

A gut microbial odd-chain fatty acid alleviates atherosclerosis in mice

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The manuscript by Yin et al. describes a series of studies suggesting that *Bacteroides uniformis* (BU) alleviates atherosclerosis via the production of the long chain fatty acid pentadecanoic acid (PA). The authors demonstrate that PA supplementation reduces atherosclerosis, which is a novel finding. They also show that both BU and PA enhance LDL receptor (Ldlr) expression, facilitating the clearance of LDL cholesterol and free cholesterol without impacting HDL cholesterol levels. Furthermore, PA supplementation inhibits HMG-CoA reductase (encoded by *Hmgcr*), the rate-limiting enzyme in cholesterol biosynthesis, indicating diminished hepatic cholesterol synthesis. However, BU colonization paradoxically increases *Hmgcr* expression, which raises questions about the mechanistic consistency. Using a small sample size, the authors provide human correlative data, where they show that PA levels are lower in cardiovascular disease (CVD) patients compared to healthy controls. Additionally, their reanalysis of publicly available datasets reveals a reduced abundance of *Bacteroides* strains in individuals with CVD. These findings contribute to our understanding of microbiome-host interactions in lipid metabolism.

While previous studies have shown that *Bacteroides* species can alleviate atherosclerosis and that certain bacteria produce PA (see comments below), these findings in isolation are not entirely novel. However, this study uniquely bridges the gap between bacterial PA production and its direct role in suppressing atherosclerosis, providing an interesting link between microbiota-derived metabolites and host lipid metabolism. The comprehensive experimental approach incorporates in vitro assays, cell lines, and mouse models to investigate BU-derived PA's impact on lipid metabolism. While the study is well-executed, there appear to be several major limitations: The reliance on correlative human data with a limited sample size, the exclusive use of male mice, and mechanistic inconsistencies weaken the generalizability of the findings. Additionally, the observed increase in PA levels with BU colonization is intriguing, but the high baseline PA levels in control mice raise questions about its origin, which remains unresolved.

Major Comments

1. The authors propose that PA inhibits HMGCR activity while BU supplementation increases *Hmgcr* expression (Fig. 2B). However, with respect to the lesion formation, cholesterol synthesis and clearance they follow similar trends. This contradiction needs to be addressed. Similarly, *del-pta* and *del-mutA* mutants, compared to WT, produce less PA, associated with reduced *Hmgcr* expression and yet exhibit increased lesion formation. These findings suggest that BU's effects may extend beyond PA production, potentially involving indirect modulation of gut microbiota or other bioactive molecules. Further mechanistic exploration is required to reconcile these inconsistencies. I'm having a hard time understanding the relationship between *Hmgcr* expression and cholesterol synthesis/levels.

2. Previous studies have demonstrated that *Bacteroides* species can mitigate atherosclerosis and hyperlipidemia through different mechanisms (PMID: 30571343, PMID: 34083551). The authors should acknowledge these findings and discuss how their work advances our understanding of *Bacteroides*-mediated effects on lipid metabolism and cardiovascular health.

3. The manuscript includes data comparing high-fat diet (HFD) and chow diet conditions. Given the well-established pro-atherogenic effects of HFD, this comparison may be redundant unless BU/PA effects are explicitly diet-dependent. Clarifying

whether the intent is to demonstrate diet-dependent effects would enhance focus.

4. While PA levels were measured in feces and liver, systemic circulation levels were not reported. Since PA's anti-atherogenic effects are presumed to act systemically, plasma or serum measurements are critical for understanding its role in reducing atherosclerosis. Additionally, in PA supplementation experiments, assessing circulating PA levels would provide proof-of-concept.

5. The study used only male mice without justification. The authors should either justify their choice or expand their study to include female mice to ensure broader applicability.

6. Control mice exhibit high baseline PA (Fig. 3K). It remains unclear whether PA is host-derived or produced by other bacteria, especially since PA production has been documented in other species (PMID: 39468026; PMID: 37386075). Conducting experiments with germ-free ApoE KO mice mono-colonized with BU WT or PA-deficient mutants would have provided mechanistic clarity to distinguish BU-specific effects from broader microbiome influences. I also recognize this is a lot of work.

7. Bacteroides species strong component of the mouse microbiota. How does a gavage with BU (>100X less Bacteroides than what is in the gut) increases PA so dramatically? Do the mouse Bacteroides not make PA? Please explain. Also, it would be useful to know the relative abundance of the gavaged BU to other Bacteroides in the gut.

Minor Comments

1. Line 59: Replace "systemic" with "systematic."

2. Line 680: Replace "confirm" with "confirm."

3. Line 110: Italicize Bacteroides.

4. Extended Data Fig. 2B: Replace "Number of gene" with "Number of genes."

5. Do the precursor levels (acetate and propionate) go down whenever there is increased PA synthesis?

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)

This manuscript by Yin et al. describes the discovery and mechanism behind the ability of Bacteroides spp. to lower cholesterol in mice and humans. It has long been known that a higher Firmicutes/Bacteroides ratio in the gut microbiome is associated with obesity and atherosclerosis, however, despite this knowledge existing for over 20 years, little functional understanding of why this ratio associates with these disease phenotypes was available. This study is highly relevant and important because it now provides a mechanistic understanding as to why Bacteroides bacteria are beneficial to these disease states. The study shows that many Bacteroides spp. produce a 15 carbon fatty acid pentadecanoic acid, which itself has been associated with metabolic health. After the initial work showing a reduction of these bacteria in the gut microbiome of those with atherosclerosis, and the ability of strains to reduce the disease in a mouse model the authors go through extensive experimentation and chemical purifications to identify and characterize the bioactive metabolite of interest. I applaud the authors in their well-rationalized and additive evidence for the role of PA in ameliorating atherosclerosis and the results of this study could lead to direct and significant medical impacts. However, there is not sufficient evidence that PA is the bioactive metabolite of interest and many of the experiments are lacking important controls.

My principal critique of this study is that the authors have not sufficiently demonstrated that PA, and only PA, is the bioactive metabolite. They themselves show that other fractions in their chemical isolations also have anti-atherosclerotic activity, but they do not investigate this further. This alone is not the only issue, it seems possible that other fatty acids of similar chain lengths may have similar inhibitory action on HMG-CoA reductase, but these are not tested. A very simple and important control that is needed throughout many of the experiments is at least a C-16 fatty acid and a C-17 fatty acid. I recognize that repeating the experiments with completely new variables is a monumental task for what is already a large study, but targeted evidence that these other fatty acids do not act similarly is required. Here are some examples where these controls would be particularly useful.

1. Are other carbon chain length fatty acids present in the other bioactive fractions? Cpd1? Cpd2? Cpd7? For example?
 2. How much C16 and C17 fatty acids are in BHIS medium with B. uniformis?
 3. What are the total cholesterol levels in HepG2 cells treated with C16 or C17 fatty acids?
 4. Do these other fatty acids inhibit HMG-CoA reductase?
 5. Do mice fed C16 or C17 fatty acids also have better atherosclerosis outcomes?
 6. Are the human fecal samples with atherosclerosis also enriched in these other fatty acids?
- These data are needed to determine the specificity of the B. uniformis action through PA.

Other critiques:

- Atherosclerosis should be described briefly in the introduction. The wide audience of Nature may not understand what this is.
- The experiments and results in lines 144-153 are difficult to follow and not explained well. This section should be expanded to aid the reader.
- The experiments treating cells with the various extracts are not very well explained. What are the controls shown in Extended data Fig. 3. Can the authors show that the chemical extracts are not harming the cells in any way? Perhaps that's what the controls are showing but it's difficult to understand.
- It is not clear that the heat killing procedure demonstrates what the authors propose it does and the details of the heat-killing are not provided. It is possible that the C15 fatty acid is part of the membrane or cell surface of *B. uniformis*. Demonstration that the heat killing has destroyed the cells is important to prove that it is in fact extirpated actively and not just part of the membranes.
- The authors statement in line 343-344 that the mutants mitigate atherosclerosis is not well supported. They show evidence that it reduces plaque formation, but other data is required to claim 'atherosclerosis' has been ameliorated.

Referee #4

(Remarks to the Author)

Yin et al. explored the impact of the bacterial metabolite pentadecanoic acid (PA), produced by *Bacteroides* species, on atherosclerosis. The study suggests that PA reduces atherosclerotic burden, likely by decreasing cholesterol synthesis through inhibition of HMG-CoA reductase and enhancing LDL receptor (LDLR)-mediated cholesterol clearance. This is a timely and interesting paper, and the authors have performed extensive work to narrow down the active metabolite among those produced by *B. uniformis*.

This manuscript presents an intriguing hypothesis and substantial experimental effort. However, major revisions are needed to improve data presentation (especially figure clarity), plaque quantification methodology, inflammation assessment, and mechanistic understanding.

Major comments:

1. Figure Clarity and Quantification Approach

- Several figure legends are too small to read clearly, and critical images (e.g., of the aortic sinus and aorta) are also presented at a scale that makes interpretation difficult.
- Plaque Quantification: A key methodological limitation is the lack of comprehensive plaque quantification throughout the entire aortic sinus. Accurate assessment requires serial sectioning and quantification across the whole region. Some images, such as Figure 3H (CHD group), appear to be taken at non-comparable anatomical levels of the sinus, which may compromise the validity of comparisons. Also, some images labeled as representative do not visibly show plaques stained in red, which is inconsistent with the quantification data reported. This discrepancy undermines the reliability of the visual data. A more rigorous, standardized method of plaque quantification (as described in references such as *Vascular Biology* 2024; 6(1):e230017, DOI: 10.1530/VB-23-0017) is recommended.

2. Mouse Model Relevance

- The authors used ApoE^{-/-} mice on a high-fat diet (HFD), a model that is highly inflammatory and may not accurately reflect moderate or chronic human disease. Validation of the findings in ApoE^{-/-} mice on a regular chow diet would be more physiologically relevant, as these mice develop significant atherosclerosis even without dietary fat overload. Notably, the representative plaque images for ApoE^{-/-} mice on chow diet appear to show almost no plaques, despite reported quantification indicating plaque areas of ~50,000–100,000 μm^2 . Again, image resolution and legend legibility make these data difficult to verify.
- Since one of the proposed mechanisms involves LDLR upregulation, the effect of PA and *B. uniformis* should be tested in *Ldlr*^{-/-} mice fed high-cholesterol diet to determine the dependence of the observed anti-atherosclerotic effects on LDLR function.

3. Inflammation Assessment

- Inflammation is insufficiently evaluated. CD68 was used as a macrophage marker, but it lacks specificity; CD68 can also be expressed by smooth muscle cells and other cell types. More appropriate markers, such as MOMA-2 or F4/80, should be included for macrophage identification.
- A comprehensive analysis of immune cell infiltration—ideally using flow cytometry on digested aortas—is recommended. This would enable precise quantification of macrophage subtypes, lymphocytes, and other immune cells involved in plaque development.

5. Mechanism of HMG-CoA Reductase Inhibition through PA

The mechanism by which PA inhibits HMG-CoA reductase (HMGCR) remains insufficiently explored. The authors report that PA inhibits HMGCR activity with an IC_{50} of 15.53 μM , whereas atorvastatin has a much lower IC_{50} of 0.05 μM . This large difference suggests that PA is a significantly weaker inhibitor; however, the implications of this disparity are not adequately discussed.

In this context, the paragraph starting at line 430 is overly speculative, asserting that PA is more favorable because it is endogenous and may be more adaptable to the host than statins. This claim lacks sufficient mechanistic support and should be substantiated or revised. Moreover, it remains unclear whether PA acts as a competitive inhibitor at the acetyl-CoA binding site or through a different mechanism. This should be experimentally investigated and clearly discussed.

Finally, the proposed mechanism of action of PA appears to resemble that of statins. However, based on the authors' own results, atorvastatin is clearly more potent in inhibiting HMGCR activity. Therefore, the authors need to reconcile how PA can exert atheroprotective effects similar to atorvastatin despite being a much weaker inhibitor.

Several bacterial species are capable of producing PA, as acknowledged by the authors. Can the authors estimate the specific contribution of *Bacteroides uniformis* to systemic PA levels in vivo, relative to other PA-producing bacteria?

Minor comments

There is a mistake in Extended data Fig. 10 L 7
The abbreviation “CHD” for chow diet is unconventional and should be reconsidered for clarity.

Referee #5

(Remarks to the Author)

In their manuscript, Yin et al. explore how gut bacteria—specifically *Bacteroides uniformis*—may help protect against atherosclerosis by producing a beneficial fatty acid called pentadecanoic acid (PA, C15:0). Using a combination of state-of-the-art microbiome analysis, cell-based tests, and mouse models, the authors show that PA lowers cholesterol levels by inhibiting HMG-CoA reductase, the key enzyme in cholesterol production, and by increasing the removal of LDL cholesterol from the blood. As a result, PA treatment significantly reduces atherosclerotic plaque development in atheroprone mice fed a high-fat diet.

The study presents an interesting idea: that certain gut bacteria can produce compounds with statin-like effects, potentially offering a new way to manage atherosclerotic cardiovascular disease. However, while the inhibitory effect of PA on HMG-CoA reductase is interesting, the novelty of the findings is substantially limited, given the previously reported LDL-cholesterol-lowering effects of PA treatment already tested in humans (e.g., Chooi, Y. C. et al., *Am J Clin Nutr* 119, 788–799, doi:10.1016/j.ajcnut.2023.11.013 (2024)).

