nature portfolio

Peer Review File

Gut microbiota-derived metabolite isovalerylcarnitine modulates salt sensitivity of blood pressure and incident hypertension: a multicenter dietary salt intervention trial

Corresponding Author: Professor Xiangfeng Lu

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)
The manuscript primarily investigates the effects of a high-salt (and low-salt) diet on blood pressure, microbiome and metabolome in a human cohort (MetaSalt), a validation cohort (GenSalt), an animal experiment in rats and a so-called prospective observational cohort. The authors show that there are changes at the microbiome and metabolome level (in addition to blood pressure) in response to salt intake and identify isovalerylcarnithine as a metabolite that is reduced by high salt and is associated with salt sensitivity. The authors' efforts with several human studies of sufficient size and a wealth of data must be recognised, and animal experiments have also been added. Nevertheless, the reviewer has a number of criticisms, most of which relate to the lack of stringency with regard to the contextualisation of the results obtained, their implications and the mechanisms involved.

1. Although the manuscript identifies various salt-responsive bacterial groups/species and metabolites, the reviewer is not satisfied with the extent to which these can be clearly assigned to the host or the bacteria. The title and the text suggest that a ‘microbiota-acylcarnitin network’ is identified here, but the manuscript does not fulfil this claim. There is a lack of mechanistic clarity, for example with regard to the main metabolite isovalerylcarnitine.
2. Given that the authors have shotgun metagenomic sequencing available, the analysis of the microbiome data is extremely insufficient because, for example, general measures of microbiome composition such as diversity measures (alpha, beta) are missing, but also the presentation of a clear analysis strategy for salt-responsive bacteria. A bacterial pathway analysis that complements the metabolomics data is missing and the use of „MetOrigin“ is insufficient in the eyes of the reviewer, given that shotgun data are available.
3. When exactly was blood pressure measured and which values have been used for the analysis? Please state in the main text and show in the study scheme. Which values were taken for the SSBP definition?
4. What thresholds were used to categorise someone as moderately or extremely salt sensitive or salt resistant?
5. Since the meals have been provided in MetaSalt, there is a lack of nutritional information in this manuscript. In how far can the authors be sure that the effects are salt-related and not confounded by the other components of the provided meals? Please show macronutrient and if possible some other micronutrients to enable evaluation of the dietary composition.
6. Regarding the habitual/accustomed diets of the participants at baseline: are nutritional protocols/dietary records available to investigate in how far the nutrition of the participants changes upon study entry? In how far does fibre content change in the study, as fibre is essential in BP regulation (SCFA)?
7. Please provide the formula with which levels of 24hr UNa and UK were calculated. As far as I could see the reference provided does not contain this information.
8. A flow chart showing enrollment, randomization and follow-up is a standard and necessary for each human study shown.
9. Table S1 (Baseline characteristics) contains important information and should not be hidden in the Excel file but rather shown in the main text.
10. Table S1: Blood pressure levels, BMI, smoking status and other blood pressure relevant measures should be shown for males and females separately.
11. Figure 1 is far too crowded and the different study regimes should be shown where the respective data are shown.
12. Figure 2 A/C volcano plots: which effect size measure did you use?
13. Figure 2A/C: consider highlighting/labeling a few data points that represent your main findings here already.
14. Figure 3: The Cytoscape-generated species-metabolites network needs to be validated somehow. For microbial species

and metabolic networks responsible/associated enzymatic pathways could be tested in a targeted manner using the available shotgun metagenomic datset.
15. Figure 3: Error bars missing on the bar graphs.
16. Figure S4 needs more information/explanation in the text e.g. regarding CAZy families, sensitivity analyses and actual meaning for the whole manuscript.
17. Page 6 lines 139-140 „…metabolites were associated with salt-related git microbial specias to some extent…". In my view, this kind of somewhat unscientific, not very stringent expression, but also analysis strategy, is emblematic of large parts of the paper, which identify potential targets, but do not sufficiently illuminate their microbial origin or their mechanistic significance.
18. Please use consitently new taxonomic nomenclature regarding Bacillota vs. Firmicutes.
19. The confirmational experiments in rats are interesting. Please disclose all components of the diets. Was a purified diet used? What was used to replace the salt in the so-called low-salt diet group - I mean the 7.55% difference in salt content? Or in other words: which component did the HSD rats have less of than the control rats?
20. In the methods section the control diet is called low-salt diet. I believe it should be called normal salt.
21. It is interesting that DSS rats treated with isovaerlycarnitine (subcutaneous osmotic minipumps) have lower blood pressure. However, few valid assumptions are made about the mechanistic cause and no conclusive data are presented. What is the albumin/creatinine ratio (albuminuria) in the urine, what is the natriuresis under isovaerlycarnitine? What do the kidneys and blood vessels look like histologically, is there less hypertensive damage in these end organs, or signs of oxidative stress? The same applies to the endothelial function experiments. The whole experiment is interesting, but in my view it has not been sufficiently analysed to draw valid mechanistic conclusions.
22. Isovalerylcarnitine was administered subcutaneously. Are similar effects to be expected with oral administration if the microbiome is also involved?
23. Isovalerylcarnitine is used diagnostically for a rare metabolic disease: isovaleric acidemia caused by a deficiency of isovaleryl-CoA dehydrogenase. Isovalerylcarnitine is elevated in this case. To what extent must this be taken into account for the translational perspective? Is the expression of isovaleryl-CoA dehydrogenase in animal experiments influenced by high salt? If so, what are the reasons?

Reviewer #3

(Remarks to the Author)
In the current manuscript, Lin and colleagues investigated the roles of the microbiome and metabolome in salt-sensitivity of blood pressure (SSBP) and hypertension. In these analyses, they identified 85 gut microbial species and 70 metabolites were altered by high salt. Among these findings, 22 microbial species and 8 metabolites were further associated with SSBP. Furthermore, from an associated gut microbiota-acyl carnitine network, the primary isovalerylcarnitine metabolite was replicated in an independent sample and experimentally validated in rats. This metabolite, which inversely associated with SSBP, was shown to reduce the risk of incident hypertension in a prospective cohort. In total, this was a well conducted study with triangulating support from human and animal studies. The findings could have potential implications for the prevention of hypertension, identifying a metabolite that could potentially be altered to decrease the risk of hypertension. However, a few minor questions/concerns should be addressed.
1.) It is unclear how thresholds for determining SSBP degree were determined. For example, salt sensitive individuals were categorized as those with greater than a 10 mmHg response to the high or low sodium diet. Were these arbitrary thresholds? Based on some sort of indicator of risk?
2.) In analyses investigating associations of SSBP with changes in metabolites, was SSBP modeled as an ordinal or categorical variable?
3.) In the volcano plots showing the microbial species and metabolite responses to high salt, there is notable asymmetry in the plot, with the vast majority of significant findings demonstrating depletion on high salt. What is the possible explanation for this finding? Is this related to the antimicrobial properties of salt? If so, why would this be more substantial for the metabolite plot?
4.) Similar to previous work in the GenSalt study, participants were categorized as 'salt sensitive', 'moderately salt-sensitive', and 'salt resistant'. In that report, salt-resistance of blood pressure was also be associated with an increased risk of incident hypertension. Were there any findings of microbiota or metabolites that were associated with decreased SSBP (or salt resistance) that increased the risk of hypertension? Is this possible to determine from your analyses? Some discussion of this issue would be of interest to the informed reader.

Version 1:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
I´ve been asked to comment on the revisions performed to Reviewer #1´s concerns.

ORIGINAL REVIEWER QUERY 1: The authors have included an FMT experiment to demonstrate that the gut microbiota is a source of isovalerylcarnitine, as per the original reviewer's request. The supplementary methods state that this was a 28 day experiment – with a control group, an antibiotic treatment and an antibiotic treatment+FMT group. The following additional method details would be of benefit:

How long was the antibiotic treatment provided for? And thus how long into the 28 day experiment was it stopped? (perhaps a schematic would assist to make this clear)
Was the decrease in bacterial load following antibiotic treatment confirmed prior to FMT treatment? Regarding the FMT preparation, was deoxygenated PBS used? Or any efforts to complete the FMT preparation in an anaerobic environment? Was the FMT delivered fresh or frozen? If frozen was any cryopreservant used? It could benefit to check the GRAFT reporting guidelines for animal FMT experiments (https://pmc.ncbi.nlm.nih.gov/articles/PMC8489962/) for ensuring an adequate level of details is reported.

ORIGINAL REVIEWER QUERY 18: The original reviewer made the point that bacterial taxonomy has been updated in 2021 (https://ncbiinsights.ncbi.nlm.nih.gov/2021/12/10/ncbi-taxonomy-prokaryote-phyla-added/), and now the name for Firmicutes is Bacillota, etc…For Firmicutes this has been updated to Bacillota in the text…however is still Firmicutes in the figures (e.g. Firmicutes bacterium CAG:124, etc). This inconsistent phyla nomenclature between the text and figures may be confusing for readers.

The remainder of the original reviewer questions I believe to be adequately addressed in this revision.

(Remarks on code availability)
Without any input data, I cannot run and evaluate the code.

Just reading the code, I can identify a few areas of concern that would limit the ability for the code to be used by others:

SECTION 2: SSBP BIOMARKERS
Line 138-303, the section “2) - Identification of SSBP-related biomarkers”:

Each subsection in this section starts with a melt of a dataframe called “chgdata” before subsequent commands are run.
e.g. line 141:
temp <- melt(chgdata, c("labid2","famid","ss","age","gender","bmi","fcc","htn","smoking","chol"), metasalt.sr.species$speid)
It’s not clear where this chgdata object is coming from or what format it is…so this section of the code is not reusable by others/cannot reproduce results.

SECTION 3: ANIMAL DATA

I understand the privacy issues related to human data – however the data for the animal experiments could be included – e.g. if alldata.wistar, alldata.dahl, alldata.sd were uploaded to the github as a .rds object, then I could run this code and check the reproducibility of this section at least.

Reviewer #5

(Remarks to the Author)
I have been asked to comment on the revisions made in response to Reviewer #3’s concerns regarding the revised manuscript by Lin et al. After thoroughly reviewing the authors’ responses and the corresponding changes in the manuscript, I can confirm that the authors have provided comprehensive and satisfactory answers to each of the reviewer’s comments. The manuscript has been appropriately amended with relevant references and additional content where necessary.

(Remarks on code availability)

Reviewer #1 (Remarks to the Author):

The manuscript primarily investigates the effects of a high-salt (and low-salt) diet on blood pressure, microbiome and metabolome in a human cohort (MetaSalt), a validation cohort (GenSalt), an animal experiment in rats and a so-called prospective observational cohort. The authors show that there are changes at the microbiome and metabolome level (in addition to blood pressure) in response to salt intake and identify isovalerylcarnithine as a metabolite that is reduced by high salt and is associated with salt sensitivity. The authors' efforts with several human studies of sufficient size and a wealth of data must be recognised, and animal experiments have also been added. Nevertheless, the reviewer has a number of criticisms, most of which relate to the lack of stringency with regard to the contextualisation of the results obtained, their implications and the mechanisms involved.

Response: We sincerely appreciate your thoughtful review of our manuscript and the constructive suggestions and comments you have provided. Accordingly, we have carefully incorporated additional analyses and experiments into the revised manuscript, and we have prepared a detailed, point-by-point response to each of your questions and comments. We hope that the following explanations and revisions will fully address your concerns and enhance the quality of our submission.

