the manuscript to ensure that our key findings and their implications are precisely articulated, with a focus on making our results and methods of machine learning more accessible to readers.

Comprehension: We have worked to simplify complex sections and replace figures where necessary to enhance understanding.

Modeling Strategy: We comprehensively changed the original strategy of "hyperparameter optimization → feature selection → hyperparameter re-optimization" into "feature selection → hyperparameter optimization" as shown below, to facilitate the parallel comparison of multiple algorithms and improve the rationality of modeling strategy.

1. Line 255: What does "SLs" refer to? I was unable to find its definition in the text.

Response: We appreciate the reviewer for this query about the definition of "SLs", which is the abbreviation of sphingolipids (Lines 126-127). In this revision, we have also checked the full form of all abbreviations at first use and updated the abbreviation list as follows:

Revision Made: SPIP, single-pass intestinal perfusion; NAC, N-Acetylcysteine; XGB, eXtreme Gradient Boosting; ML, machine learning; BCS, Biopharmaceutics Classification System; PBGI, physiologically based gastrointestinal; ACAT, advanced compartmental absorption and transit; GITA, gastrointestinal transit and absorption; ADAM, advanced dissolution, absorption and metabolism; *B. thetaiotaomicron*, *Bacteroides thetaiotaomicron*; *B. fragilis*, *Bacteroides fragilis*; SPT, serine palmitoyltransferase; PAA, palmitoyl-CoA azide; SLs, Sphingolipids; cysteine-Sa, cysteine-sphinganine; cysteine-So, cysteine-sphingosine; cysteine-DHCer, cysteine-dihydro ceramides; cysteine-Cer, cysteine-ceramides; cysteine-SM, cysteine-sphingomyelin; *F*_{sys}, fraction of dose entering the systemic circulation; ICH, International Council for Harmonisation

of Technical Requirements for Pharmaceuticals for Human Use; MLR, Multiple Linear Regression; NN, Neural Network; k-NN, k-Nearest Neighbors; RF, Random Forest; DT, Decision Tree; PLS, Partial Least Squares; SVR, Support Vector Regressor; LGBM, Light Gradient Boosting Machine; MAE, mean absolute error; ROC, Receiver operating characteristic; AUC, area under curve; SHAP, SHapley Additive exPlanations; AE, absolute error; PPV, positive predictive value; NPV, negative predictive value; NRI, net reclassification improvement; *S. paucimobilis*, *Sphingomonas paucimobilis*. (Supplementary files, Lines 88-110)

2. Line 255: What is the "risk-based model"? Was it described elsewhere in the manuscript?

Response: Thank you for your attention to the term of "risk-based model", which is a strategy to prioritize features and data resources based on its risk criticality. The strength of a risk-based model lies in its adaptability and precision. Unlike one-size-fits-all approach that treats all features equally, a risk-based model enables targeted action, thereby optimizing data resource allocation and enhancing overall risk management. However, this term was not described elsewhere in the manuscript. To address this concern raised, we have rephrased it as "multi-parameter model" to ensure it more accessible to readers.

3. Line 256: While the cohort background is detailed in the Methods section, it should also be briefly summarized in the main text. Are these participants healthy?

Response: We appreciate reviewer's suggestion to provide the summarized cohort background in the main text. In this version, we have expanded it by adding more information of enrolled participants as follows:

Revision Made: we subsequently enrolled the prospective clinical cohort of 240 healthy volunteers (aged between 22 and 69 years, with body mass index of 22.1 to 24.8 kg/m²) to exclude participants with clinically detectable gastrointestinal conditions or diseases. More participants' information was elaborated in Extended Data Table 1. (Lines 240-243)

4. Lines 257-259: What is the total number of features analyzed? Are the features categorical or continuous? Regarding the intestinal ceramide metabolome and microbiota, did you include all microbial taxa and metabolites? If so, this would result in thousands of features. What feature selection strategies were used to avoid overfitting?

Response: Thank you for this insightful question to highlight the total feature number of microbial taxa and metabolites, particularly regarding the strategies of feature selection to avoid overfitting. We greatly appreciate the opportunity to provide further explanation for addressing this concern. **Feature Number and Type:** In this study, the total number of included features is 19 and they are all continuous other than "Description" (Figure 5b).

Feature Selection: The features were selected based on their relevance to the research objectives, which focused on identifying key microbial taxa and metabolites that significantly impacted NAC permeability and its biopharmaceutics classification. Specifically, 10 microbial sphingolipid metabolites and 2 key strains were experimentally confirmed from all ceramide metabolites and microbial taxa.