The manuscript is clearly written, and the data are presented in a straightforward manner. The following points are suggested to improve the manuscript:

1. The manuscript does not adequately justify the selected dose of *B. uniformis* (10^9 CFU/mouse). The authors should clarify whether this dose was based on prior studies, pilot data, or established therapeutic thresholds. It would also be important to assess whether a dose-response relationship exists—i.e., whether lower or higher doses result in similar or enhanced atheroprotective effects. Moreover, a discussion on how this dosage translates to therapeutically relevant levels in humans would strengthen the translational relevance of the findings.
2. It also remains unclear how long *B. uniformis* persists in the host following oral gavage. The authors are encouraged to provide data, or cite existing literature, on the strain's *in vivo* stability, half-life, and colonization dynamics, including its distribution within host tissues. In addition, information on the circulating levels and pharmacokinetics of PA would be critical to understanding the duration of its therapeutic action and whether sustained exposure is necessary for efficacy.
3. The current experimental design includes measurement of fecal bacterial load at only one time point (Day 4 of Week 12, Extended Data Fig. 1G), without evaluation of baseline levels or longitudinal colonization kinetics. Including multiple time points would provide more detailed insights into bacterial colonization, persistence, and uptake efficiency. The rationale for measuring at a single time point should be clearly explained.
4. The authors are also encouraged to expand the discussion on the long-term therapeutic potential of *B. uniformis*. Given that gut microbial communities and host physiology can change over time, it is essential to address whether the beneficial effects are transient or durable. The manuscript should consider whether short-term administration is sufficient or if sustained or repeated dosing is required to maintain efficacy. Additionally, the influence of host environment on bacterial colonization and function over time should be discussed.
5. Given the reported reduction in intracellular cholesterol, plasma LDL-C levels, and atherosclerotic plaque formation, it would be useful to assess whether *B. uniformis* treatment also affects broader systemic metabolic parameters such as body weight, liver weight, or insulin sensitivity.

6. While the study includes data on monocyte/macrophage involvement in atherosclerosis and inflammation, it lacks a broader characterization of immune cell populations in the mouse model. The authors are encouraged to also explore whether *B. uniformis* and PA modulate other immune pathways, particularly T regulatory cells or atherogenic Th1/Th17 subsets. A more comprehensive immunophenotypic analysis would enhance understanding of the immune-modulatory effects of *B. uniformis* / PA

7. The manuscript notes that PA biosynthesis involves acetate and propionate as precursors (lines 318–320). Since propionate has known atheroprotective and cholesterol-lowering effects, the authors should consider whether the observed outcomes may be influenced by increased levels of these precursors. It would be informative to assess whether acetate or propionate concentrations change following *B. uniformis* administration, potentially contributing to the observed atheroprotection.

Minor points:

1. To improve the clinical interpretation of the findings, the manuscript should incorporate a detailed table of cohort characteristics, including age, sex, presence of hypertension, hyperlipidemia, diabetes, and other relevant factors. These host variables are known to influence both metabolism and gut microbiota composition, and could help determine whether the effects of *B. uniformis* are consistent across diverse patient profiles or specific to certain risk groups.
2. Quantitative analysis and statistical evaluation of the Western blot results should be included to support the conclusions and enhance the methodological rigor of the study.
3. In the figure legends, the precise concentration of PA used in each experiment should be explicitly stated. The authors should also provide a rationale for selecting this concentration and discuss whether it reflects physiologically relevant levels found in the human bloodstream, especially in the context of healthy individuals versus patients with ASCVD.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have addressed most of my previous comments, some with experimental evidence. I really appreciate the additional experiments in response to reviewer comments, which have strengthened several aspects of the study. However, a few issues remain that, in my opinion, require further clarification or textual revision.

1. Some inconsistencies in PA production suggest that microbe-microbe interactions may contribute to the observed phenotype and should be discussed: while BU supplementation increases PA levels in both germ-free and SPF backgrounds, the magnitude of PA production is dramatically higher in SPF mice. This difference implies that community context substantially amplifies PA production beyond what is achieved by BU alone. In addition, Δ pta/mutA mutant strains, which are proposed to lack the sole pathway for PA production, still exhibit substantially higher PA levels than PBS controls. If this pathway were exclusively responsible for PA synthesis, such elevations would not be expected. Together, these observations suggest that other *Bacteroides* species or community-level interactions may contribute to PA production, potentially through metabolic cross-feeding or indirect modulation of microbial pathways. A formal test of this would be ideal, at a minimum, a discussion of potential microbe-microbe interactions and community effects would provide a more balanced interpretation of the data.

2. A key concern is whether supplementation with PA can be verified within relevant biological compartments. If PA administration is effective, one would expect measurable changes in at least one compartment of the system. While the absence of changes in circulating levels is noted, it remains unclear whether PA accumulates in the gut, liver, or other tissues, or whether it is rapidly cleared or excreted. If rapid excretion occurs, corresponding changes in fecal levels would be expected. Without evidence that PA exposure is detectable in any biological compartment, it is difficult to assess confidence in the interpretation of the supplementation experiment.

3. I'm not fully convinced by the statement that host doesn't produce PA. The current data does not rule out host production, particularly given the high baseline PA detected in germ-free mice. This baseline could reflect a combination of dietary input (as cited) and host synthesis. Have PA levels in the diet been directly measured, and do those levels align with what is detected in vivo? At least, the authors should modify the text to better reflect the limitations of the current experimental evidence.

Minor comments:

There are abbreviations that need to be defined, particularly in Figure legends. For example, in Extended data Fig 1, BU-L, BU-H, 1M, 3M etc. are not immediately clear to the reader. In addition, the nature of PBS control requires clarification - whether it corresponds to a 4-week or 12-week time point. Given that atherosclerosis progression is highly time-dependent, the use of appropriately matched longitudinal controls is essential for meaningful comparisons.

Lines 87-90: The sentence repeats the same point about *Bacteroides* abundance in two ways. The authors could combine these statements to avoid redundancy.

Line 178: LDL receptor (LDLR) should be defined earlier.

Lines 281-282: Add citation(s) to "Contrary to the traditional view that dietary 282 components like milk fat and ruminant meat are the primary sources of circulating PA."

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)

I am mostly satisfied with the response to my critiques and suggestions, but still believe that a large question remains about this study that should be addressed. Is the effect from *B. uniformis* and its reduction in atherosclerosis due to PA (15:0) exclusively or is this more about odd-chain fatty acids? The authors describe evidence in their experiments and the literature that C17 also has some effects on improving dyslipidemia, though their data does not indicate that is through HMG-CoA reductase, which is an important result. This new data and the literature brings up the question whether or not this is an effect of odd chain fatty acids or purely PA 15:0. The authors did not include any of the responses to my questions in their revised manuscript (as far as I can find) which is a significant oversight.

If PA C15:0 is exclusive in its action of inhibiting HMG-CoA reductase, then how is C17:0 also lowering cholesterol in their HepG2 cells? I don't expect the authors to answer the second question, that could be a whole study on its own, but this is a very important question and the matter of odd-chain vs. even chain FAs and the specificity of PA 15:0 must be addressed where appropriate.

Referee #4

(Remarks to the Author)

Although the authors have satisfactorily addressed some of the comments, the following concerns remain and should be addressed.

The authors do not accurately quantify atherosclerotic plaques. They analyzed only a single section at the level of the valves, rather than the entire aortic sinus. Even if plaque size is maximal at this level, quantifying plaques from only one

section is not technically appropriate. Therefore, this major concern remains unresolved. The authors need to repeat at least the main experiments and section the entire aortic sinus to obtain accurate plaque quantification. This is particularly important because the previously shown images indicate that the sections were not anatomically matched across samples, which makes comparisons difficult and potentially misleading.

My point 4 also remains insufficiently addressed. Although the authors estimated total microbiota-derived PA production at approximately 175 µg/g of feces and stated that they screened around 100 *Bacteroides* species capable of producing PA, they did not adequately address my original concern regarding the specific contribution of *Bacteroides uniformis* to systemic PA levels in vivo. At minimum, the authors should estimate the contribution of *Bacteroides uniformis* to fecal PA levels to provide insight into the relative importance of PA production by this species compared with other bacteria.

Referee #5

(Remarks to the Author)

The revised manuscript addresses the major scientific and technical concerns raised in my initial review and presents a more coherent mechanistic framework linking *Bacteroides uniformis*-derived pentadecanoic acid (PA) to cholesterol metabolism and atherosclerosis. By integrating microbiota manipulation, lipidomics, pharmacokinetics, scRNA-seq, and murine atherosclerosis models, the authors provide evidence that PA functions as an endogenous inhibitor of HMGCR, leading to reduced cholesterol synthesis and atheroprotection in vivo.

Although inverse associations between circulating PA levels and cardiometabolic disease—including LDL-cholesterol-lowering effects in human cohorts—have been reported previously (e.g., Chooi, Y. C. et al., *Am. J. Clin. Nutr.* 119, 788–799 (2024)), the identification of a defined microbial source and the accompanying mechanistic analyses represent a meaningful advance beyond prior correlative studies. The experimental approach is technically sound, the datasets are of high quality, and the statistical analyses appear appropriate, with uncertainty handled in a careful and conservative manner.

The conclusions are largely supported by the data and are appropriately framed within the limitations of the murine models used. The authors also acknowledge important constraints, including sex bias, dietary context, and lesion quantification, which lends credibility to the interpretation. One minor point is that, given circulating PA can also originate from dietary sources, the Discussion would benefit from a brief clarification of how microbial and dietary contributions to the PA pool might be disentangled, particularly in human settings where diet and microbiome composition are highly variable. Addressing this point would further strengthen the translational relevance of the work without necessitating additional experiments.

Overall, the revised manuscript is clearly written, the abstract and introduction provide appropriate context, and prior literature is cited in a balanced and comprehensive manner.

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have sufficiently addressed my previous comments, mostly with experimental evidence, and the paper reads well now. I appreciate the authors' efforts for additional experiments. I have a few remaining comments/suggestions that I think are aimed at further clarification and improving interpretability.

In genetic studies such as this, complementation experiments are particularly important. Gene disruptions can lead to unintended polar effects on downstream genes, especially when they are located within the same operon or share regulatory elements. While I regret not raising this earlier, I would encourage the authors to consider complementation – if not in the current study, then at least as an important point for future validation.

Are there differences in the abundance of *Bacteroides uniformis* colonization under germ-free (GF) versus specific pathogen-free (SPF) conditions? I wonder if there are differences that could partly explain PA levels between these settings.

I appreciate the clarification regarding the involvement of alternative (and potentially additive) pathways in generating propionyl-CoA. My understanding now is that *mutA* and *pta* likely contribute through parallel routes, and in that context, a double mutant might be expected to behave like PA-null phenotype?

Minor:

Regarding the in vivo experiments described (for eg. lines 412-413), it would improve clarity to explicitly state whether these experiments were conducted in ApoE^{-/-} mice under SPF or GF conditions. Since both models appear to be used, clearly specifying this here and throughout the manuscript would help avoid confusion.

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #3

(Remarks to the Author)

I would like to thank the authors for their specific attention to my interest in the specificity of C15 pentadecanoic acid vs. the C17 in their atherosclerosis study. The authors show some new interesting results that their bacterium does not synthesize much C17. They also show that C17 does reduce cholesterol production but not through inhibition of HMG-CoA reductase, which is a fascinating result perfect for follow up studies. I applaud the authors for the rigor of this very interesting study.

Referee #4

(Remarks to the Author)

The authors have adequately addressed my comments, and I have no further comments to add

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)

authors have addressed my concerns

Referee #2

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

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1 Dear Professor Zhao,

2
3 Thank you for your patience as your manuscript, "Gut bacteria-derived odd-chain fatty acid
4 modulates cholesterol homeostasis and alleviates atherosclerosis", has been under review
5 at Nature. It has now been seen by 5 referees. You will see from their comments below
6 that while they find your work of potential interest, they have raised several substantive
7 concerns that in our view need to be addressed in full before we can consider publication
8 in Nature.

9
10 While the referees feel that the mechanism described is interesting in principle, they raise
11 several important technical concerns, including the absence of appropriate controls, lack
12 of rigour in quantifications and inconsistencies in the data. In addition, the referees find
13 several gaps in mechanistic understanding, and indicate that further experiments to
14 strengthen this would be required, such as evidence that PA from *B. uniformis* is the
15 bioactive metabolite responsible for protection against atherosclerosis (and not other lipids,
16 or PA derived from other species in the gut), further insights into the mechanism of HMGCR
17 inhibition by PA and most importantly, to demonstrate that the effects of PA are in fact
18 biologically meaningful (given reservations relating to the effect size). Should further
19 experiments allow you to address the criticisms in full, we would be happy to consider a
20 revised manuscript (unless something similar has been accepted at Nature or appeared
21 elsewhere in the meantime). Importantly, given the extent and nature of the reservations,
22 I'm afraid that we cannot promise to send a revised manuscript back to the reviewers,
23 unless we are convinced that their concerns have been addressed with a positive
24 experimental outcome.

25
26 We hope to receive your revised paper within four to six months. If you cannot complete
27 the required revisions within this time frame, please let us know when you would anticipate
28 being able to submit a revised manuscript. Any revised manuscript should conform to our
29 format instructions and publication policies (see [here](#)). We also strongly suggest that your
30 revised manuscript has tracked changes, which is increasingly requested by referees to
31 aid in their re-review.

32 Major comments

33 1. PA from *B. uniformis* is the bioactive metabolite responsible for protection against
34 atherosclerosis not other lipids.