1. *Although the manuscript identifies various salt-responsive bacterial groups/species and metabolites, the reviewer is not satisfied with the extent to which these can be clearly assigned to the host or the bacteria. The title and the text suggest that a 'microbiota-acylcarnitine network' is identified here, but the manuscript does not fulfill this claim. There is a lack of mechanistic clarity, for example, with regard to the main metabolite, isovalerylcarnitine.*

   Response:

   Thank you very much for your valuable comments. In our initial manuscript, we reported the associations between gut microbes and metabolites from the MetaSalt study and employed the "MetOrigin" tool to predict potential sources of salt-responsive metabolites. We agree that these bioinformatic predictions alone are insufficient to definitively confirm

whether a metabolite (e.g., isovalerylcarnitine) is of gut microbial origin, which inherently limits the robustness of our claim regarding the "microbiota-acylcarnitine network". To address this, we performed a validation experiment involving antibiotic intervention combined with fecal microbiota transplantation (FMT) in the revised version, specifically designed to clarify whether the key metabolite (isovalerylcarnitine) is a gut microbiota-derived metabolite. Specifically, 21 rats were randomly assigned to three groups (n=7 per group): a control group without any intervention, an antibiotic cocktail treatment (ACT) group, and an ACT followed by subsequent FMT group. Post-intervention analyses revealed remarkable changes in plasma isovalerylcarnitine levels: the levels of isovalerylcarnitine significantly decreased to 0.07 μmol/L in the ACT group (P = 0.0043), and partially recovered to 0.10 μmol/L in the ACT + FMT group (P = 0.5648), compared to the control group (0.11 μmol/L) (**Figure R1**). These results provide compelling evidence that gut microbiota play a crucial role in maintaining circulating isovalerylcarnitine levels. The reduction in isovalerylcarnitine following antibiotic-induced microbiota depletion, along with its restoration after FMT, suggests that isovalerylcarnitine is likely a gut microbiota-derived or -modulated metabolite.

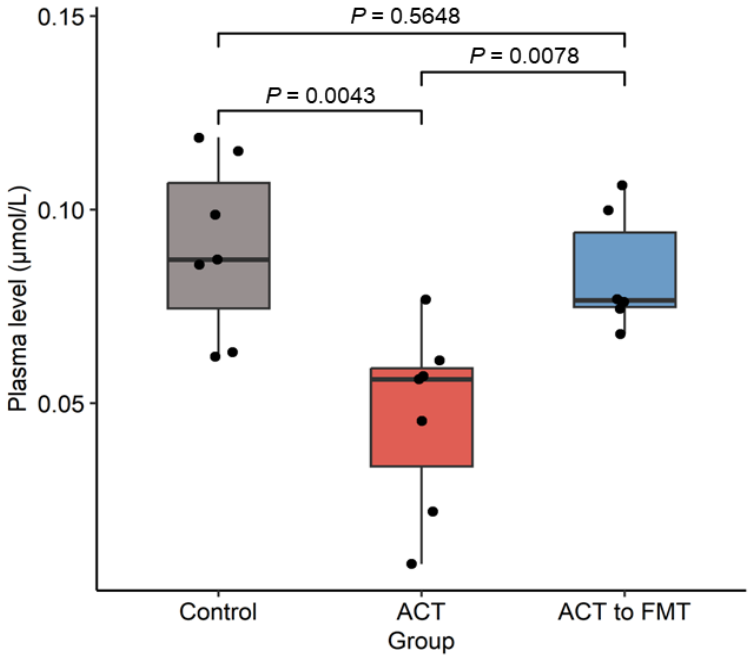


Figure R1. Difference in isovalerylcarnitine across rats in different groups of the antibiotic experiment

Twenty-one Sprague-Dawley rats were randomly allocated to three groups: control (n = 7), ACT (n = 7), and ACT to FMT (n = 7), and one rat in the ACT + FMT group was sacrificed during the intragastric administration procedure. Differences between groups were examined using t-test. ACT, antibiotic cocktail treatment; FMT, fecal microbiota transplantation.

Given that the core focus of our current study is to characterize isovalerylcarnitine as a key microbiota-derived metabolite and its association with SSBP and incident hypertension, we have revised the title to emphasize this specificity: "**Gut microbiota-derived metabolite isovalerylcarnitine modulates salt sensitivity of blood pressure and incident hypertension",** instead of stating "microbiota-metabolite network".

To address your concern about mechanistic clarification, we have made great efforts in conducting new experiments and in-depth data analyses, such as histological assessments. Our findings demonstrated that isovalerylcarnitine may protect against salt-induced damage by improving endothelial function and reducing tissue remodeling (**detailed in Question 21**). We recognize that unraveling the complete functional mechanisms underlying the role of isovalerylcarnitine would require more in-depth investigations and be time-intensive. We hope to pursue these mechanistic explorations in subsequent studies.

We have added the above contents to our revised manuscript:

Antibiotic experiment: Main text (page 13, lines 269-274 and page 25, lines 529-534); Supplementary Method; Supplementary Figures 15-16.

Histological assessment: Main text (page 12-13, lines 258-268 and page 24, lines 526-527); Supplementary Method; Figure 5.

2. *Given that the authors have shotgun metagenomic sequencing available, the analysis of the microbiome data is extremely insufficient because, for example, general measures of microbiome composition such as diversity measures (alpha, beta) are missing, but also the presentation of a clear analysis strategy for salt-responsive bacteria. A bacterial pathway analysis that the metabolomics data is missing and the use of "MetOrigin" is insufficient in the eyes of the reviewer, given that shotgun data are available.*

Response:

Thank you for your suggestion to strengthen the microbiome analyses using the shotgun metagenomic data. We have added the relevant analyses in our revised manuscript.

First, regarding diversity measures, we have supplemented analyses of gut-microbial α-diversity and β-diversity based on the Simpson index and species-level Bray-Curtis distance, respectively. We also measured inter-individual variability by calculating the median Bray-Curtis dissimilarity for each participant relative to all others within each study phase—this metric reflects the degree of compositional divergence across individuals.[1] Linear mixed models were applied to test the differences in α-diversity and median Bray-Curtis dissimilarity, and permutational ANOVA (999 permutations) was used to examine the disparity in β-diversity. No significant difference in gut-microbial β-diversity across the three study phases was observed (P = 0.3660) (Supplementary Figure 4). However, both gut-microbial α-diversity and median Bray-Curtis dissimilarity significantly increased from the baseline to the low-salt intervention (P = 0.0080 and 4.35×10^{-6}, respectively), and decreased from the low-salt to the high-salt stage (P = 0.0156 and 0.0380, respectively) (**Figure R2**). Additionally, higher SSBP was associated with increased gut-microbial α-diversity and median Bray-Curtis dissimilarity in all study phases (**Figure R3**). These findings highlight that while the overall community structure (β-diversity) remains stable, salt intake modulates microbial richness, evenness, and inter-individual divergence.

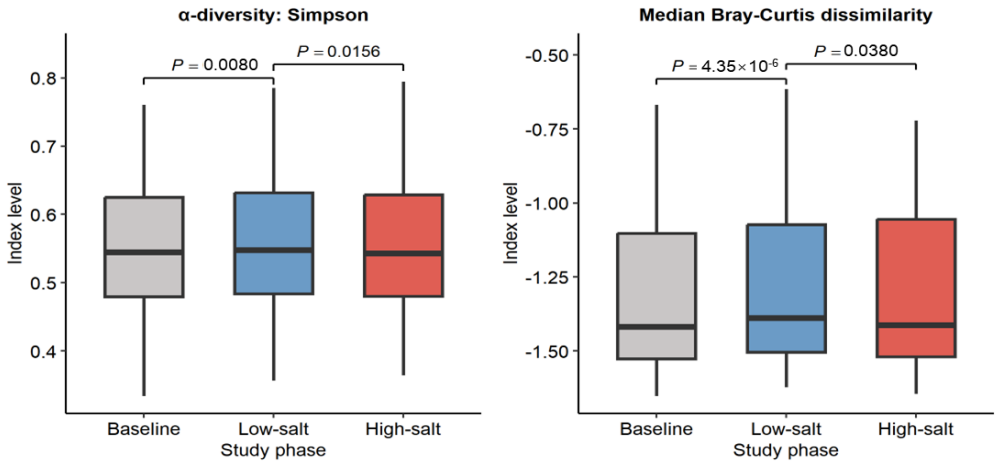


Figure R2. α-diversity and median Bray-Curtis dissimilarity across study phases

Gut-microbial diversity was assessed for the participants of the MetaSalt study (n = 503). α-diversity was estimated based on Simpon index. Median Bray-Curtis dissimilarity for each individual in each study phase was calculated as the median of distance measures against all other individuals, and log-transformed before analysis due to skewed distribution. Differences were tested using linear mixed models with the random intercepts of individuals and families.

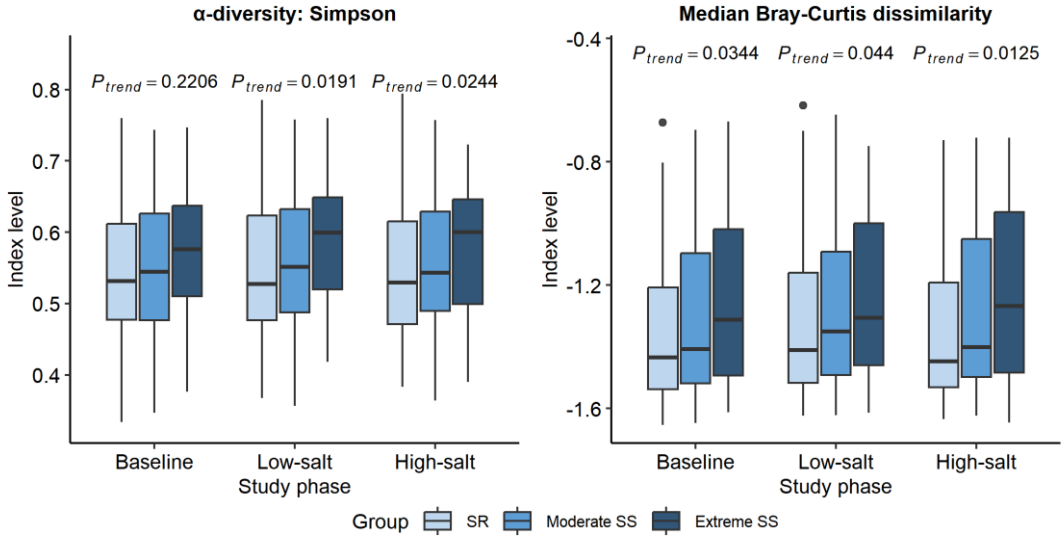


Figure R3. α-diversity and median Bray-Curtis dissimilarity across SSBP groups in each study phase

Gut-microbial diversity was assessed for the MetaSalt participants (n = 503). α-diversity was estimated based on Simpon index. Median Bray-Curtis dissimilarity for each individual in each study phase was calculated as the median of distance measures against all other individuals, and log-transformed before analysis due to skewed distribution. Differences were tested using linear mixed models with the random intercepts of individuals and families. SS, salt sensitivity; SSBP, salt sensitivity of blood pressure; SR, salt resistance.