Strategies to Avoid Overfitting: To mitigate overfitting during model training, we employed the following strategies:

1. **Mutual Information Regression:** This filter method was applied to evaluate the importance of each feature based on its mutual information with the target variable F_{sys} . Features with higher mutual information scores were considered more relevant and prioritized for inclusion in the model.
2. **5-Fold Cross-Validation:** We used 5-fold cross-validation to assess the performance of different feature combinations across various models. This helped us to identify the optimal set of features that generalized well to unseen data, thus reducing the risk of overfitting.
3. **Inclusion of the most closely relevant metabolites:** We included all sphingolipid metabolites closely related to NAC permeability, such as those derived from palmitoyl-CoA and cysteine. Including both upstream substrates and downstream products ensured a comprehensive representation of the metabolic pathways involved, preventing underfitting while maintaining the relevance to the research objective.

By combining these strategies, we aimed to build a robust machine learning model that effectively captures the complex relationships between microbial features and NAC permeability without overfitting to the training data.

5. Lines 261-262: *"The feature values of the participants in the Validation Cohort were divided into $F_{sys} \geq 85\%$ (Fig. 5b, left panel) and $F_{sys} < 85\%$ (Fig. 5b, right panel)." I do not understand what this means. Is F_{sys} the target variable (the variable to be predicted)? If so, why was it used to divide feature values (inputs)?*

Response: We sincerely appreciate the reviewer for the query why we divided the feature values by F_{sys} of 85% in Figure 5b. The rationale behind this grouping was mainly driven by the international standard of biopharmaceutics classification.

Clarity of F_{sys} : We fully agree with the reviewer that fraction of dose entering the systemic circulation (F_{sys}) of NAC is the target variable of this study.

Reason for dividing inputs by F_{sys} of 85%: As stated in International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline on M9: *Biopharmaceutics Classification System-Based Biowaivers*, drugs with $F_{sys} \geq 85\%$ are evaluated to

be highly permeable. In this study, we ultimately aimed to prompt a reevaluation of biopharmaceutics classification system by including the impact of intestinal microbiota on drug permeability. Therefore, we ranked the test set in order from high to low by true F_{sys} values rather than predicted F_{sys} values. Then inputs were divided by F_{sys} of 85% as the boundary to visualize the potential effect of each feature value on F_{sys} .

The following sentences and figure are updated as follows:

"As the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline on *M9: Biopharmaceutics Classification System-Based Biowaivers* statement that drugs with F_{sys} higher than 85% are considered to be highly permeable^{3,4}, feature values of the participants in test set were ranked in order according to their true F_{sys} values from high to low. We divided the test set into high (left panel) and low (right panel) permeability groups using $F_{sys} = 85\%$ as the boundary (Figure 5b)." (Lines 252-258)