35 **Response:** We thank you and the reviewer for raising this important point. Our data
36 indicate that PA is the principal bioactive metabolite mediating the atheroprotective effects
37 of *B. uniformis* (BU). This conclusion is supported by multiple complementary lines of
38 evidence.

39 First, administration of PA-deficient BU mutants ($\Delta mutA$ or Δpta) resulted in markedly
40 reduced PA levels in feces, liver, and serum (Fig. 5D–F in the revised manuscript),

accompanied by a substantial attenuation of the anti-atherosclerotic effects observed with wild-type BU (Fig. 5G, H and Extended Data 9D–O in the revised version). Second, direct PA supplementation robustly recapitulated the protective phenotype in HFD-fed mice, achieving an efficacy comparable to that of atorvastatin when administered at equivalent doses.

Notably, PA-deficient mutants retained the capacity to produce low levels of PA (~20% of wild-type), which may account for the residual protective effects still observed in these groups. However, this residual activity was insufficient to reproduce the full therapeutic benefit conferred by wild-type BU, supporting the conclusion that PA functions as the dominant effector molecule. While we do not exclude the possibility that additional bacterial products may exert minor or supportive effects, these factors are unable to compensate for the marked reduction in PA and remain to be identified in future studies.

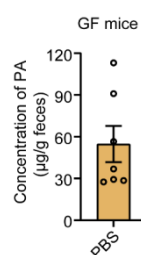
Accordingly, we have revised the *Discussion* to emphasize PA as the central mediator of BU-driven atheroprotection, while acknowledging the inherently multifactorial nature of live-bacterium interventions (Please see Line 511-513 in the revised manuscript; all revised words are colored in red).

2. PA derived from other species in the gut

Response: We appreciate your and reviewer's thoughtful question. To assess the contribution of PA from various sources, we conducted a careful quantitative analysis. Please check below.

First, we would like to clarify that PA is not synthesized by the host, as stated in the revised manuscript (Please see Line 431-432). Thus, fecal PA detected in mice originates from dietary input and/or gut microbiota. To clarify their relative contributions, we performed the following analyses.

(i) Dietary contribution to fecal PA. We observed that germ-free (GF) mice fed a HFD and treated with PBS exhibited ~55 µg/g feces of PA after 6 weeks (Supplementary Fig. 5 in the revised manuscript, which also shown as below), indicating that dietary PA contributes approximately ~55 µg/g feces under these conditions.



Supplementary Fig. 5 | PA levels in feces of HFD-fed GF *Apoe*^{-/-} mice after a 6-week gavage with PBS (n = 7). Data are presented as mean ± SEM.

(ii) **Contribution from resident gut microbiota.** In SPF mice maintained on HFD, baseline fecal PA levels were approximately ~230 µg/g (Fig. 3L in the revised manuscript, which also shown as below). Subtracting the estimated dietary contribution (~55 µg/g), the gut microbiota accounts for ~175 µg/g feces of PA. Previously we have screened approximately 100 bacterial strains and found that the majority of *Bacteroides* species possess the capacity to produce PA. These findings suggest that resident *Bacteroides* constitute a major source of baseline microbiota-derived PA in SPF mice.

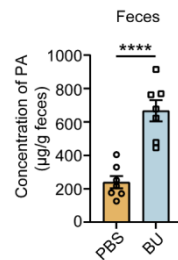


Fig. 3L | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS or BU (n = 7). Data are presented as mean ± SEM. Statistical significance was determined using two-tailed Student's t test: *****p* < 0.0001.

(iii) **Contribution of gavaged BU.** To determine the specific contribution of BU, we compared mice gavaged with BU WT vs. PA-deficient mutants (*Δpta* or *ΔmutA*). Under identical SPF and HFD feeding conditions, fecal PA levels decreased from ~670 µg/g in BU WT-treated mice to ~430 µg/g in mice receiving PA-deficient mutants, representing a reduction of ~240 µg/g feces (Fig. 5D in the revised manuscript, which also shown as below). This genotype-dependent difference provides a quantitative estimate of BU-derived PA contribution in SPF mice. Importantly, although gavaged BU is not the majority of total mice gut bacteria, this does not imply a proportional contribution to PA production. Rather, BU functions as a high-output PA producer, such that a relatively low-abundance taxon can disproportionately influence total PA levels.

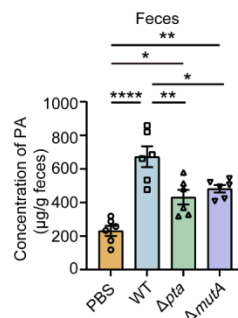


Fig. 5D | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS, BU or PA-deficient mutants (n = 6). Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

Collectively, these analyses indicate that baseline fecal PA reflects combined inputs from diet and resident microbiota, while BU gavage provides a quantitatively substantial PA

contribution. The marked reduction in PA levels upon administration of PA-deficient BU mutants, despite comparable colonization, supports BU-derived PA biosynthesis as a major and functionally relevant driver of PA elevation in SPF mice. We have made corresponding revision at Line 432-445 at *Results* and Line 1045-1060 at *Methods* section in the revised manuscript.

3. Further insights into the mechanism of HMGCR inhibition by PA

Response: We thank you for the insightful question regarding the inhibitory mechanism of PA on HMGCR. Our previous surface plasmon resonance (SPR) analysis had already established direct binding between PA and HMGCR, with a dissociation constant (K_D) of 7.41 μM . To further investigate this interaction and characterize the inhibition mechanism, we performed additional experiments. Using isothermal titration calorimetry (ITC), we independently confirmed the binding, obtaining a comparable K_D of 6.11 μM (Fig. 4F, G in the revised manuscript, which also shown as below), reinforcing the specificity of the PA-HMGCR interaction.

To elucidate the mode of inhibition, we conducted detailed enzymatic kinetic assays using purified human HMGCR. PA caused a dose-dependent increase in the apparent Michaelis constant (K_m) for the substrate HMG-CoA, while the maximal reaction rate (V_{max}) remained unchanged (Fig. 4H in the revised manuscript, which also shown as below). This kinetic signature is characteristic of competitive inhibition, suggesting that PA acts as a competitive inhibitor against HMG-CoA.

In summary, the combined SPR/ITC binding data and the competitive inhibition kinetics demonstrate that PA acts as a direct competitive inhibitor of HMGCR. We have made the corresponding revision at Line 348-355 at *Results* and Line 1160-1172 at *Methods* section in the revised manuscript.

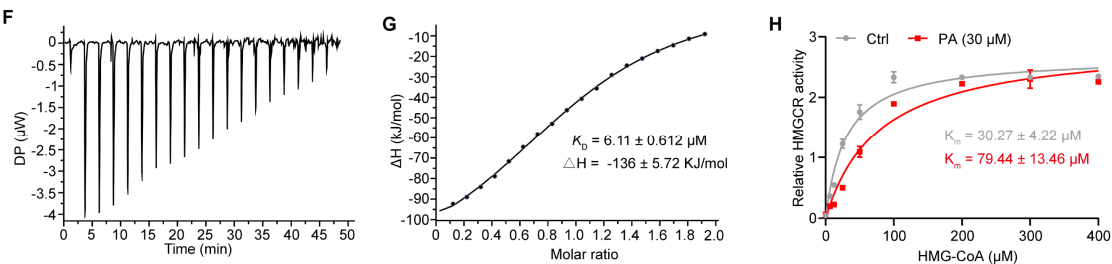


Fig. 4F-H | F and G. Isothermal titration calorimetry (ITC)-mediated determination of HMGCR binding to PA. **H.** Increasing HMG-CoA concentrations in the presence or absence of 30 μM PA were used in enzymatic inhibition assays performed as in Fig. 4D ($n = 3$). Data are presented as mean $\pm$ SEM.

4. The effects of PA are in fact biologically meaningful (given reservations relating to the effect size)

Response: We appreciate your insightful suggestion. We have expanded our cohort to

include 209 fecal donors (106 patients with dyslipidemia and 103 control individuals) and 211 serum donors (108 patients with dyslipidemia and 103 control individuals). Detailed cohort characteristics are provided in Supplementary Table 6. Consistent with prior observations, patients with dyslipidemia exhibited lower levels of PA in both fecal and serum samples compared to healthy controls (Fig. 5K in the revised manuscript, which also shown as below, and Lines 451-455, 1255-1257 in the revised manuscript).

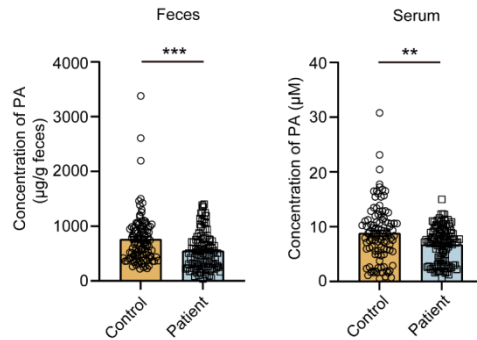


Fig. 5K | The levels of PA in fecal and serum samples. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-tailed Mann-Whitney U test: ** $p < 0.01$; *** $p < 0.001$.

Referee #1

The manuscript by Yin et al. describes a series of studies suggesting that *Bacteroides uniformis* (BU) alleviates atherosclerosis via the production of the long chain fatty acid pentadecanoic acid (PA). The authors demonstrate that PA supplementation reduces atherosclerosis, which is a novel finding. They also show that both BU and PA enhance LDL receptor (Ldlr) expression, facilitating the clearance of LDL cholesterol and free cholesterol without impacting HDL cholesterol levels. Furthermore, PA supplementation inhibits HMG-CoA reductase (encoded by *Hmgcr*), the rate-limiting enzyme in cholesterol biosynthesis, indicating diminished hepatic cholesterol synthesis. However, BU colonization paradoxically increases *Hmgcr* expression, which raises questions about the mechanistic consistency. Using a small sample size, the authors provide human correlative data, where they show that PA levels are lower in cardiovascular disease (CVD) patients compared to healthy controls. Additionally, their reanalysis of publicly available datasets reveals a reduced abundance of *Bacteroides* strains in individuals with CVD. These findings contribute to our understanding of microbiome-host interactions in lipid metabolism.

While previous studies have shown that *Bacteroides* species can alleviate atherosclerosis and that certain bacteria produce PA (see comments below), these findings in isolation are not entirely novel. However, this study uniquely bridges the gap between bacterial PA production and its direct role in suppressing atherosclerosis, providing an interesting link between microbiota-derived metabolites and host lipid metabolism. The comprehensive experimental approach incorporates in vitro assays, cell lines, and mouse models to investigate BU-derived PA's impact on lipid metabolism. While the study is well-executed, there appear to be several major limitations: The reliance on correlative human data with a limited sample size, the exclusive use of male mice, and mechanistic inconsistencies weaken the generalizability of the findings. Additionally, the observed increase in PA levels with BU colonization is intriguing, but the high baseline PA levels in control mice raise questions about its origin, which remains unresolved.

Response: We sincerely thank you for the thorough and balanced evaluation of our manuscript. We appreciate the recognition of the novelty of linking *Bacteroides uniformis*-derived pentadecanoic acid (PA) to atheroprotection and for highlighting both the strengths and limitations of the study. We have carefully considered all of the concerns raised. In response, we performed additional experiments and analyses and revised the manuscript accordingly. Below, we provide detailed, point-by-point responses to each comment and indicate where corresponding changes (marked as red) have been made in the revised manuscript.

Particularly, regarding the human cohort size, we have expanded our cohort to include **209** fecal donors (106 patients with dyslipidemia and 103 control individuals) and **211** serum donors (108 patients with dyslipidemia and 103 control individuals). Detailed cohort characteristics are provided in Supplementary Table 6. Consistent with prior observations, patients with dyslipidemia exhibited lower levels of PA in both fecal and serum samples

compared to healthy controls (Fig. 5K in the revised manuscript, which also shown as below, and Lines 451-455, 1255-1257 in the revised manuscript; all revised words are colored in red).

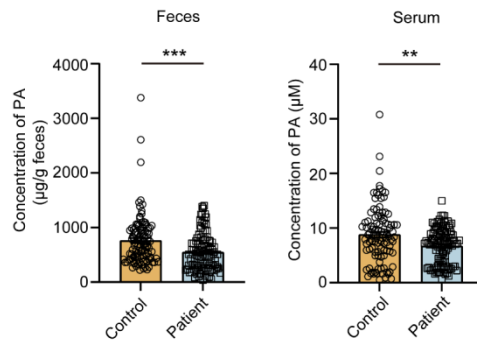


Fig. 5K | The levels of PA in fecal and serum samples. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-tailed Mann-Whitney U test: $**p < 0.01$; $***p < 0.001$.

Major Comments

1. The authors propose that PA inhibits HMGCR activity while BU supplementation increases *Hmgcr* expression (Fig. 2B). However, with respect to the lesion formation, cholesterol synthesis and clearance they follow similar trends. This contradiction needs to be addressed. Similarly, *del-pta* and *del-mutA* mutants, compared to WT, produce less PA, associated with reduced *Hmgcr* expression and yet exhibit increased lesion formation. These findings suggest that BU's effects may extend beyond PA production, potentially involving indirect modulation of gut microbiota or other bioactive molecules. Further mechanistic exploration is required to reconcile these inconsistencies. I'm having a hard time understanding the relationship between *Hmgcr* expression and cholesterol synthesis/levels.