Second, to bridge microbiome and metabolome, we expand functional analyses of the shotgun metagenomic data. We annotated gut-microbial functions using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Carbohydrate-Active EnZymes (CAZy) families, and evaluated the differences in the relative abundance of these gut-microbial functions between intervention periods. We observed that the high-salt intervention significantly influenced 36 KEGG pathways and 8 CAZy families (Figures 2c

and 2d, and Supplementary Tables 3-4). Of them, 9 KEGG pathways overlapped with those enriched pathways identified from salt-related metabolites (Figure 2f). Notably, fatty acid biosynthesis and valine, leucine and isoleucine biosynthesis—both directly linked to acylcarnitine metabolism[2]—were the top two pathways. These align with our metabolomics findings, reinforcing a functional connection between microbial activity and acylcarnitine homeostasis. In addition, we extracted microbial genes related to carnitine/acylcarnitine from metagenome data, and assessed their associations with isovalerylcarnitine and 16 isovalerylcarnitine-related species. Three genes [including *caiB* (carnitine CoA-transferase), *caiC* (carnitine-CoA ligase), and *TC.BCT* (betaine/carnitine transporter, BCCT family)] were positively associated with isovalerylcarnitine ($P < 0.05$). Of them, *caiB* and *TC.BCT* showed consistent positive associations with 15 and 13 isovalerylcarnitine-related species, respectively ($P < 0.05$) (Supplementary Table 10). These associations strengthen the link between microbial genes, taxa, and isovalerylcarnitine, complementing our metabolomic findings.

Third, the primary objective of utilizing the "MetOrigin" tool in our study was to predict potential origins of the identified metabolites. We did not use other functions offered by the tool since they were not applicable to the complex study design of the MetaSalt study (repeated measures and familial clustering). Instead, we performed integrative analyses using statistical approaches tailored to our dataset: linear mixed models were used to account for the repeated measurement, the correlation within families, as well as potential confounding factors.

We have added the above contents in our revised manuscript:

Gut-microbial diversity: Main text (page 6-7, lines 126-135 and page 29, lines 630-637); Supplementary Methods; Supplementary Figures 4-6.

Gut-microbial functional pathways and genes: Main text (page 7-8, lines 140-142 and lines 153-156 and page 10, lines 198-205); Figures 2c and 2d; Supplementary Tables 3, 4, and 10.

We also detailed the analysis strategy of salt-responsive bacteria and metabolites in the Method section. We believe these additions enhance the depth and rigor of our microbiome-metabolome integration, and hope they address your concerns.

3. *When exactly was blood pressure measured and which values have been used for the analysis? Please state in the main text and show in the study scheme. Which values were taken for the SSBP definition?*

Response:

Thank you for the questions. In the MetaSalt study, participants underwent 11 days of SBP and DBP measurements across all study phases to capture stable, representative BP values, including 3 consecutive days during the baseline stage, days 2, 8, 9, and 10 of both the low-salt and high-salt intervention stages. Days 8-10 represent the later phase of the intervention, when dietary salt effects are expected to stabilize. Referring to previous works based on the GenSalt study, we calculated the average BP levels during the 3 consecutive days of the baseline period and the final 3 days (Days 8-10) of each intervention stage. This approach helps minimize intra-day BP fluctuations and reduce potential carryover effects between study phases.

Mean arterial pressure (MAP) was derived from these averages using the formula: MAP = (1/3 × SBP) + (2/3 × DBP). MAP was uased for analyses because it integrates SBP and DBP into a single metric, which has been widely applied in prior SSBP research.[3,4] SSBP was defined based on the change in MAP between the high-salt and low-salt phases, following criteria adapted from prior literature.[3-6]

We stated the above contents in the Result section briefly (Main text: page 5, lines 104-108) and in the Method section (Main text: page 21, lines 442-469) in detail. We also showed them in the study scheme (Figure 2a) and as follows (**Figure R4**):

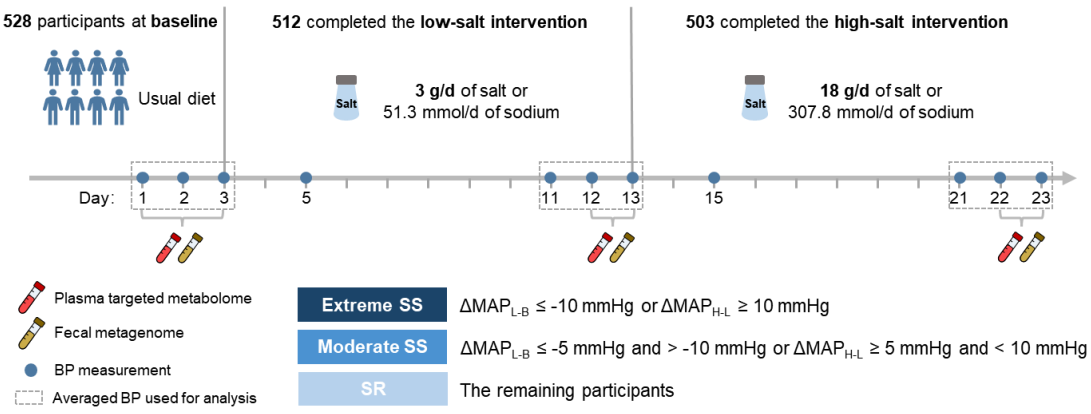


Figure R4. Study design of the MetaSalt study

BP, blood pressure; MAP, mean arterial pressure. SR, salt resistance; SS, salt sensitivity.

4. *What thresholds were used to categorise someone as moderately or extremely salt sensitive or salt resistant?*

Response:

Thank you for the questions. Referring to previous studies,[3-6] we established a criterion to determine the SSBP degree in our study as follows:

Extreme SS individuals: $\Delta MAP_{L-B} \leq -10$ mmHg or $\Delta MAP_{H-L} \geq 10$ mmHg;

Moderate SS individuals: $\Delta MAP_{L-B} \leq -5$ mmHg and > -10 mmHg or $\Delta MAP_{H-L} \geq 5$ mmHg and < 10 mmHg;

SR individuals: the remaining participants.

The definition was presented in the Method section of our revised manuscript: Main text: page 21, lines 453-459.

5. *Since the meals have been provided in MetaSalt, there is a lack of nutritional information in this manuscript. In how far can the authors be sure that the effects are salt-related and not confounded by the other components of the provided meals? Please show macronutrient and if possible some other micronutrients to enable evaluation of the dietary composition.*

Response:

Thanks for your constructive suggestions. We agree that other nutritional components may influence the levels of gut microbiota and metabolites. To clarify the dietary composition during interventions, we have compiled detailed nutritional information, including macronutrients, key micronutrients, and total energy, based on food records during both the 10-day low-salt and 10-day high-salt intervention periods for each participant. We compared the differences in these nutritional components between the two study phases, and observed that most were slightly lower during the low-salt intervention (**Table R1**), mainly because the saltiness of the diet in the low-salt intervention differed from the usual diet, leading participants to eat less food in this study phase.

Table R1. Total intake of nutrients during the low-salt and high-salt interventions

Component	Low salt	High salt
Energy, kcal	26599.96 ± 6994.08	29182.18 ± 9592.67
Protein, g	460.39 ± 129.04	558.28 ± 178.49
Fat, g	614.60 ± 173.52	725.06 ± 274.34
Carbohydrate, g	4855.78 ± 1447.24	5176.37 ± 1755.48
Fiber, g	88.50 (76.30, 112.78)	86.94 (65.74, 219.48)
Cholesterol, mg	6735.73 (6257.63, 7411.97)	6539.72 (6284.10, 7106.73)
Vitamin A, μg RAE	3945.33 ± 1195.99	4764.57 ± 1819.97
Vitamin B1, mg	7.31 ± 1.35	9.36 ± 2.92
Vitamin B2, mg	6.86 (6.04, 7.93)	7.25 (6.78, 14.43)
Vitamin B3, mg	82.44 ± 21.29	118.99 ± 43.78
Vitamin C, mg	1307.42 (1054.60, 1488.95)	1422.11 (1025.88, 2656.34)
Vitamin E, mg	286.08 ± 82.83	351.53 ± 186.92
Ca, mg	3843.93 (2921.32, 5165.12)	4165.01 (3281.15, 7580.85)
P, mg	5089.60 ± 1750.03	5813.85 ± 2503.12
Mg, mg	2085.77 (1681.95, 2559.21)	2338.39 (1736.67, 3467.97)
Fe, mg	139.51 (121.25, 270.82)	154.10 (130.68, 332.18)
Zn, mg	40.32 (32.69, 47.40)	41.32 (30.96, 59.34)
Se, mg	88.05 (64.50, 127.22)	152.39 (70.93, 190.24)
Cu, mg	8.89 (7.22, 10.30)	10.13 (7.60, 13.82)
Mn, mg	22.62 (17.64, 27.89)	19.59 (14.74, 39.71)

Normally distributed variables are presented as mean ± standard deviation; skewed distributed variables are presented as median (inter-quartile range).

To address your concern, we further adjusted for total energy and each nutritional component intake in the models to evaluate whether they could confound the effects of salt on biomarkers and the association of biomarkers with SSBP. We found that the vast majority of results (99.09%) remain robust. Notably, the association between salt intake and isovalerylcarnitine—our key metabolite—was unaffected by adjustments for these nutritional factors (**Figure R5**). This result confirmed that our findings were mainly driven by salt rather than other dietary factors.

We have added the above results to our revised manuscript: Supplementary Figure 11.

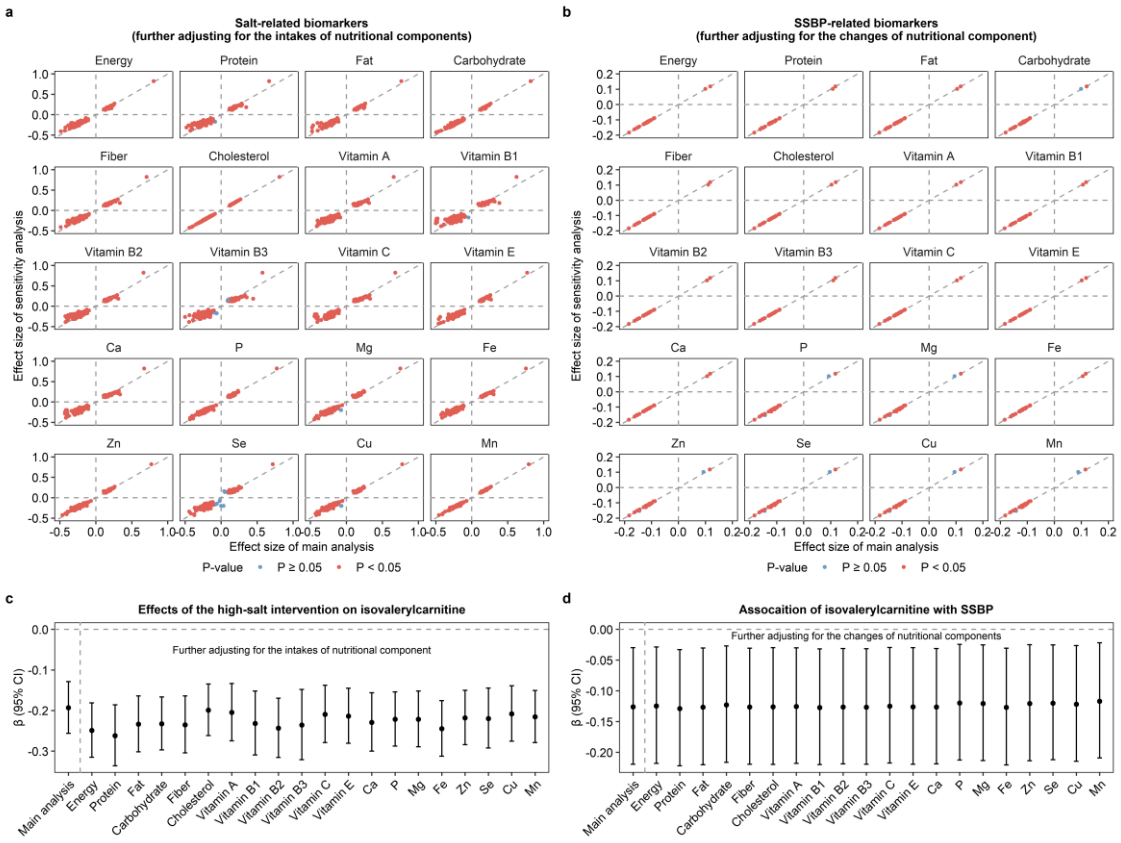


Figure R5. Sensitivity analyses of further adjusting for nutritional components during the intervention stages