Figure 5b

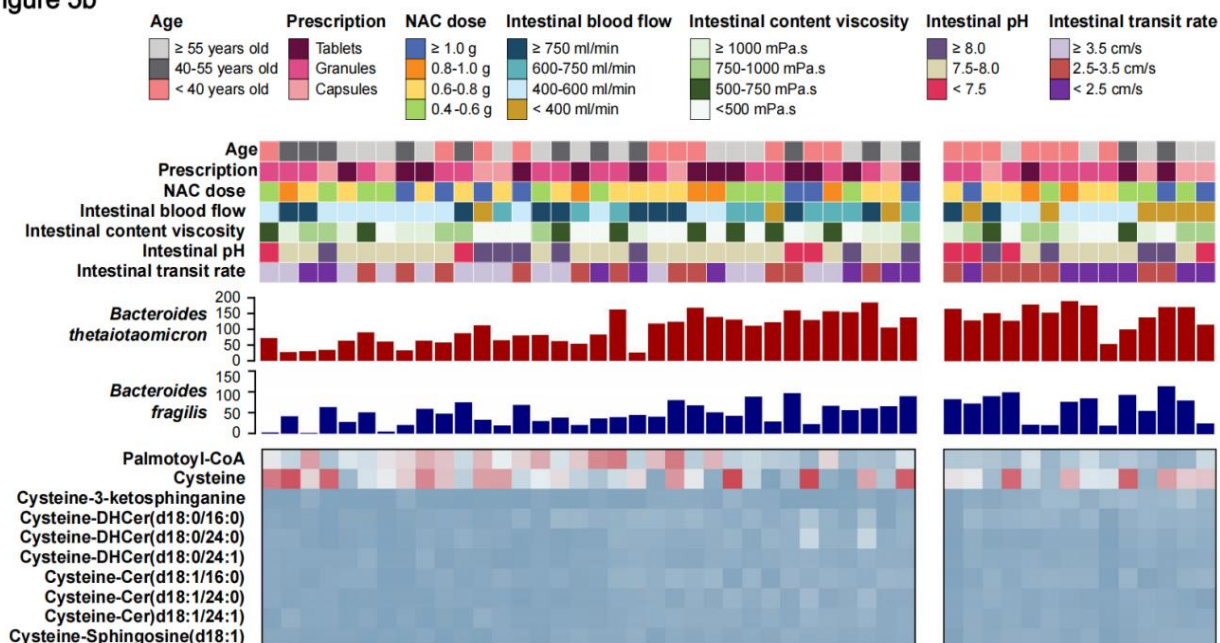


Fig. 5 (b) Participant characteristics of test set. Each column represents an individual participant with the potential determinants correlated with NAC absorption. Participants were ranked by true values of NAC F_{sys} from high to low (left to right) and divided with F_{sys} of 85% as the boundary.

6. Line 264: It is unclear how the machine learning model improves the stabilization and precision of the risk-based model. Again, what is the risk-based model?

Response: This is right, we greatly appreciate the reviewer for pointing this out. As we mentioned in the response to Question 2, "the strength of a risk-based model lies in its adaptability and precision. Unlike one-size-fits-all approach that treats all features equally, a risk-based model enables targeted action, thereby optimizing data resource allocation and enhancing overall risk management". However, we have not compared the machine learning model with the conventional

approach in our preceding paragraphs. We thus rephrased "refined" as "trained and evaluated" in this sentence. For the term of "risk-based model", we have replaced it with "multi-parameter model".

Revision Made: We then trained and evaluated nine different ML regression algorithms. (Line 259)

7. Lines 268-275: Did you use validation data during model development? Do you have three datasets (train, validation, test), or only two (train, test)? If there were three, how was the validation dataset used for? Also, what criteria were used to divide all participants into discovery, validation, and test cohorts?

Response: We sincerely thank the reviewer for this question regarding the number and criteria of datasets groupings. To address this, we have updated Fig. 5a to outline the distribution and categorization of 240 clinical participants for the training set and test set.

Figure 5a

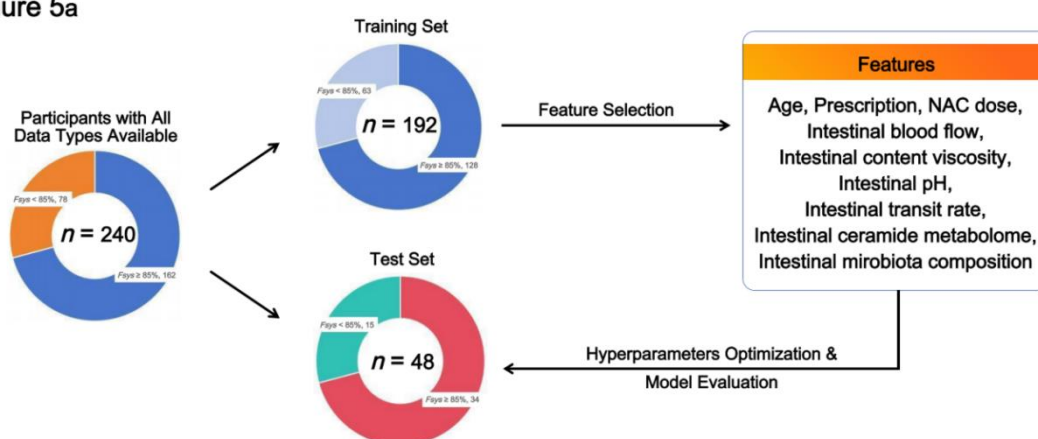


Fig. 5 (a) A total of 240 participants were enrolled for the training and test of ML models, with 192 (80%) participants as training set for feature selection and hyperparameter optimization, and 48 (20%) participants as test set for evaluation.