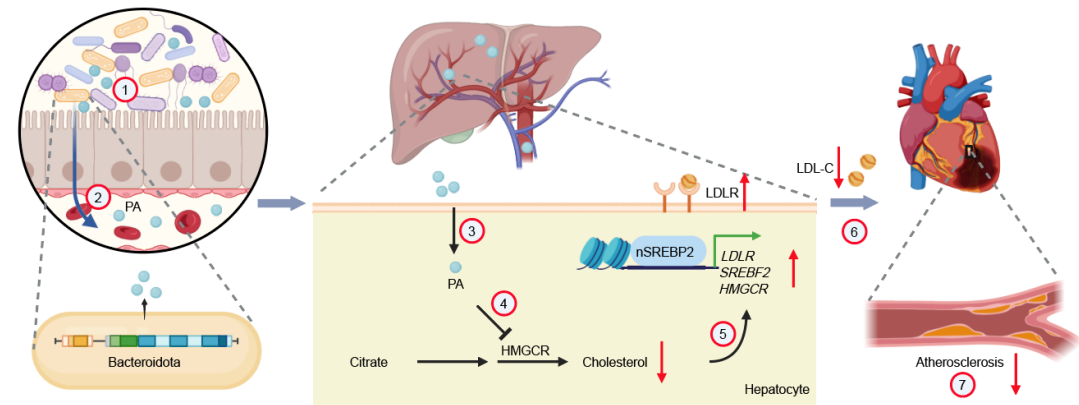
Response: We appreciate your thoughtful and insightful question and the opportunity to clarify the apparent discrepancy between HMGCR activity, *Hmgcr* expression, and cholesterol-related phenotypes. Importantly, HMGCR enzymatic activity and *Hmgcr* gene expression are regulated at distinct levels and do not necessarily change in parallel. Our proposed mechanistic framework is as follows:

(i) Direct inhibition of HMGCR activity by PA. BU-derived PA directly inhibits HMGCR enzymatic activity (Fig. 4D in the revised manuscript), leading to reduced *de novo* cholesterol synthesis in hepatocytes (Fig. 4A-C in the revised manuscript) (Please see Graphic abstract ④ below).

(ii) Compensatory transcriptional feedback via SREBP2. The resulting decrease in intracellular cholesterol activates a canonical feedback response, promoting the processing of precursor SREBP2 into its mature nuclear form (nSREBP2) (Fig. 4A, B in

the revised manuscript). nSREBP2 subsequently activates target gene promoters and transcriptionally upregulates cholesterol metabolism related genes, including *Hmgcr*² (Please see Graphic abstract ⑤ below).

(iii) Net effect of BU supplementation. As BU secretes PA, BU supplementation enhances this SREBP2-mediated compensatory response, resulting in increased *Hmgcr* transcription and expression (Fig. 2B in the revised manuscript), despite an overall reduction in HMGCR enzymatic activity and cholesterol synthesis.



Extended data Fig. 10C | Summary diagram depicting the role of PA in the modulation of hepatic cholesterol metabolism and atherosclerosis progression.

Regarding the Δpta and $\Delta mutA$ mutants, although their capacity to produce PA is reduced compared with the wild-type BU strain, they still retain a minimal level of PA production (Fig. 5B in the revised manuscript). The reduced PA output in these mutants leads to attenuated inhibition of HMGCR activity and weaker activation of the SREBP2-dependent transcriptional feedback pathway, resulting in lower *Hmgcr* expression compared with the wild-type strain (Extended data Fig. 9M-O in the revised manuscript). Consistent with this, the diminished PA-mediated inhibition of cholesterol synthesis contributes to increased lesion formation observed in the mutant-treated groups (Fig. 5G, H in the revised manuscript).

Taken together, these findings indicate that the observed changes in *Hmgcr* expression reflect a compensatory transcriptional response to altered cholesterol homeostasis. While we agree that BU may exert additional microbiota-mediated or indirect effects, our data support PA-dependent regulation of the HMGCR–SREBP2 axis as a primary mechanism underlying the observed phenotypes. We have made corresponding revision and clarification in the revised manuscript (Please see Line 206-209).

2. Previous studies have demonstrated that *Bacteroides* species can mitigate atherosclerosis and hyperlipidemia through different mechanisms (PMID: 30571343, PMID:

34083551. The authors should acknowledge these findings and discuss how their work advances our understanding of *Bacteroides*-mediated effects on lipid metabolism and cardiovascular health.

Response: We sincerely appreciate your suggestion. We have now incorporated and discussed prior studies demonstrating the protective roles of *Bacteroides* species in atherosclerosis and hyperlipidemia (PMID: 30571343; PMID: 34083551). In the revised *Discussion*, we highlight how these studies identify distinct microbiota-mediated mechanisms, and we further clarify how our findings extend the knowledge by revealing a previously unrecognized PA-dependent regulatory axis linking *Bacteroides* to hepatic cholesterol metabolism and atherosclerosis. The relevant discussion has been added to the manuscript (Please see Line 504-509 in the revised manuscript).

3. The manuscript includes data comparing high-fat diet (HFD) and chow diet conditions. Given the well-established pro-atherogenic effects of HFD, this comparison may be redundant unless BU/PA effects are explicitly diet-dependent. Clarifying whether the intent is to demonstrate diet-dependent effects would enhance focus.

Response: We sincerely appreciate your valuable suggestion. As the primary focus of this study is on the effects of BU/PA under pro-atherogenic conditions, and the inclusion of both diet groups did not add substantial mechanistic insight, we have removed the chow diet (CHD) group from the manuscript to improve clarity and focus. The relevant figures have been revised accordingly (Figures 1 and 3; Extended Data Figures 1 and 6).

4. While PA levels were measured in feces and liver, systemic circulation levels were not reported. Since PA's anti-atherogenic effects are presumed to act systemically, plasma or serum measurements are critical for understanding its role in reducing atherosclerosis. Additionally, in PA supplementation experiments, assessing circulating PA levels would provide proof-of-concept.

Response: We sincerely appreciate your valuable suggestions regarding the assessment of circulating PA levels. In response, we have now measured serum PA concentrations in HFD-fed *Apoe*^{-/-} mice treated with BU or its mutant strains ($\Delta mutA$ or Δpta). Consistent with our observations in fecal and hepatic samples, BU gavage for 12 weeks significantly increased serum PA levels compared with PBS treatment, whereas serum PA levels were markedly reduced in mice receiving Δpta or $\Delta mutA$ strains (Please see the updated version of Figures 3L and 5F, which also shown as below).

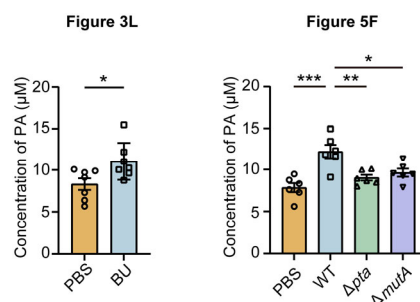


Fig. 3L and Fig. 5F | PA levels in serum of HFD-fed *Apoe*^{-/-} mice after a 12-week gavage with PBS (n = 6 or 7), BU (n = 6 or 7), or BU mutant strains (n = 6). Data are presented as mean ± SEM. Statistical significance was determined using two-tailed Student's t test (Figure 3L), or one-way ANOVA with Tukey's post hoc test (Figure 5F): **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

In addition, we assessed circulating PA levels in the PA supplementation experiments. No significant difference in PA levels was found in serum between PA-supplemented and control mice (Please see below) (Specimen sampling took place 48 hours after PA supplementation). This finding is consistent with our pharmacokinetic analyses (Supplementary Fig. 4 in the revised manuscript), which demonstrate that orally administered PA is metabolized and largely cleared within 24 hours. This turnover likely accounts for the absence of sustained increases in circulating PA levels despite repeated oral supplementation.

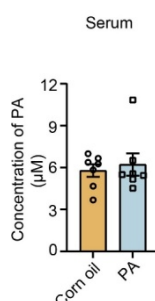


Figure legend | PA levels in serum of HFD-fed *Apoe*^{-/-} mice after 8-week gavage with Corn oil (n = 7), or PA (n = 7). Data are presented as mean ± SEM. Statistical significance was determined using two-tailed Mann-Whitney U test.

Together, these data provide direct evidence that BU-mediated elevation of PA leads to increased systemic availability, supporting its role in modulating atherosclerosis, while also highlighting distinct pharmacokinetic properties of exogenously administered PA. We have made the corresponding revision at Lines 299-300, 410-412 of the updated manuscript.

5. The study used only male mice without justification. The authors should either justify their choice or expand their study to include female mice to ensure broader applicability.

Response: We sincerely appreciate the reviewer's insightful comment. In this study, we

exclusively used male mice to minimize potential variability associated with the estrous cycle, which has been reported to influence cardiometabolic parameters and the progression of atherosclerosis (Morselli et al. Nat Rev Endocrinol. 2017¹). In addition, the cardioprotective effects of estrogen and sex-dependent differences in atherosclerosis development have been well documented¹.

Focusing on male mice allowed us to reduce hormonal variability and improve experimental consistency in this mechanistic study. This approach is consistent with many prior mechanistic studies of atherosclerosis that aimed to isolate causal pathways under controlled hormonal conditions. We fully recognize sex as an important biological variable, and future studies will extend these findings to female mice to evaluate potential sex-specific effects and translational relevance. We have acknowledged this at *Discussion* section Line 549-551.

6. Control mice exhibit high baseline PA (Fig. 3K). It remains unclear whether PA is host-derived or produced by other bacteria, especially since PA production has been documented in other species (PMID: 39468026; PMID: 37386075). Conducting experiments with germ-free ApoE KO mice mono-colonized with BU WT or PA-deficient mutants would have provided mechanistic clarity to distinguish BU-specific effects from broader microbiome influences. I also recognize this is a lot of work.

Response: We greatly appreciate your insightful suggestion. To directly distinguish BU-specific, PA-dependent effects from broader microbiome influences, we conducted complementary experiments.

First, germ-free (GF) *ApoE*^{-/-} mice were mono-colonized with either BU wild-type (WT) or the PA-deficient Δpta mutant strain. BU WT colonization elevated fecal PA levels from ~55 $\mu\text{g/g}$ (PBS control) to ~150 $\mu\text{g/g}$, while the Δpta mutant failed to do so (Please see below). This provides direct proof-of-principle that BU intrinsically produces PA *in vivo*.

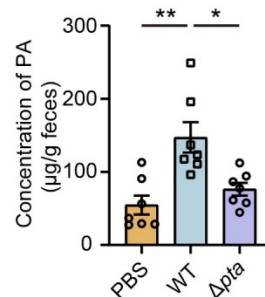


Figure legend | PA levels in feces of HFD-fed GF *ApoE*^{-/-} mice after a 6-week gavage with PBS (n = 7), BU (n = 7), and Δpta mutant strain (n = 7). Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test: **p* < 0.05; ***p* < 0.01.

In order to assess the contribution of PA from various sources, we conducted a careful

quantitative analysis. First, we would like to clarify that PA is not synthesized by the host, as stated in the manuscript (Please see Line 431-432). Thus, fecal PA detected in mice originates from dietary input and/or gut microbiota. To clarify their relative contributions, we performed the following analyses.

(i) Dietary contribution to fecal PA. We observed that germ-free (GF) mice fed a HFD and treated with PBS exhibited ~55 µg/g feces of PA after 6 weeks, indicating that dietary PA contributes approximately ~55 µg/g feces under these conditions.

(ii) Contribution from resident gut microbiota. In SPF mice maintained on HFD, baseline fecal PA levels were approximately ~230 µg/g (Fig. 3L in the revised manuscript, which also shown as below). Subtracting the estimated dietary contribution (~55 µg/g), the gut microbiota accounts for ~175 µg/g feces of PA. Previously we have screened approximately 100 bacterial strains and found that the majority of *Bacteroides* species possess the capacity to produce PA. These findings suggest that resident *Bacteroides* constitute a major source of baseline microbiota-derived PA in SPF mice.

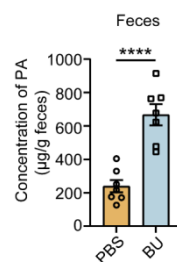


Fig. 3L | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS or BU (n = 7). Data are presented as mean ± SEM. Statistical significance was determined using two-tailed Student's t test: *****p* < 0.0001.

(iii) Contribution of gavaged BU. To determine the specific contribution of BU, we compared mice gavaged with BU WT vs. PA-deficient mutants (*Δpta* or *ΔmutA*). Under identical SPF and HFD feeding conditions, fecal PA levels decreased from ~670 µg/g in BU WT-treated mice to ~430 µg/g in mice receiving PA-deficient mutants, representing a reduction of ~240 µg/g feces (Fig. 5D in the revised manuscript, which also shown as below). This genotype-dependent difference provides a quantitative estimate of BU-derived PA contribution in SPF mice. Importantly, although gavaged BU is not the majority of total mice gut bacteria, this does not imply a proportional contribution to PA production. Rather, BU functions as a high-output PA producer, such that a relatively low-abundance taxon can disproportionately influence total PA levels.

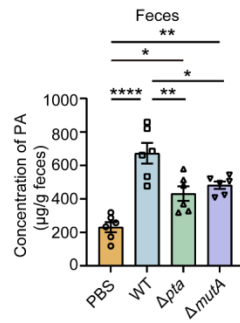


Fig. 5D | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS, BU or PA-deficient mutants (n = 6). Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test: **p* < 0.05; *p* < 0.01; ****p* < 0.001; *****p* < 0.0001.**

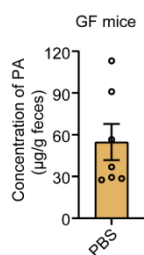
Therefore, while baseline PA pools arise from diet and resident bacteria, BU acts as a high-output PA producer that significantly augments the total pool. The marked reduction in PA levels upon colonization with PA-deficient BU mutants—despite comparable bacterial loads—definitively identifies BU-derived PA biosynthesis as the key, specific driver of the PA elevation and linked metabolic effects observed in our model. We have made corresponding revision at Line 432-445 at *Results* and Line 1045-1060 at *Methods* section in the revised manuscript.