The intakes of energy and nutrients in the low-salt and high-salt intervention stages were calculated for the MetaSalt participants (n = 503). Sensitivity analyses of further adjusting for total energy or each nutrient were conducted, which aimed to evaluate whether the effects of dietary salt on biomarkers and the associations of biomarkers with SSBP were confounded by other nutritional components. **a and b**, The effect sizes of sensitivity analyses and main analyses for all salt-related and SSBP-related biomarkers. The effect sizes estimated in the main analyses and the sensitivity analyses were illustrated as scatters, with red and blue dots indicating $P < 0.05$ and $P \geq 0.05$ in the results of the sensitivity analyses, respectively. **c and d**, The effect sizes of sensitivity analyses and main analyses for isovalerylcarnitine.

6. *Regarding the habitual/accustomed diets of the participants at baseline: are nutritional protocols/dietary records available to investigate in how far the nutrition of the participants*

changes upon study entry? In how far does fiber content change in the study, as fiber is essential in BP regulation (SCFA)?

Response:

Thank you very much for the questions. In our study, participants were instructed to maintain their usual diet at baseline. A simplified food frequency questionnaire was used to document the consumption frequency of major food groups over the previous year, including rice, grain, meat, fish, egg, dairy, fruit, vegetable, bean food, nut, pickle, pepper, beverage, and tea. However, since we did not collect specific food items and portion sizes at baseline, we cannot accurately estimate individual nutrient intake. Instead, we utilized a standard food exchange list to roughly estimate total energy intake.[7] We found the average total energy at baseline was higher than that during intervention periods. (baseline: 3287.91 ± 1469.91 kcal/day; low-salt: 2660.00 ± 699.41 kcal/day; high-salt: 2918.22 ± 959.27 kcal/day). This may be due to the controlled meal setting during the intervention. However, a more likely explanation is that the food frequency questionnaire tends to overestimate actual food intake.[8]

To minimize the potential impacts of dietary and nutritional changes on our findings, we undertook the following measures throughout the study:

(1) **Before the intervention**, professional dietitians designed recipes tailored to the dietary habits of local residents in each study community.

(2) **During the intervention**, participants were required to consume all meals at designated study canteens under the supervision of study staff. To ensure nutritional adequacy, sufficient quantities of food were provided, and participants were instructed not to consume any additional food outside the trial.

(3) **After the intervention**, we did not include metagenome and metabolome data at baseline in our study. Instead, we focused exclusively on comparing the differences in gut microbiota and metabolites between the low-salt and the high-salt phases.

With respect to dietary fiber intake, although we could not assess baseline fiber consumption, fiber intake during the low-salt and high-salt intervention periods was comparable (88.50 mg vs. 86.94 mg) (**Table R1**). To further address your concern, we also compared the plasma levels of SCFA across study phases to indirectly evaluate how much

dietary fiber changed in the study. We did not observe significant differences in SCFAs across study phases (**Table R2**). Thus, we considered that fiber intake may vary little in our study.

Table R2. Differences in the plasma levels of SCFAs across study phases

SCFA	Baseline (μmol/L)	Low salt (μmol/L)	High salt (μmol/L)	*P*
Acetic acid	95.38 ± 46.44	90.67 ± 19.32	90.27 ± 20.99	0.1484
Propionic acid	7.10 ± 1.17	7.05 ± 1.09	7.04 ± 1.27	0.5662
Butyric acid	1.60 ± 0.42	1.66 ± 0.45	1.69 ± 0.48	0.2035
Isobutyric acid	0.94 ± 0.40	0.94 ± 0.35	0.95 ± 0.54	0.8560
Isovaleric acid	0.47 ± 0.19	0.46 ± 0.18	0.45 ± 0.18	0.2207
Caproic acid	1.10 ± 0.57	1.10 ± 0.35	1.10 ± 0.57	0.5080
Isocaproic acid	0.32 ± 0.22	0.31 ± 0.16	0.30 ± 0.20	0.2144

Differences in SCFA levels among study phases of the MetaSalt study (n = 503) were tested using repeated measurement analysis of variance. SCFA, short-chain fatty acid.

7. *Please provide the formula with which levels of 24hr UNa and UK were calculated. As far as I could see the reference provided does not contain this information.*

Response:

Thank you for the suggestion. The formulas for converting overnight UNa and UK to 24-hour values were developed based on 238 participants in the GeneSalt study who had collected overnight and 24-hour urine on the same day during each study phase (**Table R3**).[9]

We have provided these formulas in our revised manuscript: Supplementary Methods.

Table R3. Formulas for converting overnight UNa and UK to 24-hour values.

Study phase	Formula
UNa, mmol	
Baseline	24 hour UNa = 111.62 + 1.77 × overnight UNa
Low-salt intervention	24 hour UNa = 23.53 + 1.56 × overnight UNa
High-salt intervention	24 hour UNa = 155.00 + 1.05 × overnight UNa
UK, mmol	
Baseline	24 hour UK = 18.40 + 1.76 × overnight UK
Low-salt intervention	24 hour UK = 16.67 + 1.49 × overnight UK
High-salt intervention	24 hour UK = 20.38 + 1.44 × overnight UK

8. *A flow chart showing enrollment, randomization and follow-up is a standard and necessary for each human study shown.*

   Response:

   Thanks for your suggestion. We have presented the flow charts for the MetaSalt study, the GenSalt study, and the prospective cohort study in our revised manuscript (Supplementary Figures 1**,** 12, and 17).

9. *Table S1 (Baseline characteristics) contains important information and should not be hidden in the Excel file but rather shown in the main text.*

   Response:

   Thanks for your kind reminder. We have presented the baseline characteristics across different degrees of SSBP in the main text of our revised manuscript (Table 1).

10. *Table S1: Blood pressure levels, BMI, smoking status and other blood pressure relevant measures should be shown for males and females separately.*

    Response:

    Thanks for your advice. We have presented the baseline characteristics for males and females separately in Supplementary Table 1.

11. *Figure 1 is far too crowded and the different study regimes should be shown where the respective data are shown.*

    Response:

    Thanks for your suggestions. We have simplified Figure 1, with only presenting the overall flow of the whole study. We have shown the detailed study regimes for each study separately, i.e., Figure 2a for the MetaSalt study, Figure 4a for the GenSalt study, Figures 5a and 5d for animal experiments, and Figure 6a for the prospective cohort study.

12. *Figure 2 A/C volcano plots: which effect size measure did you use?*

    Response:

    Thanks for your question. The effect sizes represent the relative changes of biomarkers in the high-salt period, compared with those in the low-salt period, which were estimated by using linear mixed models to compare the differences between the two intervention periods. We have stated the effect size in the legend of Figure 2.

13. *Figure 2A/C: consider highlighting/labeling a few data points that represent your main findings here already.*

    Response:

    Thank you for the suggestions. We have labeled some data points in the volcano plots in Figure 2.

14. *Figure 3: The Cytoscape-generated species-metabolites network needs to be validated somehow. For microbial species and metabolic networks responsible/associated enzymatic pathways could be tested in a targeted manner using the available shotgun metagenomic dataset.*

    Response:

    Thank you for your insightful suggestions. We extracted microbial genes that were involved in the biosynthesis and metabolism of carnitine/acylcarnitine, and assessed their

associations with isovalerylcarnitine and 16 isovalerylcarnitine-related microbes. We observed that *caiB* (carnitine CoA-transferase), *caiC* (carnitine-CoA ligase), and *TC.BCT* (betaine/carnitine transporter, BCCT family) were positively associated with the plasma levels of isovalerylcarnitine ($P < 0.05$). Of them, *caiB* and *TC.BCT* showed consistent positive associations with 15 and 13 isovalerylcarnitine-related species, respectively ($P < 0.05$). The reduction in microbial abundance may downregulate *caiB* and *TC.BCT* expression, thereby limiting carnitine uptake into bacterial cells and CoA transfer, and subsequently impairing the biosynthesis of acylcarnitines. These findings indicated the probability of gut microbiota involved in the production or metabolism of acylcarnitines.

We have added the results in our revised manuscript: Main text (page 10, lines 198-205); Supplementary Table 10.

15. *Figure 3: Error bars missing on the bar graphs.*

Response:

Thanks for this reminder. We have added error bars in the bar graphs of Figure 3.

16. *Figure S4 needs more information/explanation in the text e.g. regarding CAZy families, sensitivity analyses and actual meaning for the whole manuscript.*

Response:

Thank you for the suggestion. We have added information in the Figure legend of this figure (Supplementary Figure 11 in the revised manuscript) as follows:

"Sensitivity analyses were performed to test the robustness of our findings regarding salt-related and SSBP-related biomarkers.

For **salt-related biomarkers** [including gut-microbial species, microbial functions (KEGG pathways and CAZy families), and metabolites], we conducted two sensitivity analyses:

a. adjusting for cardiometabolic risk factors (BMI, MAP, TC, and glucose levels) during both the low-salt and high-salt interventions, and

b. excluding participants with dyslipidemia to assess the influence of pre-existing metabolic conditions.

For **SSBP-related biomarkers**, we further conducted:

c. additional adjustment for potential baseline confounders, including dietary score, work-related physical activity, alcohol consumption, education, and eGFR, and

d. exclusion of individuals with dyslipidemia.

Effect sizes from the main and sensitivity analyses were visualized as scatter plots. Red dots indicate associations that remained significant ($P < 0.05$) in sensitivity analyses, while blue dots indicate those that did not ($P \geq 0.05$).

BMI, body mass index; CAZy, the Carbohydrate-Active EnZymes database; KEGG, the Kyoto Encyclopedia of Genes and Genomes database; MAP, mean arterial pressure; TC, total cholesterol; SSBP, salt sensitivity of blood pressure."

17. *Page 6 lines 139-140 „…metabolites were associated with salt-related gut microbial species to some extent…". In my view, this kind of somewhat unscientific, not very stringent expression, but also analysis strategy, is emblematic of large parts of the paper, which identify potential targets, but do not sufficiently illuminate their microbial origin or their mechanistic significance.*

Response:

Thanks for your comments. We have revised such sentences to improve scientific rigor and clarity as follows: "A total of 67 salt-related metabolites showed nominally significant associations with various salt-related gut-microbial species and functional pathways ($P < 0.05$), indicating that the changes in gut microbiota may contribute to the changes in metabolite". In addition, we carefully reviewed the manuscript and revised similar statements to strengthen the overall scientific stringency of our descriptions.