Number of Datasets: In the original version of Fig. 5a, we had three datasets for the model development: training set, validation set, test set. 240 participants were randomly divided into *training and validation sets* (80%, 192 participants) and *test set* (20%, 48 participants). Then we trained the model of 192 participants with a 5-fold cross-validation approach for feature selection and hyperparameter optimization. As illustrated in the schematic diagram below (Diagram 1), *training and validation sets* of 192 participants were randomly divided into 5 subsets for feature selection or hyperparameter optimization by cross-validation, where every subset was rotated for evaluation (*validation set*), leaving with 4 subsets for model training (*training set*). But recognizing the diverse readership of *Nature Communications*, we decided to define only two datasets in this revision, namely *training set* (80%, 192 participants) and *test set* (20%, 48 participants), as both *training set* and *validation set* were utilized to train this ML model.

Criteria for Datasets Grouping: To ensure homogeneity of variance for all cohorts, we divided the dataset randomly using random seed (*random_state=0*) in *train_test_split* method of *sklearn.model_selection*. This method could be referenced from several studies such as "For each dataset and method, we ran 10 repetitions with different random seeds and train-test splits (90% train, 10% test)." (*Nature*, 2025, 637(8045), 319-326) and "ML models were built using Scikit-Learn (0.24.2), a 80/20 train-test data split (random split) to train baseline models..." (*Cell*, 2024, 187(23), 6687-6706).

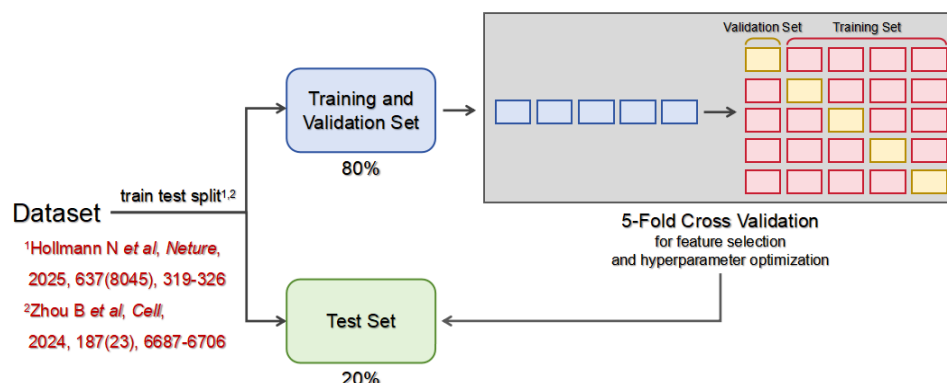


Diagram 1 Data-splitting strategy by 5-fold cross-validation approach

8. Line 280: What does "the classifier algorithm" refer to? Did you mean the classification counterpart of the regression algorithms?

Response: We thank the reviewer for prompting us to clarify "the classifier algorithm". In original Figure 5c, we employed the classification algorithm to compare four best-performing regression algorithms. Identifying the *Fsys* threshold of 85% as the criterion to differentiate high and low NAC permeability (as mentioned in Question 5), we then predicted classification results to generate ROC curves (Figure 5d). As per the reviewer's suggestion on the rationality of utilizing unoptimized classification models, in this version, we have directly compared the predicted *Fsys* values from the 4 optimized regression models and true *Fsys* values to graph the ROC curves as shown below.

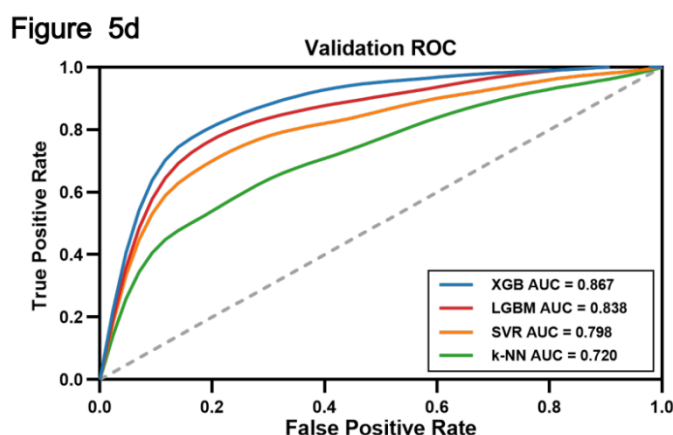


Fig. 5 (d) Receiver operating curve (ROC) for predicting NAC *Fsys* by XGB, LGBM, SVR and k-NN, with the area under the curve (AUC) values of 0.867, 0.838, 0.798 and 0.720, respectively.

9. Fig. S7b: What does the y-axis score represent? Why is the score equal to 1.0 even with the lowest number of training samples?

Response: We thank the reviewer for this reminding to elucidate the y-axis score in Fig. S7b.