7. *Bacteroides* species strong component of the mouse microbiota. How does a gavage with BU (>100X less *Bacteroides* than what is in the gut) increases PA so dramatically? Do the mouse *Bacteroides* not make PA? Please explain. Also, it would be useful to know the relative abundance of the gavaged BU to other *Bacteroides* in the gut.

Response: We appreciate your and reviewer's thoughtful question. To assess the contribution of PA from various sources, we conducted a careful quantitative analysis. Please check below.

First, we would like to clarify that PA is not synthesized by the host, as stated in the manuscript (Please see Line 431-432). Thus, fecal PA detected in mice originates from dietary input and/or gut microbiota. To clarify their relative contributions, we performed the following analyses.

(i) Dietary contribution to fecal PA. We observed that germ-free (GF) mice fed a HFD and treated with PBS exhibited ~55 µg/g feces of PA after 6 weeks (Supplementary Fig. 5 in the revised manuscript, which also shown as below), indicating that dietary PA contributes approximately ~55 µg/g feces under these conditions.



Supplementary Fig. 5 | PA levels in feces of HFD-fed GF *Apoe*^{-/-} mice after a 6-week gavage with PBS (n = 7). Data are presented as mean ± SEM.

(ii) Contribution from resident gut microbiota. In SPF mice maintained on HFD, baseline fecal PA levels were approximately ~230 µg/g (Fig. 3L in the revised manuscript, which also shown as below). Subtracting the estimated dietary contribution (~55 µg/g), the gut microbiota accounts for ~175 µg/g feces of PA. Previously we have screened approximately 100 bacterial strains and found that the majority of *Bacteroides* species possess the capacity to produce PA. These findings suggest that resident *Bacteroides* constitute a major source of baseline microbiota-derived PA in SPF mice.

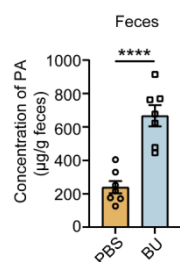


Fig. 3L | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS or BU (n = 7). Data are presented as mean ± SEM. Statistical significance was determined using two-tailed Student's t test: ***p* < 0.0001.**

(iii) Contribution of gavaged BU. To determine the specific contribution of BU, we compared mice gavaged with BU WT vs. PA-deficient mutants (*Δpta* or *ΔmutA*). Under identical SPF and HFD feeding conditions, fecal PA levels decreased from ~670 µg/g in BU WT-treated mice to ~430 µg/g in mice receiving PA-deficient mutants, representing a reduction of ~240 µg/g feces (Fig. 5D in the revised manuscript, which also shown as below). This genotype-dependent difference provides a quantitative estimate of BU-derived PA contribution in SPF mice. Importantly, although gavaged BU is not the majority of total mice gut bacteria, this does not imply a proportional contribution to PA production. Rather, BU functions as a high-output PA producer, such that a relatively low-abundance taxon can disproportionately influence total PA levels.

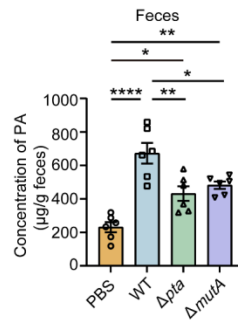


Fig. 5D | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS, BU or PA-deficient mutants (n = 6). Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test: **p* < 0.05; *p* < 0.01; ****p* < 0.001; *****p* < 0.0001.**

Collectively, these analyses indicate that baseline fecal PA reflects combined inputs from diet and resident microbiota, while BU gavage provides a quantitatively substantial PA contribution. The marked reduction in PA levels upon administration of PA-deficient BU mutants, despite comparable colonization, supports BU-derived PA biosynthesis as a major and functionally relevant driver of PA elevation in SPF mice. We have made corresponding revision at Line 432-445 at *Results* and Line 1045-1060 at *Methods* section in the revised manuscript.

Minor Comments

1. Line 59: Replace "systemic" with "systematic."

Response: Thanks for your suggestion. We have corrected it in the revised manuscript. Please see Line 62.

2. Line 680: Replace "comfirm" with "confirm."

Response: Sorry for this error. We have corrected it in the revised manuscript. Please see Line 799.

3. Line 110: Italicize *Bacteroides*.

Response: Sorry for this error. We have corrected it in the revised manuscript. Please see Line 144

4. Extended Data Fig. 2B: Replace "Number of gene" with "Number of genes."

Response: Sorry for this error. We have corrected it in the revised manuscript.

5. Do the precursor levels (acetate and propionate) go down whenever there is increased PA synthesis?

Response: We appreciate your thoughtful question regarding precursor availability during increased PA synthesis. To address this point, we measured acetate and propionate levels in feces, liver tissues, and serum from PBS- and BU-treated HFD-fed *Apoe*^{-/-} mice.

Consistent with increased PA production, propionate levels were modestly elevated in fecal, hepatic, and serum samples from BU-treated mice compared with the PBS group (Please see Panel D-F below). In contrast, acetate levels remained largely unchanged across all compartments (Please see Panel A-C below). This differential response likely reflects distinct metabolic roles and regulatory controls of these short-chain fatty acids. While propionate levels may partially track microbial PA-producing activity, acetate serves as a central substrate in host energy metabolism and is tightly regulated through multiple endogenous and microbial pathways, thereby maintaining a relatively stable steady-state concentration despite increased PA synthesis.

These findings indicate that enhanced PA production does not necessarily result in a depletion of precursor pools, but rather occurs within a buffered metabolic network that preserves acetate homeostasis.

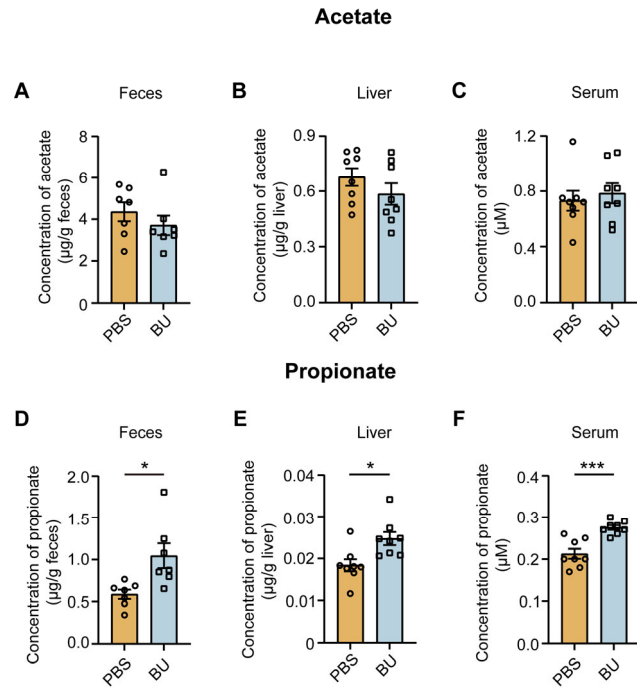


Figure legend | A-F. Precursor levels of PA in feces (A and C), liver tissues (B and E), and serum (C and F) of HFD-fed *Apoe*^{-/-} mice after a 12-week gavage with PBS (n = 7), BU (n = 7). Data are presented as mean ± SEM. Statistical significance was determined using two-tailed Student's t test (A-C, E and F), or two-tailed t-tests with Welch's correction (D): **p* < 0.05; ****p* < 0.001.

459 **Referee #2**

460 I co-reviewed this manuscript with one of the reviewers who provided the listed reports

461

Referee #3

This manuscript by Yin et al. describes the discovery and mechanism behind the ability of *Bacteroides* spp. to lower cholesterol in mice and humans. It has long been known that a higher Firmicutes/*Bacteroides* ratio in the gut microbiome is associated with obesity and atherosclerosis, however, despite this knowledge existing for over 20 years, little functional understanding of why this ratio associates with these disease phenotypes was available. This study is highly relevant and important because it now provides a mechanistic understanding as to why *Bacteroides* bacteria are beneficial to these disease states. The study shows that many *Bacteroides* spp. produce a 15 carbon fatty acid pentadecanoic acid, which itself has been associated with metabolic health. After the initial work showing a reduction of these bacteria in the gut microbiome of those with atherosclerosis, and the ability of strains to reduce the disease in a mouse model the authors go through extensive experimentation and chemical purifications to identify and characterize the bioactive metabolite of interest. I applaud the authors in their well-rationalized and additive evidence for the role of PA in ameliorating atherosclerosis and the results of this study could lead to direct and significant medical impacts. However, there is not sufficient evidence that PA is the bioactive metabolite of interest and many of the experiments are lacking important controls.

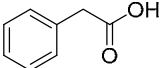
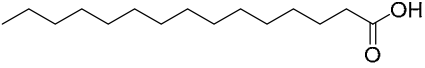
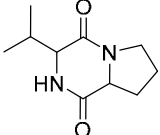
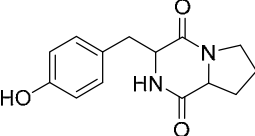
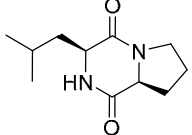
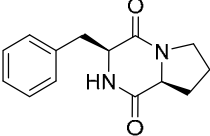
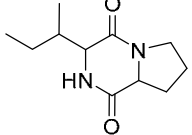
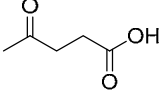
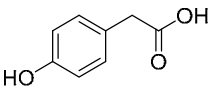
My principal critique of this study is that the authors have not sufficiently demonstrated that PA, and only PA, is the bioactive metabolite. They themselves show that other fractions in their chemical isolations also have anti-atherosclerotic activity, but they do not investigate this further. This alone is not the only issue, it seems possible that other fatty acids of similar chain lengths may have similar inhibitory action on HMG-CoA reductase, but these are not tested. A very simple and important control that is needed throughout many of the experiments is at least a C-16 fatty acid and a C-17 fatty acid. I recognize that repeating the experiments with completely new variables is a monumental task for what is already a large study, but targeted evidence that these other fatty acids do not act similarly is required. Here are some examples where these controls would be particularly useful.

Response: We are grateful for your detailed and constructive comments, which have greatly helped to strengthen the scientific rigor, mechanistic clarity, and overall impact of our study. In response to the concerns raised during review, we have substantially revised the manuscript and performed extensive new experiments and analyses. All comments have been addressed in detail with point-by-point responses (please see below), and corresponding revisions have been highlighted in red in the revised manuscript. We hope that the revised version satisfactorily addresses your questions, and we would be pleased to further revise the manuscript should additional concerns arise.

1. Are other carbon chain length fatty acids present in the other bioactive fractions? Cpd1? Cp2? Cpd7? For example?

Response: Thank you for the question. We have carefully re-evaluated the compound

data and found that Cpd1 was not a single pure molecule but rather a mixture of multiple compounds. Sorry for this error and we have therefore corrected Cpd1 to **F1-1** (Fig. 3A in the revised manuscript). Within all our resolved structures (Cpd2 ~ 10, listed below), there are no other fatty acid.

ID #	Chemical Name	Structure
Cpd 1 (corrected) to F1-1)	Mixture	—
Cpd 2	Phenylacetic acid	
Cpd 3	PA	
Cpd 4	Cyclo(Ile-Pro)	
Cpd 5	Cyclo(Tyr-Pro)	
Cpd 6	Cyclo(L-Leu-L-Pro)	
Cpd 7	Cyclo(L-Phe-L-Pro)	
Cpd 8	Cyclo(Val-Pro)	
Cpd 9	Levulinic acid	
Cpd 10	4-Hydroxyphenylacetic acid	

Compared to PA, the bioactivity of F1-1 and the other compounds in the assay are minor or absent. We therefore focused on PA and did not pursue further analyses on them.

2. How much C16 and C17 fatty acids are in BHIS medium with *B. uniformis*?

Response: Thank you for the question. To address the question of whether *B. uniformis* can synthesize C16:0 and C17:0 fatty acids, we supplemented its culture medium with ^{13}C -labeled acetate. LC-HRMS analysis revealed the incorporation of the ^{13}C label into C16:0, confirming the *de novo* biosynthetic capability of *B. uniformis* for this fatty acid (Please see Panel A, B below). In contrast, C17:0 was not detected in either the *B. uniformis* fermentation supernatant or the sterile BHIS control medium. These results demonstrate that *B. uniformis* is capable of synthesizing C16:0, but not C17:0. Notably, the *in vitro* production of C16:0 was not accompanied by a significant increase in fecal C16:0 levels following *B. uniformis* gavage in mice (Please see Panel C below). This observation is likely attributable to the substantial background of host-derived C16:0, which may mask the contribution from the bacterium².