Regarding the microbial origin of metabolites, we agree that our current results do not provide sufficient evidence supporting the microbiota source of metabolites. The metabolite of our interest (isovalerylcarnitine) was identified following a stepwise strategy across multiple datasets: discovery in the MetaSalt study, replication in the GenSalt study,

and validation in both animal models and an independent cohort. Through this approach, we identified isovalerylcarnitine as a potential gut microbiota-derived metabolite. To strengthen the causal inference regarding microbial origin of isovalerylcarnitine, we performed an antibiotic experiment in revision. After the intervention for rats, we observed that, compared to rats in the control group, the plasma levels of isovalerylcarnitine significantly decreased in rats with antibiotic cocktail treatment (ACT), and restored in rats with ACT and subsequent fecal microbiota transplantation (FMT). These changes parallelled alterations gut-microbial richness (Supplementary Figure 15-16). These results suggest that gut microbiota are involved in maintaining circulating isovalerylcarnitine levels. Its reduction after antibiotic treatment and restoration following FMT indicate that isovalerylcarnitine is likely a gut microbiota-derived or -modulated metabolite.

Regarding the mechanistic significance of isovalerylcarnitine, our initial experiments results and additional new data suggested that isovalerylcarnitine may exert protective effects by improving endothelial function and mitigating salt-induced tissue remodeling (**as detailed in our response to Question 21**).

We have revised some unscientific expressions and added new experiments in our revised manuscript:

Antibiotic experiment: Main text (page 13, lines 269-274 and page 25, lines 529-534); Supplementary Method; Supplementary Figures 15-16.

Histological assessment: Main text (page 12-13, lines 258-268 and page 24, lines 526-527); Supplementary Method; Figure 5.

18. *Please use consistently new taxonomic nomenclature regarding Bacillota vs. Firmicutes.*

Response:

Thank you for the advice. We have used the new taxonomic nomenclature regarding *Bacillota* vs. *Firmicutes* and other gut microbes at varying taxonomic levels.

19. *The confirmational experiments in rats are interesting. Please disclose all components of the diets. Was a purified diet used? What was used to replace the salt in the so-called low-*

salt diet group - I mean the 7.55% difference in salt content? Or in other words: which component did the HSD rats have less of than the control rats?

Response:

Thank you for the questions. We used non-purified diets for rats in our study, by referring to previous studies.[10,11] According to information disclosed by the supplier, the components in the normal-salt and high-salt diet were identical except for the content of NaCl (**Table R4**):

Table R4. Component of diet used in the control and HSD groups

Component	Control	HSD
Energy density, kcal/kg	3790	
Protein, %	21.5	
Fat, %	11.1	
Carbohydrate, %	67.4	
NaCl, g/kg	4.5	80
Protein source	Fish meal, Chicken meal, Soybean meal	
Fat source	Soybean oil	
Carbohydrate source	Corn, Wheat	
Vitamin	Vitamin A, Vitamin D3, α-tocopheryl acetate, Menadione Sodium bisulfite, Thiamine nitrate, Riboflavin, Pyridoxine hydrochloride, Cyanocobalamin, Niacinamide, D-calcium pantothenate, Folic acid, D-biotin	
Mineral and other micronutrient	Limestone powder, Dicalcium phosphate, Choline chloride, Tribasic copper chloride, Ferrous sulfate, Manganese sulfate, Hydroxy methionine analog chelated zinc, Selenium yeast	

To prepare the 8% NaCl diet, 87 g of NaCl was added to 1000 g of the standard control diet ingredients, resulting in a total weight of 1087 g. The additional NaCl did not replace any specific component of the original formulation. Instead, the increase in total weight led to a slight proportional reduction in all non-salt ingredients, making NaCl account for 8% of the final diet composition.

We have added the components of diets in our revised manuscript: Supplementary Method.

20. *In the methods section the control diet is called low-salt diet. I believe it should be called normal salt.*

Response:

Thank you for the suggestion. We have replaced the description of "low salt" with "normal salt" for the animal experiment in our manuscript.

21. *It is interesting that DSS rats treated with isovalerylcarnitine (subcutaneous osmotic minipumps) have lower blood pressure. However, few valid assumptions are made about the mechanistic cause and no conclusive data are presented. What is the albumin/creatinine ratio (albuminuria) in the urine, what is the natriuresis under isovalerylcarnitine? What do the kidneys and blood vessels look like histologically? Is there less hypertensive damage in these end organs, or signs of oxidative stress? The same applies to the endothelial function experiments. The whole experiment is interesting, but in my view, it has not been sufficiently analysed to draw valid mechanistic conclusions.*

Response:

Thank you for the insightful comments and questions regarding the mechanism of isovalerylcarnitine. In the revised manuscript, the levels of urinary indicators were determined, and the histological assessments of the kidney and aorta were performed in Dahl SS rats.

First, both natriuresis and the albumin/creatinine ratio were significantly elevated in the HSD group compared to controls ($P < 0.05$), with no significant difference between the HSD and HSD + isovalerylcarnitine groups ($P > 0.05$) (**Table R5**).

Table R5. Differences in urinary indicators among different groups of Dahl SS rats

Variable	Group A: Control (n = 7)	Group B: HSD (n = 11)	Group C: HSD + isovalerylcarnitine (n = 11)	*P* A vs. B	*P* A vs. C	*P* B vs. C
Urinary sodium, mmol	29.17 ± 3.61	256.88 ± 64.75	243.40 ± 70.46	1.47×10^{-7}	9.18×10^{-7}	0.6613
Urinary albumin, g/L	1.44 ± 0.37	2.95 ± 1.00	3.19 ± 0.44	0.0033	2.02×10^{-6}	0.5142
Urinary creatinine, μmol/L	2747.00 ± 880.62	2768.50 ± 1364.36	2861.11 ± 1401.04	0.9731	0.8627	0.8857
Albumin/creatinine ratio, mg/g	4.79 ± 1.17	10.37 ± 2.92	11.61 ± 4.25	0.0006	0.0023	0.4464

Urinary indicators were measured for Dahl SS rats, and differences between groups were examined using t-test. HSD, high-salt diet.

Second, we performed immunofluorescence staining to evaluate the expression of endothelial nitric oxide synthase (eNOS) in the aorta, and observed that the expression of eNOS was decreased in the HSD group (P = 0.0014), and restored in the HSD + isovalerylcarnitine group (P = 0.9039), compared with the control group (**Figure R6**). Together with our initial findings in mesenteric arteries, these results suggest that isovalerylcarnitine improves endothelial function.

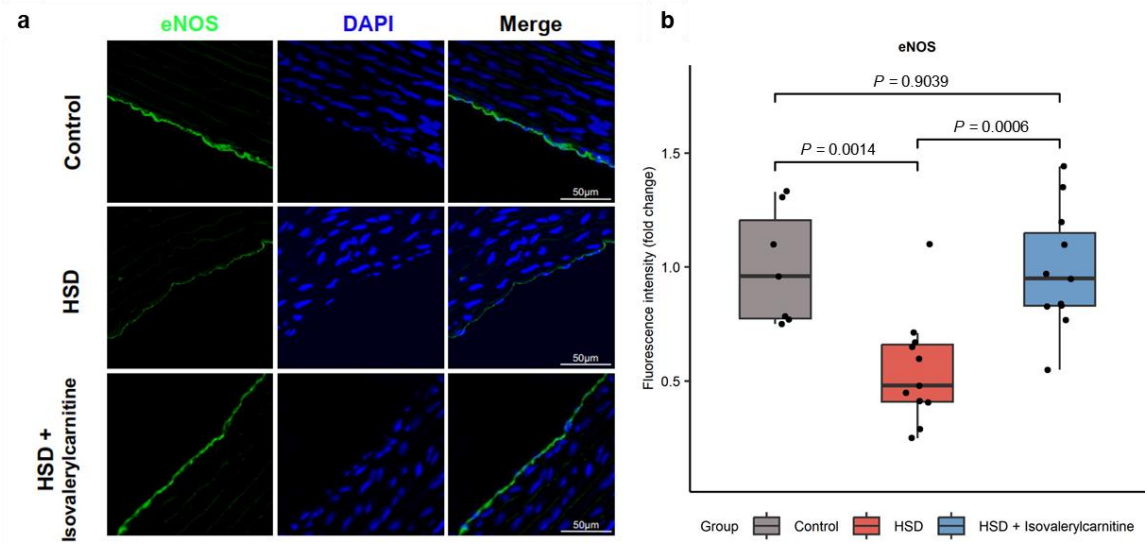


Figure R6. Difference in the fluorescence intensity of eNOS in aorta among different groups of Dahl SS rats

Immunofluorescence staining to evaluate the expression of eNOS in the aorta of Dahl SS rats in the control (n = 7), HSD (n = 11), and HSD + isovalerylcarnitine (n = 11) groups. Differences between groups were tested using t-test. eNOS, endothelial nitric oxide synthase. Bar, 50 μm. eNOS, endothelial nitric oxide synthase; HSD, high-salt diet.

Third, we conducted Masson's and Sirius red staining to assess the pathological changes. Masson's staining revealed a significant increase in collagen deposition in the thoracic aorta of the HSD group compared with the control group ($P = 6.29\times10^{-5}$), which were also reported in previous research,[12] and in the HSD + isovalerylcarnitine group, a marginal reduction in collagen fraction was observed (**Figure R7a and b).** Sirius red staining showed that high-salt intake increased renal cortical fibrosis (P for control vs. HSD = 0.0083), consistent with previous studies.[13-15] Whereas, isovalerylcarnitine inhibited these alterations (P for HSD vs. HSD + isovalerylcarnitine = 0.0192) (**Figure R7a and c**). The finding indicates that isovalerylcarnitine improves HSD-induced kidney injury and exerts protective effects against salt-induced tissue remodeling.

Fourth, we agree on the valuable suggestion regarding the assessment of oxidative stress markers. The accuracy of results from fluorescent probes for key markers of oxidative stress depended on the status of tissue samples. Considering the long storage time of our tissue samples, we failed to perform the assessment of oxidative stress level in this revision, and further work is needed to clarify this issue.

We have added the above results to our revised manuscript:

Histological assessment: Main text (page 12-13, lines 258-268 and page 24, lines 526-527); Supplementary Method; Figure 5.

Urinary indicators: Main text (page 12, lines 246-248); Supplementary Table 15.