Y-axis of Fig. S7b: Herein the y-axis score represents r^2 value to evaluate the model fitness between predicted F_{sys} values and true F_{sys} values. The pink dots showcase the training set scores, and the blue dots represent test set scores. By expanding the sample size, the r^2 values of test sets gradually increased and stabilized, in which the inflection point indicated the sufficient sample size.

Reason for the consistent score of training set: For training samples, we obtained high r^2 (equal to 1.0) even with small sample size because the training set itself was utilized to establish the machine learning model. We thus apologize for these meaningless curves of training set in Fig. S7b. Now that these panels of Fig. S7b were only assessed for the sufficient sample size, we deleted them in case of the distraction of main content.

Additional Clarity: Considering that the ultimate output model was restructured by the modeling strategy 1 as mentioned below, the feature "prescription" was filtered out by the 15-feature XGB model. This feature was originally described as categorical variables in Figures S7c-e. We thus removed Figure S7 in this revision.

10. Lines 287-288: What insights does the agglomerative hierarchical clustering provide?

Response: We thank the reviewer for the feedback on hierarchical clustering of original Fig. 5e. Herein the agglomerative hierarchical clustering was utilized to evaluate the collinearity between each two included features, facilitating the identification of potentially redundant features. However, since we have now changed the model training strategy to filter the features before optimizing hyperparameters as suggested in Question 11, the heat-map of agglomerative hierarchical clustering analyses has been omitted in this revision.

11. Lines 288-289: From a methodological perspective, any feature selection process, including the recursive feature elimination approach mentioned in the manuscript, should be embedded within the training procedure. Applying feature elimination after model fitting and hyperparameter selection is less rigorous.

Response: We fully agree with the reviewer on these invaluable suggestions. As illustrated below (Diagram 2), two types of strategies are mainly applied to the model training of machine learning: **Strategy 1:** Directly use the default hyperparameters to select features, then train the model to optimize hyperparameters; **Strategy 2:** Pre-train the model to optimize hyperparameters, then select features from the optimized model, and finally retrain the model to refine hyperparameters.

In the original version, we leveraged strategy 2 to pre-train the models and horizontally compare the models with optimal hyperparameter combinations, then identified the model with superior predictive performance for further optimization. Followed by the feature elimination, we re-optimized all hyperparameters to ensure whether modelling strategy is reasonable. As per the reviewer's suggestion on the less rigorous strategy 2, we ultimately implemented the strategy 1 of model training, which advances feature selection before hyperparameters optimization. This strategy not only streamlines the training process, but also enables each algorithm to compare with others by its own optimal model. More details of feature selection process could be found in the source code and its README.md file.

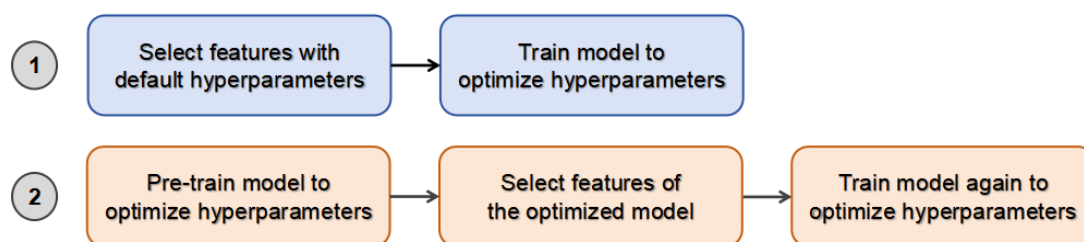


Diagram 2 Two different model-training strategies.

12. Lines 293-295: "Removing any of these features leads to a reduction in model accuracy." How significant were these reductions when each highly correlated feature was removed? Could you investigate the underlying cause? This may indicate overfitting.

Response: Thank you for your question about the significance of accuracy reductions when removing highly correlated features and the potential causes. We have conducted a detailed analysis to address this concern.

Significance of Reductions: When each highly correlated feature was removed, we observed a measurable reduction in model accuracy. The specific reductions of XGB model are listed in Supplementary Table 4 (Those of other ML models' are listed in Extended Table 3). For instance, removing Cysteine-Cer(d18:1/24:0), Cysteine-Cer(d18:1/16:0), Cysteine-DHCer(d18:0/16:0) from XGB model resulted in an accuracy drop of 6.392%, 2.565%, 2.817%, respectively.

Underlying Causes: The accuracy reductions are primarily due to the biological significance of these features within sphingolipid metabolism pathways. Despite their high correlations, each feature contributes unique information about the metabolic processes influencing NAC permeability. Removing any single feature disrupts the comprehensive representation of these pathways, leading to less accurate predictions.