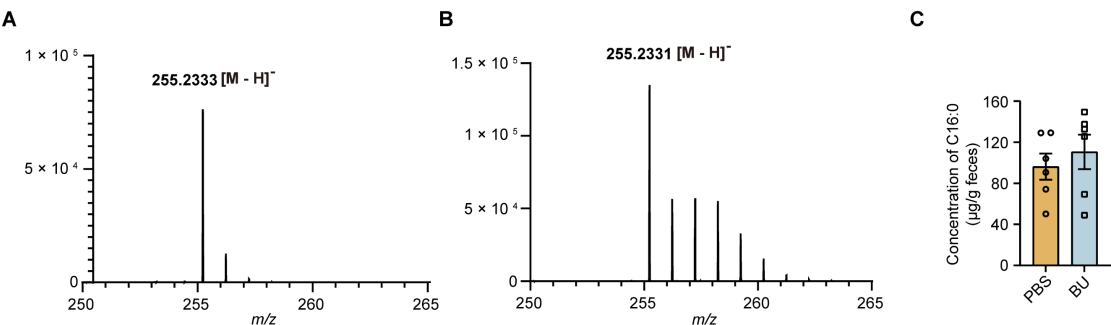


Figure legend | A and B. High-resolution mass spectrometry (HRMS) of C16:0 in BHIS medium (A) and *B. uniformis* fermentation (B) supplemented with ^{13}C -labeled acetate. **C.** C16:0 levels in feces of HFD-fed *Apoe*^{-/-} mice after a 12-week gavage with PBS (n = 6) or BU (n = 6). Data are presented as mean ± SEM (C). Statistical significance was determined using two-tailed Student's t test (C).

3. What are the total cholesterol levels in HepG2 cells treated with C16 or C17 fatty acids?

Response: Thank you for the question. Consistent with previous findings³, the treatment of C16:0 was able to enhance the total cholesterol level, while C17:0 exhibited the similar effect to reduce the total cholesterol level, compared to PA (C15:0; Please see below).

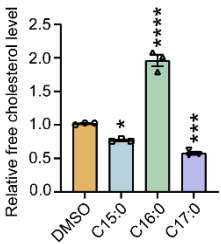


Figure legend | Free total cholesterol (FTC) in HepG2 cells treated with DMSO, long chain fatty acids (C15:0, C16:0 or C17:0; 80µM). Data are presented as mean ± SEM. Statistical significance was determined using two-sided Welch ANOVA with Dunnett's T3 test: * $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$.

4. Do these other fatty acids inhibit HMG-CoA reductase?

Response: Thank you for the question. We performed enzyme activity assays using C16 and C17 fatty acids at their maximum soluble concentrations. The results demonstrated no significant inhibitory activity against HMG-CoA reductase under these conditions (Please see below).

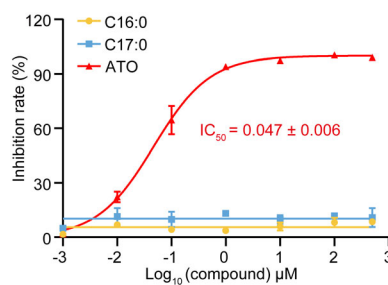


Figure legend | Inhibition curve of C16:0, C17:0 and atorvastatin (ATO) against HMG-CoA reductase (n = 3). Data are presented as mean ± SEM.

5. Do mice fed C16 or C17 fatty acids also have better atherosclerosis outcomes?

Response: Thank you for the question. While our study did not directly test the effects of feeding C16:0 or C17:0 fatty acids in mice, existing literature provides insight. C16:0 has been reported to promote atherosclerosis by accelerating endothelial cell injury⁴, whereas higher circulating levels of C17:0 are associated with lower cardiovascular disease risk in humans⁵. Our cellular data confirm that C17:0 reduces cholesterol accumulation, supporting the hypothesis that it may attenuate disease progression. Thus, based on current evidence, one would predict divergent effects: C17:0 supplementation might improve atherosclerosis outcomes, while C16:0 would not.

6. Are the human fecal samples with atherosclerosis also enriched in these other fatty acids?

Response: Thank you for the question. Alongside the measurement of PA (C15:0), we analyzed fecal samples from a cohort comprising 90 patients with dyslipidemia and 90 healthy controls. Consistent with the previously reported reduction in PA (C15:0) levels, the dyslipidemia group also exhibited significantly lower fecal levels of C17:0 compared to the control group (Please see below).

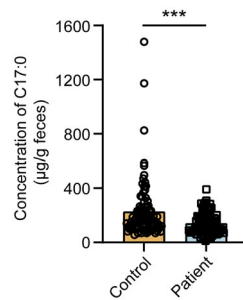


Figure legend | The levels of C17:0 in fecal samples. Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-tailed Mann-Whitney U test: *** $p < 0.001$.

Other critiques:

1. Atherosclerosis should be described briefly in the introduction. The wide audience of Nature may not understand what this is.

Response: Thank you for this helpful suggestion. We have revised the introduction accordingly and have incorporated a brief description of atherosclerosis for a broader readership. Please see Line 50-52 of the revised manuscript.

2. The experiments and results in lines 144-153 are difficult to follow and not explained well. This section should be expanded to aid the reader.

Response: Thank you for this helpful suggestion. We have expanded and clarified the description of the experiments and results in that section to improve readability, as detailed in Line 179-188 of the revised manuscript.

3. The experiments treating cells with the various extracts are not very well explained. What are the controls shown in Extended data Fig. 3. Can the authors show that the chemical extracts are not harming the cells in any way? Perhaps that's what the controls are showing but it's difficult to understand.

Response: Thank you for raising these important points regarding experimental clarity and controls. We have revised the manuscript to address them as follows:

Explanation of Controls in Extended Data Fig. 3: The controls shown are vehicle controls (e.g., solvent extracts from blank medium, dissolved in DMSO), which are now explicitly stated in the revised figure legend (Please Line 612-615).

Assessment of Cytotoxicity and Safety: To directly evaluate whether the extracts cause cell harm, we performed cell viability assays (CCK-8). The half-maximal inhibitory

concentration (IC₅₀) values for the key extracts are listed below. Crucially, in all functional experiments described, the treatment concentrations used were substantially lower than the determined IC₅₀ values. This confirms that the observed biological effects are not due to cytotoxic effects. We have made corresponding revision at the updated manuscript (Please see Line 255-256).

We apologize for the initial lack of clarity and hope the revised text and this clarification fully address your concerns.

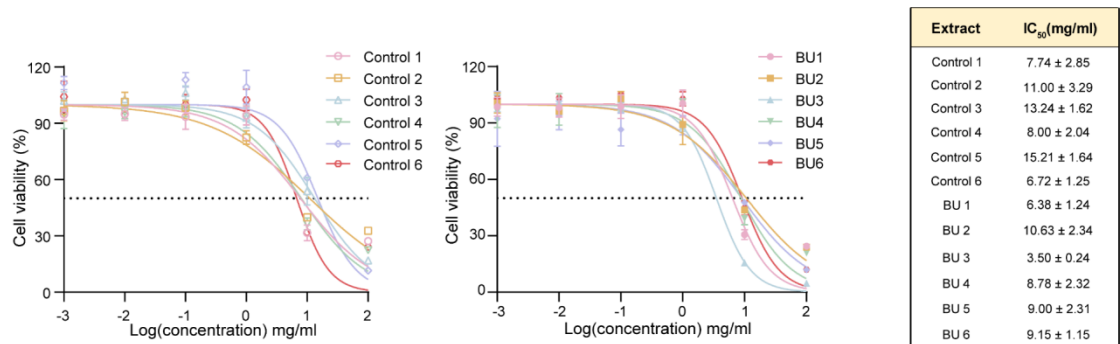


Figure legend | Determination of IC₅₀ values for the indicated extracts using CCK-8 cell viability assays. Data are presented as mean ± SD.

4. It is not clear that the heat killing procedure demonstrates what the authors propose it does and the details of the heat-killing are not provided. It is possible that the C15 fatty acid is part of the membrane or cell surface of *B. uniformis*. Demonstration that the heat killing has destroyed the cells is important to prove that it is intact extreted actively and not just part of the membranes.

Response: Thank you for this critical point. We have now provided the methodological details for heat-killing (65 °C for 30 minutes) in the revised *Methods* section (Please see Line 783-786).

We agree that it is essential to demonstrate that the heat-killing procedure effectively neutralized bacterial activity. Our data in Extended Data Fig. 4 show that, in contrast to live *B. uniformis*, the heat-killed preparation (HKBU):

- did not reduce atherosclerotic plaque area.
- had no significant effect on plasma total cholesterol or LDL-C levels.
- did not lessen macrophage infiltration or systemic inflammation.

The complete loss of atheroprotective efficacy in HKBU strongly suggests that the beneficial effect is not mediated by a structural component, but depends on an active bacterial process, consistent with the requirement for active synthesis or secretion of the soluble fatty acid (PA). This interpretation has been clarified in the revised text (Please see Line 235-241).

617 5. The authors statement in line 343-344 that the mutants mitigate atherosclerosis is not
618 well supported. They show evidence that it reduces plaque formation, but other data is
619 required to claim 'atherosclerosis' has been ameliorated.

620 **Response:** We thank you for this observation. Our data demonstrate that the mutants
621 reduce plaque formation but do not fully justify the broad claim that they "mitigate
622 atherosclerosis".

623 To clarify: while the mutants exhibited a residual atheroprotective effect compared to the
624 PBS group, this effect was significantly weaker than that of the wild-type strain. This is
625 consistent with a substantial reduction, but not a complete elimination, of PA production.
626 We have made the corresponding revision at the updated version (Please see Line 414-
627 418).

628

Referee #4

Yin et al. explored the impact of the bacterial metabolite pentadecanoic acid (PA), produced by *Bacteroides* species, on atherosclerosis. The study suggests that PA reduces atherosclerotic burden, likely by decreasing cholesterol synthesis through inhibition of HMG-CoA reductase and enhancing LDL receptor (LDLR)-mediated cholesterol clearance. This is a timely and interesting paper, and the authors have performed extensive work to narrow down the active metabolite among those produced by *B. uniformis*.

This manuscript presents an intriguing hypothesis and substantial experimental effort. However, major revisions are needed to improve data presentation (especially figure clarity), plaque quantification methodology, inflammation assessment, and mechanistic understanding.

Response: We are grateful for your detailed and constructive comments, which have greatly helped to strengthen the scientific rigor, mechanistic clarity, and overall impact of our study. In response to the concerns raised during review, we have substantially revised the manuscript and performed extensive new experiments and analyses. All comments have been addressed in detail with point-by-point responses (please see below), and corresponding revisions have been highlighted in red in the revised manuscript. We hope that the revised version satisfactorily addresses your questions, and we would be pleased to further revise the manuscript should additional concerns arise.

Major comments:

1. Figure Clarity and Quantification Approach

1.1 Several figure legends are too small to read clearly, and critical images (e.g., of the aortic sinus and aorta) are also presented at a scale that makes interpretation difficult.

Response: Thank you for pointing this out. We apologize for the sizing and clarity issues in the figures and have improved all relevant images in the revised manuscript to ensure optimal readability.

1.2 Plaque Quantification: A key methodological limitation is the lack of comprehensive plaque quantification throughout the entire aortic sinus. Accurate assessment requires serial sectioning and quantification across the whole region. Some images, such as Figure 3H (CHD group), appear to be taken at non-comparable anatomical levels of the sinus, which may compromise the validity of comparisons. Also, some images labeled as representative do not visibly show plaques stained in red, which is inconsistent with the quantification data reported. This discrepancy undermines the reliability of the visual data. A more rigorous, standardized method of plaque quantification (as described in references such as *Vascular Biology* 2024; 6(1):e230017, DOI: 10.1530/VB-23-0017) is recommended.

Response: We appreciate your valuable suggestions. We have carefully reviewed all histological data and made corrections to ensure that all comparison were made at comparable levels, and that all images were consistent with their quantification data.

Regarding your suggestion of carrying out a comprehensive plaque quantification throughout the entire aortic sinus, thank you for pointing out this very comprehensive and detailed protocol. We have read it very carefully. Unfortunately, we are not able to carry out the quantification throughout the entire aortic sinus with our existing cross sections. In this manuscript, we aimed to quantify the lipid deposition at the valve region (where all three entire leaflets are seen), a widely utilized landmark of atherosclerotic plaques^{6,7}. As such, we did not save cross sections that does not have all three entire leaflets. We have stated that quantification throughout the entire aortic sinus should be carried out in future studies in *Discussion* (Please see Line 553-555 in the revised manuscript; all revised words are colored in red).

2. Mouse Model Relevance

2.1 The authors used ApoE^{-/-} mice on a high-fat diet (HFD), a model that is highly inflammatory and may not accurately reflect moderate or chronic human disease. Validation of the findings in ApoE^{-/-} mice on a regular chow diet would be more physiologically relevant, as these mice develop significant atherosclerosis even without dietary fat overload. Notably, the representative plaque images for ApoE^{-/-} mice on chow diet appear to show almost no plaques, despite reported quantification indicating plaque areas of ~50,000–100,000 μm^2 . Again, image resolution and legend legibility make these data difficult to verify.

Response: We appreciate your thoughtful comment regarding the physiological relevance of the HFD-fed *ApoE*^{-/-} model. We agree that chow-fed *ApoE*^{-/-} mice develop a more indolent form of atherosclerosis and may be informative for studying long-term disease progression. The primary goal of the present study, however, was to dissect the mechanistic role of *B. uniformis* and PA under conditions of established hyperlipidemic stress, for which the widely used HFD-fed *ApoE*^{-/-} model⁶⁻⁸ provides robust and reproducible lesion development within 8-12 weeks. Importantly, validation in chow diet-fed *ApoE*^{-/-} mice would require long-term feeding (typically more than 24-30 weeks) to achieve sufficient and quantifiable lesion burden, particularly in the aortic root and valve regions. Given the revision timeframe and the extensive duration required to conduct and approve long-term animal studies, such experiments were not feasible within the scope of the current revision. We have now noted that future studies will be required to validate our findings under chow-fed conditions in *Discussion* (Please see Line 551-553).