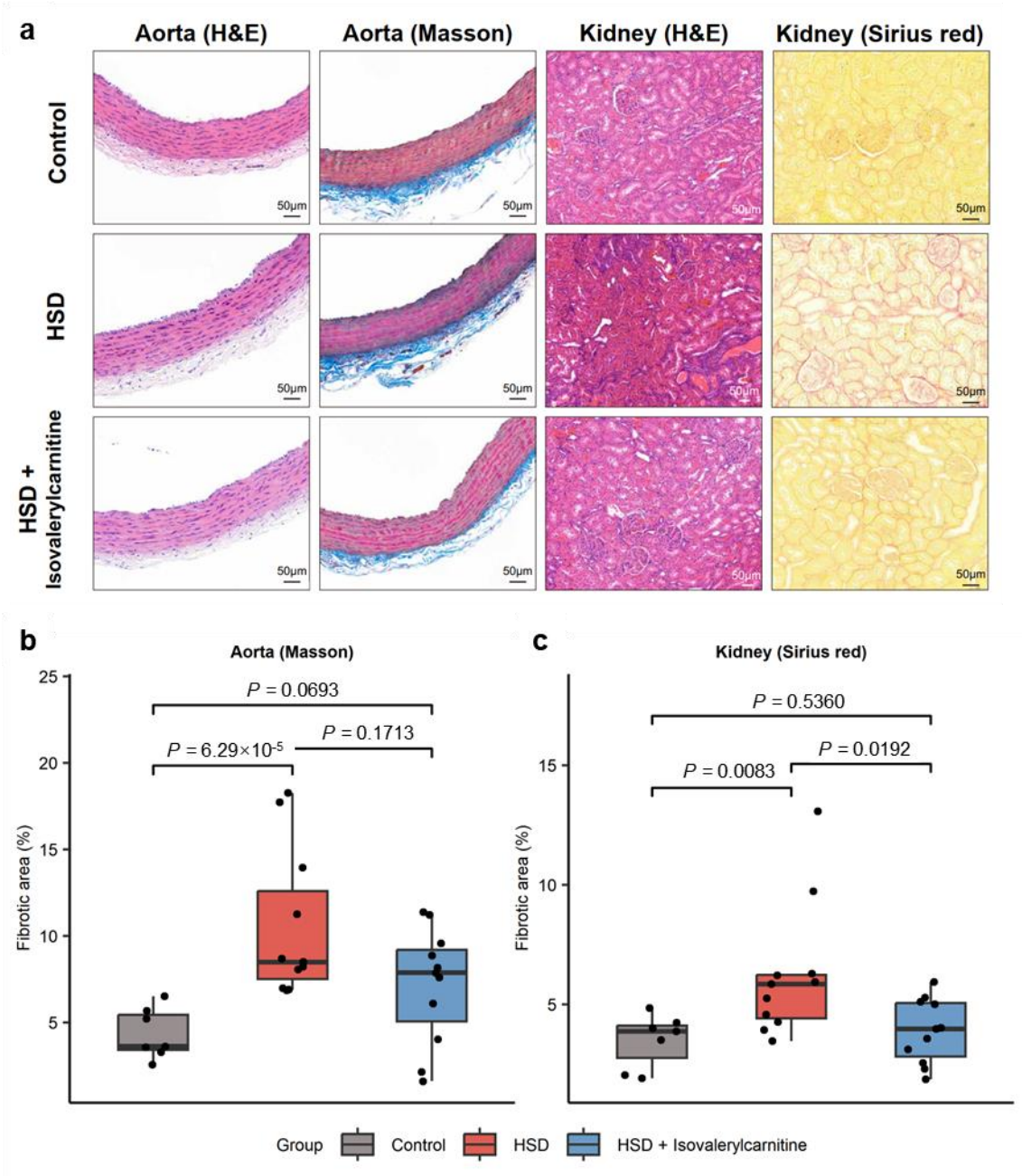


Figure R7. Difference in the histological assessment for kidney and aorta among different groups of Dahl SS rats

a, Sirius red staining for kidney. **b,** Masson's staining for aorta. Staining was performed for the kidney and aorta of rats in the control (n = 7), HSD (n = 11), and HSD + isovalerylcarnitine (n = 11) groups. Differences between groups were tested using Wilcoxon rank sum test. Bar, 50 μm HSD, high-salt diet.

22. *Isovalerylcarnitine was administered subcutaneously. Are similar effects to be expected with oral administration if the microbiome is also involved?*

Response:

Thank you for the question. In our revision, we conducted an additional intervention experiment in which subcutaneous administration of isovalerylcarnitine was replaced with oral delivery. To determine an oral dosage, we referred to previous studies involving L-carnitine or acylcarnitine,[2,16] and performed a pharmacokinetic analysis in which rats received either oral or subcutaneous administration of isovalerylcarnitine. Plasma concentration-time curves were recorded from 0 to 4 hours. When comparing 2 mg/kg oral administration to 200 μg/kg subcutaneous injection (the dosage used in our initial experiment), the plasma profiles were comparable (P = 0.2480) (**Figure R8a**), suggesting that an oral dose of 2 mg/kg could approximate the plasma concentration achieved by subcutaneous delivery. For the subsequent intervention study, the compound was administered via drinking water. Given an average body weight of 0.3 kg and a daily water intake of 80 ml per rat, the required drug concentration in drinking water was calculated as:

$$2\ \text{mg/kg} \times 0.3\ \text{kg} \div 80\ \text{ml} = 7.5\ \mu\text{g/ml}.$$

Following by pharmacokinetic analysis, 20 Dahl-SS rats were randomly allocated to three groups: A, control group (0.45% NaCl, n = 4); B, HSD group (8% NaCl, n = 8); and C, HSD + oral isovalerylcarnitine (8% NaCl in diet and 7.5 μg/ml isovalerylcarnitine in drinking water, n = 8) (**Figure R8b**). All rats were intervened for 28 days, and BP levels were measured on days 0, 7, 14, 21, and 28 using a tail-cuff system. We observed that, compared with the HSD group, rats in the HSD + oral isovalerylcarnitine group showed a significant SBP decline (161.82 ± 8.89 mmHg vs. 151.31 ± 5.55 mmHg, P = 0.0132) (**Figure R8c**). This result indicates that the oral administration of isovalerylcarnitine shows a similar effect to subcutaneous administration through an osmotic mini-pump.

We have added the experiment to our revised manuscript: Main text (page 12, lines 248-251 and page 25, lines 529-534); Supplementary Methods; Supplementary Figure 14.

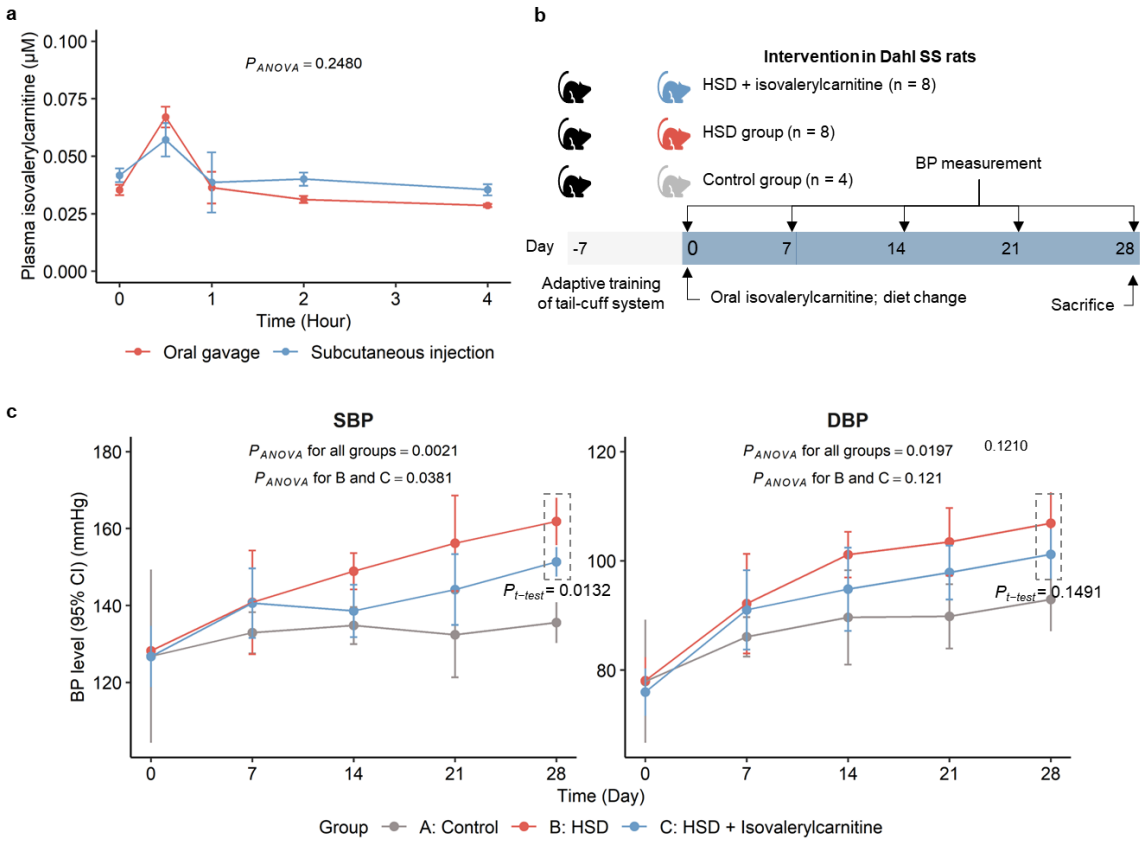


Figure R8. Flow chart and results of the intervention experiment of oral isovalerylcarnitine in Dahl SS rats

a, Plasma concentrations of isovalerylcarnitine after oral gavage (20 mg/kg) or subcutaneous injection (200 μg/kg) in a pharmacokinetic analysis (n = 3 per group). **b,** Flow chart of the intervention experiment of oral isovalerylcarnitine. **c,** Difference in BP levels between groups. Twenty Dahl SS rats were randomly allocated to three groups: control (n = 4), HSD (n = 8), and HSD + oral isovalerylcarnitine (n = 8) groups. Differences among groups were tested using repeated measurement ANOVA or t-test. ANOVA, analysis of variance; DBP, diastolic blood pressure; HSD, high-salt diet; SBP, systolic blood pressure.

23. *Isovalerylcarnitine is used diagnostically for a rare metabolic disease: isovaleric acidemia caused by a deficiency of isovaleryl-CoA dehydrogenase. Isovalerylcarnitine is elevated in this case. To what extent must this be taken into account for the translational perspective? Is the expression of isovaleryl-CoA dehydrogenase in animal experiments influenced by high salt? If so, what are the reasons?*

Response:

Thanks for your insightful question. Isovalerylcarnitine is known to increase in isovaleric acidemia due to isovaleryl-CoA dehydrogenase (IVD) deficiency. In contrast, we observed a decrease in plasma isovalerylcarnitine after the high-salt intervention. To explore whether this change was linked to IVD, immunofluorescence staining was performed to measure IVD expression in the kidney tissue of Dahl SS rats (**Figure R9a**). We found that high-salt intake did not significantly affect IVD expression, nor did isovalerylcarnitine supplementation alter IVD levels (all $P > 0.05$) (**Figure R9b**). The plasma isovalerylcarnitine levels and IVD fluorescence intensity showed no significant correlation ($R^2 = 0.0052$, $P = 0.7367$). In addition, we also measured isovaleric acid (the precursor of isovaleryl-CoA) and observed no significant differences among these groups (all $P > 0.05$) (**Figure R9c**). These findings suggest that the observed reduction in isovalerylcarnitine occurs independently of IVD and isovaleric acid. Therefore, we considered that supplementing isovalerylcarnitine to prevent hypertension is less likely to cause the symptoms of isovaleric acidemia.