Overfitting Consideration: To avoid the overfitting that these accuracy reductions may cause, we used recursive feature elimination with 5-fold cross-validation (RFECV) in original version. This method iteratively eliminates features while maintaining model performance across different data subsets. The consistent accuracy drops across validation folds suggest the reductions are genuine and not merely artifacts of overfitting.

```
# Apply recursive feature elimination cross-validation to select the optimal feature combination
xgb = XGBRegressor(subsample=0.6, reg_lambda=100, reg_alpha=0.01, n_estimators=500, min_child_weight=4,
                    max_depth=9, learning_rate=0.15, gamma=0.2, colsample_bytree=0.7, random_state=0)
selector = RFECV(xgb, step=1, cv=5, scoring='neg_mean_absolute_error').fit(X_train, y_train)
X_train_selected = selector.transform(X_train)
score_ori = cross_val_score(forest, X_train, y_train, cv=5, scoring='neg_mean_absolute_error')
score_sel = cross_val_score(forest, X_train_selected, y_train, cv=5, scoring='neg_mean_absolute_error')
print("Original score: ", score_ori)
print("Score after feature selection: ", score_sel)
print("Best feature combination: ", selector.support_)
print("Number of selected features: ", selector.n_features_)
print("The order of feature exclusion: ", selector.ranking_)
```

Diagram 3 RFECV was applied in original strategy to select the best feature combination.

13. Line 296: What is meant by "suitability" ?

Response: We sincerely appreciate the reviewer's scrutiny of this wording. Herein we had intended to state that "To confirm that suitable hyperparameters were selected for 15-feature XGB model...". Due to the change of model training strategy, however, the sentence containing "suitability" has been removed from this revised manuscript.

14. Lines 297-298: Once again, the feature selection algorithm should be incorporated into the training process.

Response: We thank the reviewer for this valuable feedback to highlight the timing of feature selection algorithm. In this version, we have incorporated feature selection into model training process (strategy 1) as mentioned above.

15. Line 55: The manuscript lacks clarity on how commercial models (ACAT, GITA, ADAM) make predictions. What are the inputs and outputs of these models? Are they mechanism-based and data-free? Providing some background information on these models would improve understanding.

Response: Thank you for your question about the commercial models used in our study. We have provided more detailed information about three commercial models to enhance clarity.

Background on Commercial Models: The ACAT, GITA, and ADAM models are established transit compartment models used commercially to predict oral drug absorption. These mechanism-based models incorporate several key inputs:

- Physicochemical properties: Such as drug solubility and pKa.
- Dosage form: Including dose amount and type of preparation.
- Physiological parameters: Such as intestinal transit time and intestinal pH.

The outputs of these models are predictions of drug absorption characteristics, such as the fraction of dose absorbed or systemic exposure levels. But none of these models quantitatively incorporate gut microbial characteristics, which is a gap that our study aims to address.

Integration with Our ML Approach: To compensate for the limitations of these commercial models, we introduced our 15-feature XGB model to incorporate intestinal microbial features along with other relevant parameters. We have enrolled an additional prospective cohort of 79 participants to validate the prediction accuracy. For these participants, we calculated predicted *F*_{sys} values using both the commercial models (ACAT, GITA, ADAM) and XGB model. The input parameters used for these simulations are detailed in Supplementary Table 5. This revision should provide a clearer understanding of how the commercial models function and how our ML model enhances predictive capabilities by integrating microbial characteristics.

16. Lines 686-688: *The code availability statement is unacceptable. Without access to the code, it is impossible to fully evaluate the machine learning approach.*

Response: Thank you for your inquiry regarding the code availability of this study. We had intended to protect the copyright of this model construction without full disclosure. For the reviewer's request of fully evaluating the machine learning approach, we have uploaded the README.md file and source code containing fixed feature and hyperparameter combinations identified in this study to ensure reproducibility for the diverse readership of *Nature Communications*. The code availability statement was revised as follows:

Revision Made: The source code containing fixed features and hyperparameter combinations identified in this study to ensure reproducibility is openly available in the cloud-based executable platform Code Ocean at <https://codeocean.com/capsule/8060667/tree>.

We trust that the changes we have made in response to the reviewers' feedback have adequately addressed their concerns and that the manuscript is now suitable for publication in *Nature Communications*.

Thank you once again for your valuable input.

Yours Sincerely

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