Regarding the specific concerns about image quality and data presentation, we have replaced all relevant figures with higher-resolution versions and have rigorously re-verified the corresponding plaque area measurements to ensure consistency and accuracy.

2.2 Since one of the proposed mechanisms involves LDLR upregulation, the effect of PA and *B. uniformis* should be tested in *Ldlr*^{-/-} mice fed high-cholesterol diet to determine the dependence of the observed anti-atherosclerotic effects on LDLR function.

Response: We appreciate your valuable suggestion. To investigate the dependence of the observed anti-atherosclerotic effects on LDLR function, we tested the effect of PA and *B. uniformis* in hepatocytes-specific *Ldlr*-deficient *Apoe*^{-/-} mice (Fig. 2E, F, Fig. 3I-K, Extended Data Fig. 2 and Extended Data Fig. 7A-K in the revised manuscript). In these mice, *Ldlr* was specifically knocked out in the liver using single-guide RNA driven by a hepatocytes-specific promoter. This approach allows us to clearly delineate the role of LDLR function in the liver, as opposed to the total *Ldlr*^{-/-} model, which cannot distinguish the contributions of LDLR in hepatocytes vs. peripheral cells, such as endothelial cells and smooth muscle cells. These findings confirm that the anti-atherosclerotic effects are indeed dependent on LDLR function in hepatocytes. We have rephrased some sentences in *Result* section to clarify (Please see Line 179-188 in the revised manuscript).

3. Inflammation Assessment

3.1 Inflammation is insufficiently evaluated. CD68 was used as a macrophage marker, but it lacks specificity; CD68 can also be expressed by smooth muscle cells and other cell types. More appropriate markers, such as MOMA-2 or F4/80, should be included for macrophage identification.

Response: We agree that markers such as F4/80 offer greater cellular specificity, although CD68 is a widely utilized marker for identifying macrophages in atherosclerotic plaques (for example, Zhang et al. Nature 2025⁹). To address this concern, we performed additional immunohistochemical staining for F4/80 on aortic root sections. The results confirmed a reduction in macrophage infiltration, consistent with the trend observed using CD68 staining (Please see below). These findings support the conclusion that CD68 is effective for assessing macrophage levels in this context. Nonetheless, we agree that F4/80 provides greater specificity and will incorporate it in future studies to further refine macrophage identification.

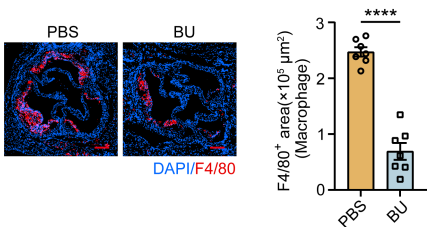
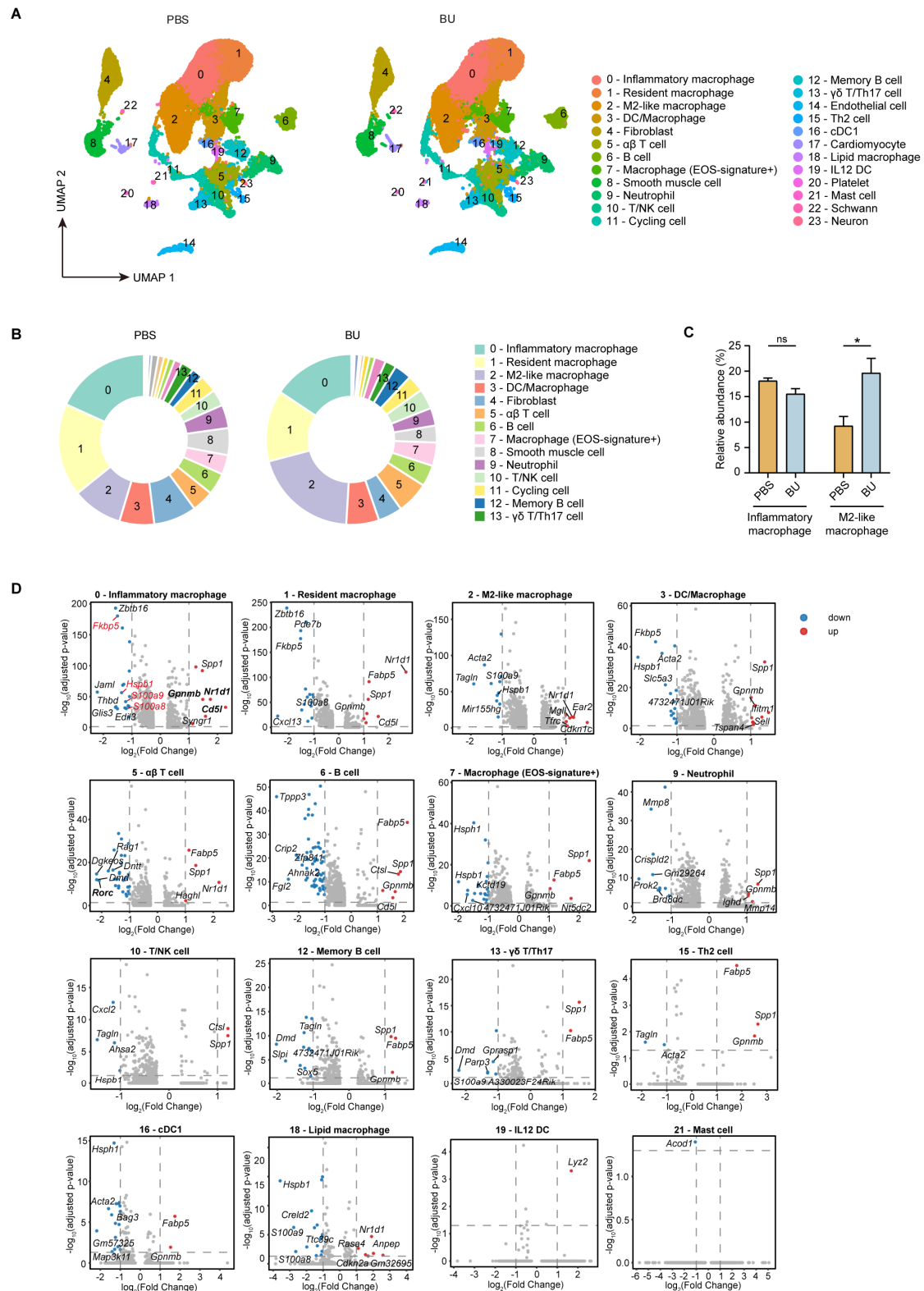


Figure legend | Representative images of F4/80 immunofluorescence staining used to detect macrophages in the sections of aortic roots (left). Quantification of CD68-positive area (right). Scale bar: 200 μm. HFD-fed *Apoe*^{-/-} mice were gavaged with PBS and BU three times per week for 12 weeks. Data are presented as mean ± SEM.

Statistical significance was determined using two-tailed Student's t test: **** $p < 0.0001$.

3.2 A comprehensive analysis of immune cell infiltration—ideally using flow cytometry on digested aortas—is recommended. This would enable precise quantification of macrophage subtypes, lymphocytes, and other immune cells involved in plaque development.

Response: We appreciate your valuable suggestion. To conduct a more comprehensive analysis, we carried out single-cell RNA sequencing within CD45⁺ cells dissociated from the whole aorta from both groups, respectively. Our panoramic analysis annotated 24 distinct cell clusters including macrophage subtypes, lymphocytes, other immune cells, and some non-immune cells (Supplementary Fig. 1A in the revised manuscript, which also shown as below). In both groups, inflammatory, M2-like and residential macrophages consist a substantial portion (~45%) of all CD45⁺ cells (Supplementary Fig. 1B in the revised manuscript, which also shown as below). The relative abundance of M2-like macrophage in BU-gavaged mice increased significantly compared to PBS-gavaged mice (Supplementary Fig 1C in the revised manuscript, which also shown as below; the revised Supplementary Table 2A). Further, we identified differentially expressed genes (DEGs) by each of the annotated 16 immune cell clusters between the two groups. A total number of 67 DEGs were upregulated and 238 were down-regulated in the BU group ($|\text{Fold Change}| \geq 2$; adjusted $p < 0.05$; Supplementary Fig 1D in the revised manuscript, which also shown as below; the revised Supplementary Table 2B). Notably, although the relative abundance of inflammatory macrophages did not differ significantly between the two groups, the inflammatory macrophages from the BU group exhibited a less inflammatory transcriptional profile, characterized by the downregulation of inflammation-associated genes (*S100a8*, *S100a9*, *Fkbp5*, and *Hspb1*) and the upregulation of genes previously reported to exert anti-inflammatory or inflammation-resolving functions (*Gpnmb*, *Nr1d1*, and *Cd5l*). Interestingly, $\alpha\beta$ T cells from the BU group exhibited reduced expression of *Rorc*, suggesting a diminished propensity toward Th17 differentiation. Together, these shifts indicate that *B. uniformis* remodels the plaque immune microenvironment toward a less inflammatory state.



Supplementary Fig. 1 | Single-cell RNA sequencing of CD45⁺ cells dissociated from the whole aorta of PBS- and BU-gavaged mice. A. Uniform Manifold Approximation and Projection (UMAP) visualization of 24 annotated cell types. B. Relative abundance of each of the 24 cell types in CD45⁺ cells. C. Relative abundance of M2 macrophages. Data are the mean $\pm$ SEM of 3 samples. * $p < 0.05$ (two sided Welch's two-sample t-

test). D. Differentially expressed genes by designated cell type of the BU group compared with the PBS group (cut off line: |fold change| ≥ 2 ; adjusted $p < 0.05$). In each volcano plot, the 5 most down-regulated genes and 5 most up-regulated genes are labeled in black. Additional genes of interest are labeled in red.

4. Mechanism of HMG-CoA Reductase Inhibition though PA

4.1 The mechanism by which PA inhibits HMG-CoA reductase (HMGCR) remains insufficiently explored. The authors report that PA inhibits HMGCR activity with an IC_{50} of 15.53 μM , whereas atorvastatin has a much lower IC_{50} of 0.05 μM . This large difference suggests that PA is a significantly weaker inhibitor; however, the implications of this disparity are not adequately discussed.

Response: We appreciate your valuable suggestion. We have carried out additional pharmacokinetic experiments to answer this question. Please see our detailed response under question #4.3.

4.2 In this context, the paragraph starting at line 430 is overly speculative, asserting that PA is more favorable because it is endogenous and may be more adaptable to the host than statins. This claim lacks sufficient mechanistic support and should be substantiated or revised. Moreover, it remains unclear whether PA acts as a competitive inhibitor at the acetyl-CoA binding site or through a different mechanism. This should be experimentally investigated and clearly discussed.

Response: We appreciate your valuable suggestion. we have revised the relevant sentences (Please see Line 524-526) to temper those claims and focus on the established findings.

Our prior surface plasmon resonance (SPR) analysis had already established direct binding, yielding a dissociation constant (K_D) of 7.41 μM (Fig .4E in the revised manuscript). To further characterize this interaction and define the precise inhibitory mechanism, we performed additional experiments. Using isothermal titration calorimetry (ITC), we independently confirmed the binding, measuring a comparable K_D of 6.11 μM (Fig. 4F, G in the revised manuscript, which also shown as below; Please see line 348-351), reinforcing the specificity of the PA-HMGCR interaction. To elucidate the mode of inhibition, we conducted detailed enzymatic kinetic assays. With purified human HMGCR, PA caused a dose-dependent increase in the apparent Michaelis constant (K_m) for the substrate HMG-CoA, while the maximal reaction rate (V_{max}) remained unchanged (Fig. 4H in the revised manuscript, which also shown as below; Please see line 351-355). This kinetic signature is diagnostic of competitive inhibition, indicating that PA acts as a competitive inhibitor against HMG-CoA. In summary, the combined SPR/ITC binding data and the competitive inhibition kinetics establish that PA acts as a direct competitive inhibitor of HMGCR. We have made the corresponding revision at Line 348-355 at *Results* and Line 1160-1172 at *Methods* section in the revised manuscript.