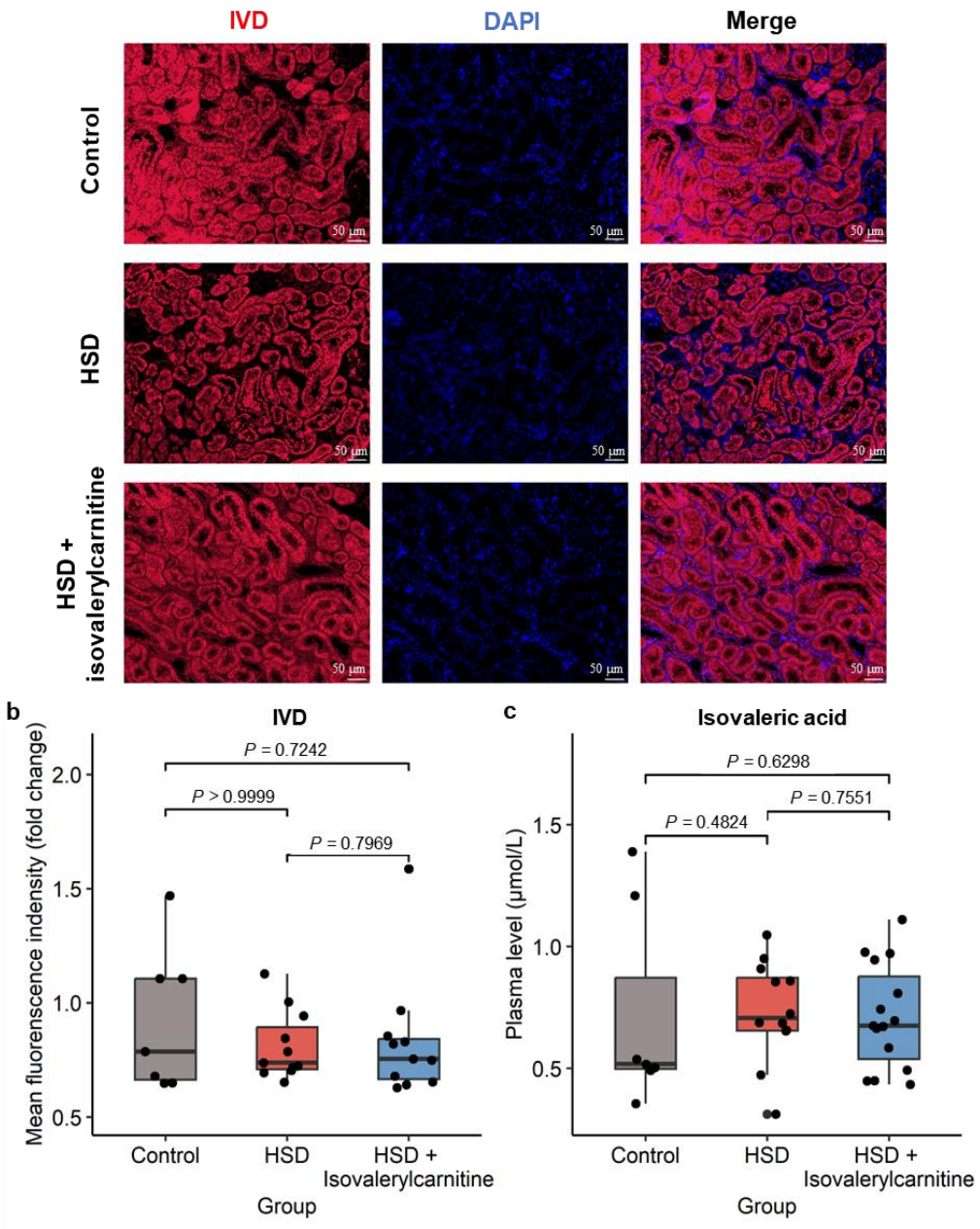


Figure R9. Differences in the expression of kidney IVD and the plasma level of isovaleric acid among different groups of Dahl SS rats

IVD expression and isovaleric acid level were measured for Dahl SS rats in the control (n = 8), HSD (n = 11), and HSD + isovalerylcarnitine (n = 11) groups. Differences between groups were tested using Wilcoxon rank sum test. Bar, 50 μm. HSD, high-salt diet. IVD, isovaleryl-CoA dehydrogenase

Reviewer #3 (Remarks to the Author):

In the current manuscript, Lin and colleagues investigated the roles of the microbiome and metabolome in salt-sensitivity of blood pressure (SSBP) and hypertension. In these analyses, they identified 85 gut microbial species and 70 metabolites that were altered by high salt. Among these findings, 22 microbial species and 8 metabolites were further associated with SSBP. Furthermore, from an associated gut microbiota-acyl carnitine network, the primary isovalerylcarnitine metabolite was replicated in an independent sample and experimentally validated in rats. This metabolite, which is inversely associated with SSBP, was shown to reduce the risk of incident hypertension in a prospective cohort. In total, this was a well-conducted study with triangulating support from human and animal studies. The findings could have potential implications for the prevention of hypertension, identifying a metabolite that could potentially be altered to decrease the risk of hypertension. However, a few minor questions/concerns should be addressed.

Response:

We are grateful for your review of our manuscript and for providing constructive suggestions and comments. We have provided point-by-point responses to each question as follows. We hope these responses could address your concerns.

1. *It is unclear how thresholds for determining SSBP degree were determined. For example, salt-sensitive individuals were categorized as those with greater than a 10 mmHg response to the high or low sodium diet. Were these arbitrary thresholds? Based on some sort of indicator of risk?*

   Response：

   Thank you for the questions. We referred to some previous studies,[3-6] and established the following criterion to determine the SSBP degrees in our study as follows:

   Extreme SS individuals: $\Delta MAP_{L-B} \leq -10$ mmHg or $\Delta MAP_{H-L} \geq 10$ mmHg;

   Moderate SS individuals: $\Delta MAP_{L-B} \leq -5$ mmHg and > -10 mmHg or $\Delta MAP_{H-L} \geq 5$ mmHg and < 10 mmHg;

   SR individuals: the remaining participants.

This definition was presented in the Method section **(Main text: page 21, lines 453-459**). It must be acknowledged that there is currently no universal criterion for determining SSBP.[17] The SSBP trait in humans exhibits a Gaussian distribution, which requires the selection of arbitrarily chosen cutoffs for BP change magnitudes based on salt-intervention trials.[17] Different studies have adopted varying threshold to classify SS and SR individuals, using either absolute or relative changes in MAP following salt loading (high-salt) or depletion (low-salt).[3,4]

A 5 mmHg change in MAP was commonly used as a threshold by previous studies.[6,18] Notably, the GenSalt study, conducted by our team using a protocol similar to the MetaSalt study, also adopted a 5 mmHg change to categorize SS and SR individuals, and demonstrated a significant association between metabolic syndrome and SS status.[5] This prior finding provided a foundation for investigating metabolic signatures underlying SSBP in the present study. To ensure consistency and comparability with the GenSalt study, we thus adopted the 5 mmHg threshold. Moreover, a more stringent threshold of 10 mmHg was applied to identify individuals with more extreme SS status, thereby enhancing the statistical power and robustness of downstream analyses.

2. *In analyses investigating associations of SSBP with changes in metabolites, was SSBP modeled as an ordinal or categorical variable?*

Response:

Thanks for your question. We first included SSBP as an ordinal variable in models, and screened SSBP-related biomarkers according to $P < 0.05$. We also included SSBP as a categorical variable to further assess the differences in SSBP-related biomarkers across different groups of SSBP, with the SR group as the reference, and the adjusted average changes of gut microbes and metabolites in each group were estimated.

We have detailed these contents in the revised manuscript: Main text (page 30, lines 647, 651-653).

3. *In the volcano plots showing the microbial species and metabolite responses to high salt, there is notable asymmetry in the plot, with the vast majority of significant findings demonstrating depletion on high salt. What is the possible explanation for this finding? Is this related to the antimicrobial properties of salt? If so, why would this be more substantial for the metabolite plot?*

Response:

Thank you for the insightful questions. We considered that the asymmetry observed in the volcano plots was more likely attributable to the antimicrobial properties of salt. High-salt intake may suppress the growth of most gut microbes, thereby reducing the production of gut microbiota-derived metabolites. In our study, we observed that 67 of 70 salt-related metabolites were significantly associated with salt-related species (Supplementary Figure 8), and 52 of them were annotated to a microbiota source using "MetOrigin" tool based on known databases (Supplementary Table 6). These findings demonstrate that the observed reductions in metabolite levels may, at least in part, result from salt-induced shifts in the gut microbiota.

Regarding your question on why the metabolites plot exhibited more pronounced changes, we thought that this may be due to the multiple pathways through which high-salt intake affects circulating metabolites, including both microbial and host-mediated factors:

(1) Many metabolites originate not only from gut microbes but also directly from dietary sources (Supplementary Table 6). High-salt diet may alter digestive enzymes activity and pancreatic secretion, impairing gastrointestinal digestion and absorption.[19] This may lead to reduced systemic levels of certain metabolites.

(2) High-salt diet increases the metabolic and excretory burden on the kidneys, leading to a decrease in the absorption of some metabolites.

In contrast, the microbiome data have inherent statistical limitations. The compositional nature (relative abundance ranging from 0 to 1), sparsity, skewed distribution, and limited dynamic range—despite transformation—may reduce the sensitivity to detect changes, especially at the species level. These characteristics may partly explain the less substantial findings in the microbial volcano plots.

We have added some discussion in our revised manuscript as follows:

"Interestingly, the majority of these metabolites were decreased by the high-salt intervention, possibly due to the antimicrobial properties of salt. A large proportion of them were associated with salt-related gut microbiota and could be annotated to microbial origins using the MetOrigin tool. In addition to microbiota-derived pathways, dietary components also contribute to the plasma metabolome. High-salt intake may impair digestion and absorption by altering enzyme activity and pancreatic function, thereby affecting protein and lipid metabolism. Most of the salt-related metabolites identified in our study were amino acids or fatty acids, and enrichment of relevant KEGG pathways, such as fatty acid biosynthesis, supports this explanation." (Main text: page 16, line 333-341).

4. *Similar to previous work in the GenSalt study, participants were categorized as 'salt sensitive', 'moderately salt-sensitive', and 'salt resistant'. In that report, salt-resistance of blood pressure was also associated with an increased risk of incident hypertension. Were there any findings of microbiota or metabolites that were associated with decreased SSBP (or salt resistance) that increased the risk of hypertension? Is it possible to determine from your analyses? Some discussion of this issue would be of interest to the informed reader.*

Response:

Thanks for your insightful questions and suggestions. As you mentioned, the recent work based on the GenSalt study provided an interesting and novel observation—the positive association between SR and the risk of incident hypertension.[20] Since the participants of the MetaSalt study have not yet been followed up, we are currently unable to validate this association. Thus, in our study, we continued to assume the linear relationship between SSBP degrees and hypertension.

In response to your suggestion, we further evaluated the relationships of the eight SS-related metabolites identified in our study with incident hypertension. We observed that carnitine—a metabolite that was inversely associated with SSBP (β = -0.12; 95% CI: -0.21, -0.03)—was positively associated with incident hypertension (HR = 1.197; 95% CI: 1.069, 1.341). However, the effects of carnitine on cardiovascular health may show dual effects and context-dependent,[21-23] which are beyond the scope of the discussion in the present

study. Additionally, its association with SSBP was not validated using samples from the GenSalt study.

We believe that SR may exert certain impacts on the cardiovascular system, and we will conduct follow-up for the MetaSalt participants and elucidate potential biological pathways in the future.

Reference

1. Olsson, L.M.*, et al.* Dynamics of the normal gut microbiota: A longitudinal one-year population study in Sweden. *Cell Host Microbe* **30**, 726-739 e723 (2022).
2. Dambrova, M.*, et al.* Acylcarnitines: Nomenclature, biomarkers, therapeutic potential, drug targets, and clinical trials. *Pharmacol. Rev.* **74**, 506-551 (2022).
3. Elijovich, F.*, et al.* Salt sensitivity of blood pressure: A scientific statement from the American Heart Association. *Hypertension* **68**, e7-e46 (2016).
4. Liu, Y., Shi, M., Dolan, J. & He, J. Sodium sensitivity of blood pressure in Chinese populations. *J. Hum. Hypertens.* **34**, 94-107 (2020).
5. Chen, J.*, et al.* Metabolic syndrome and salt sensitivity of blood pressure in non-diabetic people in China: a dietary intervention study. *Lancet* **373**, 829-835 (2009).
6. Gupta, D.K.*, et al.* Effect of Dietary Sodium on Blood Pressure. *JAMA* **330**, 2258-2266 (2023).
7. Xiao, X.*, et al.* Dietary patterns and cardiometabolic risks in diverse less-developed ethnic minority regions: results from the China Multi-Ethnic Cohort (CMEC) Study. *Lancet Reg. Health West Pac.* **15**, 100252 (2021).
8. Bingham, S.A.*, et al.* Validation of dietary assessment methods in the UK arm of EPIC using weighed records, and 24-hour urinary nitrogen and potassium and serum vitamin C and carotenoids as biomarkers. *Int. J. Epidemiol.* **26 Suppl 1**, S137-151 (1997).
9. He, J.*, et al.* Gender difference in blood pressure responses to dietary sodium intervention in the GenSalt study. *J. Hypertens.* **27**, 48-54 (2009).
10. Yan, X.*, et al.* Intestinal flora modulates blood pressure by regulating the synthesis of intestinal-derived corticosterone in high salt-induced hypertension. *Circ. Res.* **126**, 839-853 (2020).
11. Bier, A.*, et al.* A High Salt Diet Modulates the Gut Microbiota and Short Chain Fatty Acids Production in a Salt-Sensitive Hypertension Rat Model. *Nutrients* **10**, 1154 (2018).

12. Grigorova, Y.N., *et al.* Aortic Fibrosis, Induced by High Salt Intake in the Absence of Hypertensive Response, is Reduced by a Monoclonal Antibody to Marinobufagenin. *Am. J. Hypertens.* **29**, 641-646 (2016).
13. Wang, Y., *et al.* Salt-induced epithelial-to-mesenchymal transition in Dahl salt-sensitive rats is dependent on elevated blood pressure. *Braz. J. Med. Biol. Res.* **47**, 223-230 (2014).
14. Chakraborty, S., *et al.* Salt-responsive metabolite, beta-hydroxybutyrate, attenuates hypertension. *Cell Rep.* **25**, 677-689 e674 (2018).
15. Yang, P., *et al.* Protective effect of oral histidine on hypertension in Dahl salt-sensitive rats induced by high-salt diet. *Life Sci.* **270**, 119134 (2021).
16. Mate, A., Miguel-Carrasco, J.L. & Vázquez, C.M. The therapeutic prospects of using L-carnitine to manage hypertension-related organ damage. *Drug Discov. Today* **15**, 484-492 (2010).
17. Afolabi, J., *et al.* Salt Sensitivity of Blood Pressure. *Circ. Res.* **134**, 1234-1239 (2024).
18. Zhang, F., *et al.* Association of Serum Metabolites and Salt Sensitivity of Blood Pressure in Chinese Population: The EpiSS Study. *Nutrients* **15**, 690 (2023).
19. Wang, C., *et al.* High-salt diet has a certain impact on protein digestion and gut microbiota: A sequencing and proteome combined study. *Front. Microbiol.* **8**, 1838 (2017).
20. He, J., *et al.* Sodium Sensitivity, Sodium Resistance, and Incidence of Hypertension: A Longitudinal Follow-Up Study of Dietary Sodium Intervention. *Hypertension* **78**, 155-164 (2021).
21. Zhao, J.V., Burgess, S., Fan, B. & Schooling, C.M. L-carnitine, a friend or foe for cardiovascular disease? A Mendelian randomization study. *BMC Med.* **20**, 272 (2022).
22. DiNicolantonio, J.J., Lavie, C.J., Fares, H., Menezes, A.R. & O'Keefe, J.H. L-carnitine in the secondary prevention of cardiovascular disease: systematic review and meta-analysis. *Mayo Clin. Proc.* **88**, 544-551 (2013).
23. Ussher, J.R., Lopaschuk, G.D. & Arduini, A. Gut microbiota metabolism of L-carnitine and cardiovascular risk. *Atherosclerosis* **231**, 456-461 (2013).

Reviewer #4 (Remarks to the Author):

I´ve been asked to comment on the revisions performed to Reviewer #1´s concerns.

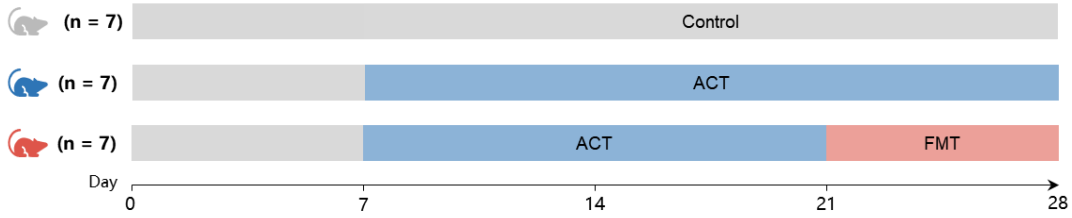
ORIGINAL REVIEWER QUERY 1: The authors have included an FMT experiment to demonstrate that the gut microbiota is a source of isovalerylcarnitine, as per the original reviewer's request. The supplementary methods state that this was a 28 day experiment – with a control group, an antibiotic treatment and an antibiotic treatment+FMT group. The following additional method details would be of benefit:

How long was the antibiotic treatment provided for? And thus how long into the 28 day experiment was it stopped? (perhaps a schematic would assist to make this clear)

Was the decrease in bacterial load following antibiotic treatment confirmed prior to FMT treatment? Regarding the FMT preparation, was deoxygenated PBS used? Or any efforts to complete the FMT preparation in an anaerobic environment? Was the FMT delivered fresh or frozen? If frozen was any cryopreservant used? It could benefit to check the GRAFT reporting guidelines for animal FMT experiments (https://pmc.ncbi.nlm.nih.gov/articles/PMC8489962/) for ensuring an adequate level of details is reported.

Response:

Thanks for your insightful comments regarding the FMT experiment. The study design included a 1-week adaptation feeding period followed by a 3-week intervention. The antibiotic treatment group received antibiotics for 3 weeks, and the antibiotic + FMT group received 2 weeks of antibiotic treatment followed by 1 week of FMT. To improve clarity, we have provided a schematic of the experimental timeline in the revised manuscript (**Supplementary Fig. 15a**).



The antibiotic cocktail (0.5 g/L vancomycin, 1 g/L neomycin sulfate, 1 g/L metronidazole, 1 g/L ampicillin) was administered via drinking water, and this antibiotic regimen has been previously validated to effectively deplete detectable commensal bacteria (*Blood 129, 729-739 (2017)*). As expected, we observed a reduced bacterial colony on the petri dishes using fecal specimens collected on day 21, when compared with baseline. For FMT preparation, we used

pre-reduced PBS supplemented with 0.05% L-cysteine hydrochloride to minimize oxygen exposure, and all procedures were performed within an anaerobic chamber to preserve microbial viability. In our study, the FMT suspension was administered in fresh status, without freezing or the use of cryopreservants.

Furthermore, we have carefully reviewed the GRAFT reporting guidelines and ensured that our revised manuscript (**Supplementary Methods, Pages 9-10**) now includes all relevant details regarding the FMT procedures and experimental design to meet the recommended standards.

ORIGINAL REVIEWER QUERY 18: The original reviewer made the point that bacterial taxonomy has been updated in 2021 (https://ncbiinsights.ncbi.nlm.nih.gov/2021/12/10/ncbi-taxonomy-prokaryote-phyla-added/), and now the name for Firmicutes is Bacillota, etc…For Firmicutes this has been updated to Bacillota in the text…however is still Firmicutes in the figures (e.g. Firmicutes bacterium CAG:124, etc). This inconsistent phyla nomenclature between the text and figures may be confusing for readers.

Response:

We thank the reviewer for pointing out the inconsistency in phylum nomenclature. In the last version of manuscript, we have re-annotated all taxa using the latest NCBI taxonomy, in which *Firmicutes* has been reassigned to the phylum *Bacillota*. Accordingly, the text has been updated to use *Bacillota* throughout. Regarding the names in the figures (e.g., "*Firmicutes bacterium CAG:124*"), these labels originate from the original placeholder names assigned to metagenome-assembled genomes (CAG: Cluster of Assembled Genomes). For some of these gut-microbial taxa, the 2021 taxonomy update revised only the phylum-level classification, whereas their species-level placeholder names remain unchanged in current databases. This is illustrated by *Firmicutes bacterium CAG:124*, whose latest taxonomy record still retains the original species label (https://www.ncbi.nlm.nih.gov/datasets/taxonomy/tree/?taxon=1263002). Therefore, while we have updated the phylum annotation from *Firmicutes* to *Bacillota* in our analyses, the species labels (e.g., "*Firmicutes bacterium CAG:XXX*") are retained to maintain consistency with the NCBI taxonomy and established metagenomic nomenclature.

The remainder of the original reviewer questions I believe to be adequately addressed in this revision.

Response:

We are grateful for the time and effort in reviewing our manuscript and the original reviewer's questions.

Reviewer #4 (Remarks on code availability):

Without any input data, I cannot run and evaluate the code.

Just reading the code, I can identify a few areas of concern that would limit the ability for the code to be used by others:

SECTION 2: SSBP BIOMARKERS

Line 138-303, the section "2) - Identification of SSBP-related biomarkers":

Each subsection in this section starts with a melt of a dataframe called "chgdata" before subsequent commands are run.

e.g. line 141: temp <− melt(chgdata, c("labid2", "famid", "ss", "age", "gender", "bmi", "fcc", "htn", "smoking", "chol"), metasalt.sr.species$speid)

It's not clear where this chgdata object is coming from or what format it is…so this section of the code is not reusable by others/cannot reproduce results.

SECTION 3: ANIMAL DATA

I understand the privacy issues related to human data – however the data for the animal experiments could be included – e.g. if alldata.wistar, alldata.dahl, alldata.sd were uploaded to the github as a .rds object, then I could run this code and check the reproducibility of this section at least.

Response:

We sincerely thank the reviewer for the comments and careful evaluation of our code availability. Following your suggestions, we have separately uploaded a randomly selected subset of the MetaSalt, GenSalt, and cohort participants (20%) as example datasets to enable verification of the analytical code used in this study. Of note, the results generated from these data do not correspond to the true findings reported in the article. Regarding the animal experiments, we have uploaded all datasets used in this study as .rds files to GitHub

(https://github.com/Shinarlin/SSBP-and-multiomics). Analyses based on these files fully reproduce the results presented in the manuscript.

Reviewer #5 (Remarks to the Author):

I have been asked to comment on the revisions made in response to Reviewer #3's concerns regarding the revised manuscript by Lin et al. After thoroughly reviewing the authors' responses and the corresponding changes in the manuscript, I can confirm that the authors have provided comprehensive and satisfactory answers to each of the reviewer's comments. The manuscript has been appropriately amended with relevant references and additional content where necessary.

Response:

We are grateful for the time and effort in reviewing our manuscript and the original reviewer's questions.