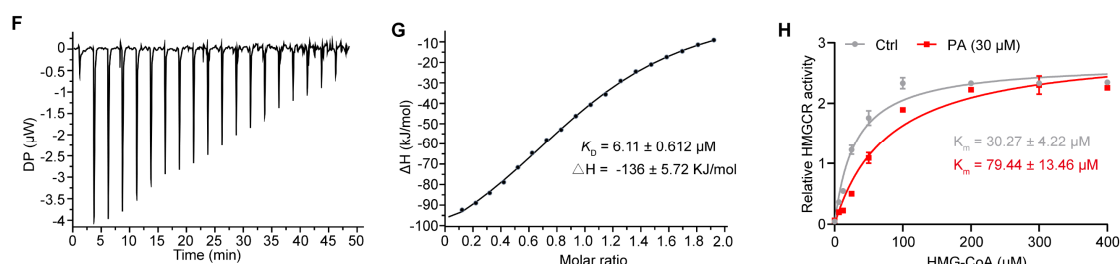
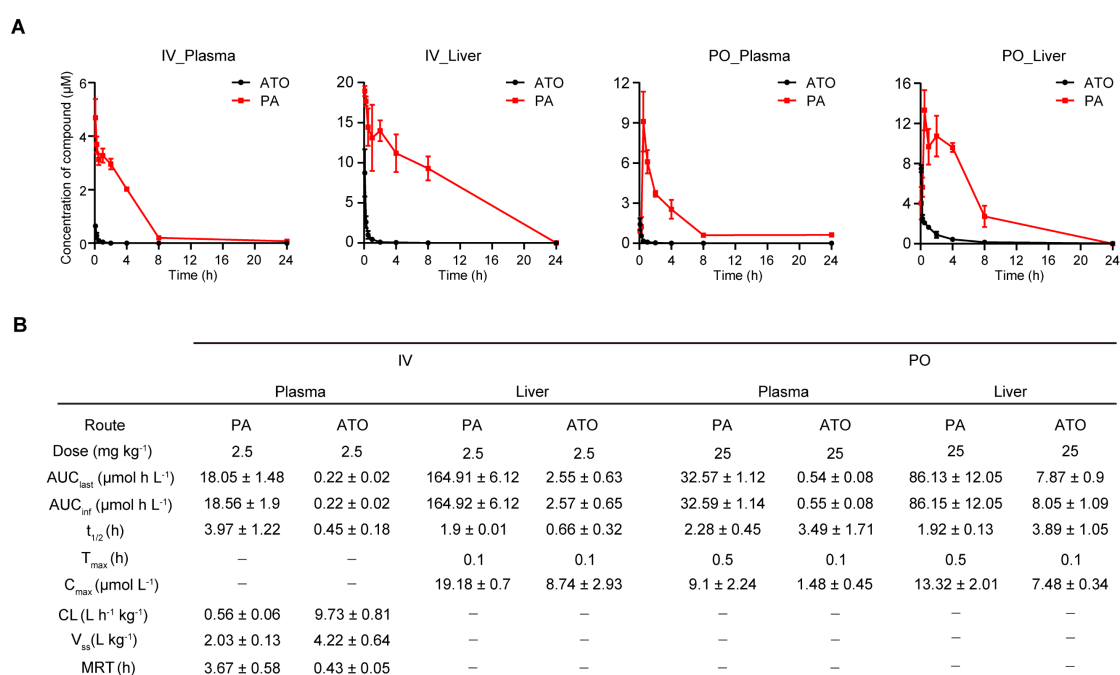


Fig. 4F-H | F and G. Isothermal titration calorimetry (ITC)–mediated determination of HMGR binding to PA. **H.** Increasing HMG-CoA concentrations in the presence or absence of 30 μM PA were used in enzymatic inhibition assays performed as in Fig. 4D ($n = 3$). Data are presented as mean $\pm$ SEM.

4.3 Finally, the proposed mechanism of action of PA appears to resemble that of statins. However, based on the authors' own results, atorvastatin is clearly more potent in inhibiting HMGR activity. Therefore, the authors need to reconcile how PA can exert atheroprotective effects similar to atorvastatin despite being a much weaker inhibitor.

Response: We appreciate your valuable suggestion. To address how PA can exert atheroprotective effects similar to atorvastatin despite being a much weaker inhibitor, we conducted a pharmacokinetic comparison between PA and atorvastatin. Interestingly, PA demonstrated a significantly higher area under the curve (AUC) in both plasma and liver (Supplementary Fig. 4A in the revised manuscript, which also shown as below). Furthermore, PA exhibited a much lower clearance rate (CL) and a much higher mean residence time (MRT), indicating that PA persists longer in the system compared to atorvastatin (Supplementary Fig. 4B in the revised manuscript, which also shown as below). These pharmacokinetic properties may help compensate for PA's weaker IC_{50} against HMGR. We have made the corresponding revision at Line 359-363 at *Results* and Line 1174-1182 at *Methods* section in the revised manuscript.



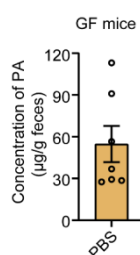
Supplementary Fig. 4 | Murine pharmacokinetic (PK) parameters for PA and atorvastatin. **A.** Comparison of total plasma and liver concentration profiles of PA and ATO administered as IV (2.5 mg/kg), or PO (25 mg/kg) (n = 3). **B.** Male C57BL/6 mice were dosed for the IV and PO arms alternating sampling per time point was performed to limit the number of times blood was collected per mice, therefore we were unable to calculate error for these treatments. AUC: area under the curve; $t_{1/2}$: half-life; T_{max} = time of peak blood concentration; CL: clearance; C_{max} = maximum concentration reached; MRT: Mean residence time; V_{ss} : volume of distribution; Data shown are mean $\pm$ SD (A and B).

4.4 Several bacterial species are capable of producing PA, as acknowledged by the authors. Can the authors estimate the specific contribution of *Bacteroides uniformis* to systemic PA levels in vivo, relative to other PA-producing bacteria?

Response: We appreciate your thoughtful question. To assess the contribution of PA from various sources, we conducted a careful quantitative analysis. Please check below.

First, we would like to clarify that PA is not synthesized by the host, as stated in the revised manuscript (Please see Line 431-432). Thus, fecal PA detected in mice originates from dietary input and/or gut microbiota. To clarify their relative contributions, we performed the following analyses.

(i) Dietary contribution to fecal PA. We observed that germ-free (GF) mice fed a HFD and treated with PBS exhibited ~55 $\mu\text{g/g}$ feces of PA after 6 weeks (Supplementary Fig. 5 in the revised manuscript, which also shown as below), indicating that dietary PA contributes approximately ~55 $\mu\text{g/g}$ feces under these conditions.



Supplementary Fig. 5 | PA levels in feces of HFD-fed GF *Apoe*^{-/-} mice after a 6-week gavage with PBS (n = 7). Data are presented as mean $\pm$ SEM.

(ii) Contribution from resident gut microbiota. In SPF mice maintained on HFD, baseline fecal PA levels were approximately ~230 $\mu\text{g/g}$ (Fig. 3L in the revised manuscript, which also shown as below). Subtracting the estimated dietary contribution (~55 $\mu\text{g/g}$), the gut microbiota accounts for ~175 $\mu\text{g/g}$ feces of PA. Previously we have screened approximately 100 bacterial strains and found that the majority of *Bacteroides* species possess the capacity to produce PA. These findings suggest that resident *Bacteroides* constitute a major source of baseline microbiota-derived PA in SPF mice.

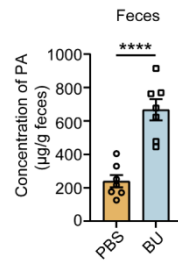


Fig. 3L | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS or BU (n = 7). Data are presented as mean ± SEM. Statistical significance was determined using two-tailed Student's t test: ***p* < 0.0001.**

(iii) Contribution of gavaged BU. To determine the specific contribution of BU, we compared mice gavaged with BU WT vs. PA-deficient mutants (Δ *pta* or Δ *mutA*). Under identical SPF and HFD feeding conditions, fecal PA levels decreased from ~670 µg/g in BU WT-treated mice to ~430 µg/g in mice receiving PA-deficient mutants, representing a reduction of ~240 µg/g feces (Fig. 5D in the revised manuscript, which also shown as below). This genotype-dependent difference provides a quantitative estimate of BU-derived PA contribution in SPF mice. Importantly, although gavaged BU is not the majority of total mice gut bacteria, this does not imply a proportional contribution to PA production. Rather, BU functions as a high-output PA producer, such that a relatively low-abundance taxon can disproportionately influence total PA levels.

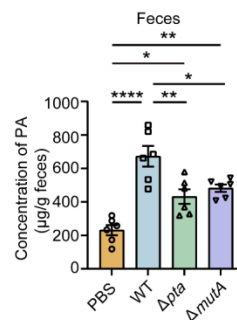


Fig. 5D | PA levels in feces of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS, BU or PA-deficient mutants (n = 6). Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test: **p* < 0.05; *p* < 0.01; ****p* < 0.001; *****p* < 0.0001.**

Collectively, these analyses indicate that baseline fecal PA reflects combined inputs from diet and resident microbiota, while BU gavage provides a quantitatively substantial PA contribution. The marked reduction in PA levels upon administration of PA-deficient BU mutants, despite comparable colonization, supports BU-derived PA biosynthesis as a major and functionally relevant driver of PA elevation in SPF mice. We have made corresponding revision at Line 432-445 at *Results* and Line 1045-1060 at *Methods* section in the revised manuscript.

Minor comments

895 1. There is a mistake in Extended data Fig. 10 L 7.

896 **Response:** Sorry for this error. We found the figure caption not appropriate and have
897 removed it.

898 2. The abbreviation “CHD” for chow diet is unconventional and should be reconsidered
899 for clarity.

900 **Response:** We appreciate your valuable suggestion. We agree that the abbreviation “CHD”
901 for the chow diet group was unconventional and could cause confusion. We have removed
902 the “CHD” designation and now refer this group as the “chow diet” or “control diet” group
903 throughout the text to ensure clarity.

Referee #5

In their manuscript, Yin et al. explore how gut bacteria—specifically *Bacteroides uniformis*—may help protect against atherosclerosis by producing a beneficial fatty acid called pentadecanoic acid (PA, C15:0). Using a combination of state-of-the-art microbiome analysis, cell-based tests, and mouse models, the authors show that PA lowers cholesterol levels by inhibiting HMG-CoA reductase, the key enzyme in cholesterol production, and by increasing the removal of LDL cholesterol from the blood. As a result, PA treatment significantly reduces atherosclerotic plaque development in atheroprone mice fed a high-fat diet.

The study presents an interesting idea: that certain gut bacteria can produce compounds with statin-like effects, potentially offering a new way to manage atherosclerotic cardiovascular disease. However, while the inhibitory effect of PA on HMG-CoA reductase is interesting, the novelty of the findings is substantially limited, given the previously reported LDL-cholesterol-lowering effects of PA treatment already tested in humans (e.g., Chooi, Y. C. et al., *Am J Clin Nutr* 119, 788–799, doi:10.1016/j.ajcnut.2023.11.013 (2024)).

The manuscript is clearly written, and the data are presented in a straightforward manner. The following points are suggested to improve the manuscript:

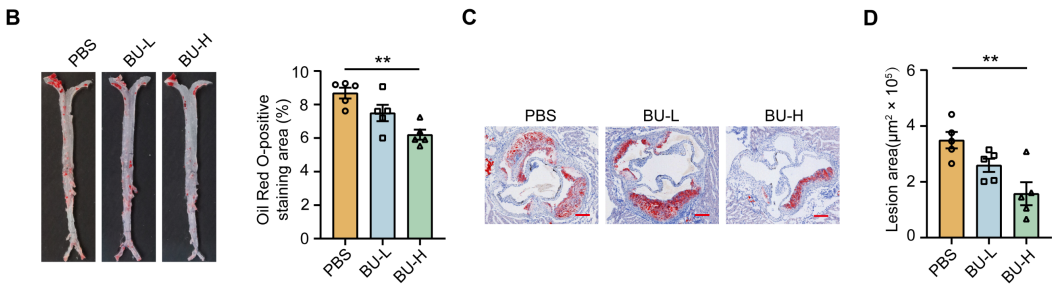
Response: We are grateful for your detailed and constructive comments, which have greatly helped to strengthen the scientific rigor, mechanistic clarity, and overall impact of our study. In response to the concerns raised during review, we have substantially revised the manuscript and performed extensive new experiments and analyses. All comments have been addressed in detail with point-by-point responses (please see below), and corresponding revisions have been highlighted in red in the revised manuscript. We hope that the revised version satisfactorily addresses your questions, and we would be pleased to further revise the manuscript should additional concerns arise.

1. The manuscript does not adequately justify the selected dose of *B. uniformis* (10^9 CFU/mouse). The authors should clarify whether this dose was based on prior studies, pilot data, or established therapeutic thresholds. It would also be important to assess whether a dose-response relationship exists—i.e., whether lower or higher doses result in similar or enhanced atheroprotective effects. Moreover, a discussion on how this dosage translates to therapeutically relevant levels in humans would strengthen the translational relevance of the findings.

Response: We appreciate your thoughtful questions. The dose of 10^9 CFU/mouse was selected based on previous studies¹⁰ and validated by our pilot studies. Specifically, a comparison between 10^8 CFU/mouse (Low concentration BU, BU-L) and 10^9 CFU/mouse (High concentration BU, BU-H) revealed that the lower dose did not produce a significant atheroprotective effect, whereas the 10^9 CFU/mouse dose did, confirming a dose-dependent response and justifying our choice of dose for the main study (Extended data

Fig. 1B-D in the revised manuscript, which also shown below). We have added relevant details and figure panels to the *Results* section (Please see Line 103-105 in the revised manuscript; all revised words are colored in red).

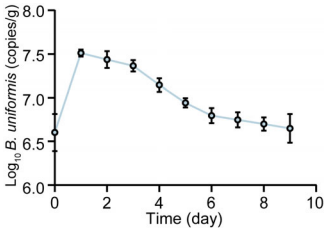
To address the translational relevance, we have also included a discussion regarding dose extrapolation to humans in *Discussion* section (Please see line 537-541 in the revised manuscript).



Extended data Fig. 1B-D | B. Representative images of Oil Red O-stained plaques in whole aorta with quantifications. **C and D.** Representative images of Oil Red O-stained plaques in the sections of aortic roots with quantifications. Scale bar: 200 μm. **B-D.** HFD-fed *Apoe*^{-/-} mice were gavaged with PBS and *B. uniformis* three times per week for 12 weeks. Data are presented as mean ± SEM (B and D). Statistical significance was determined using one-way ANOVA with Dunnett's test (B and D): ***p* < 0.01.

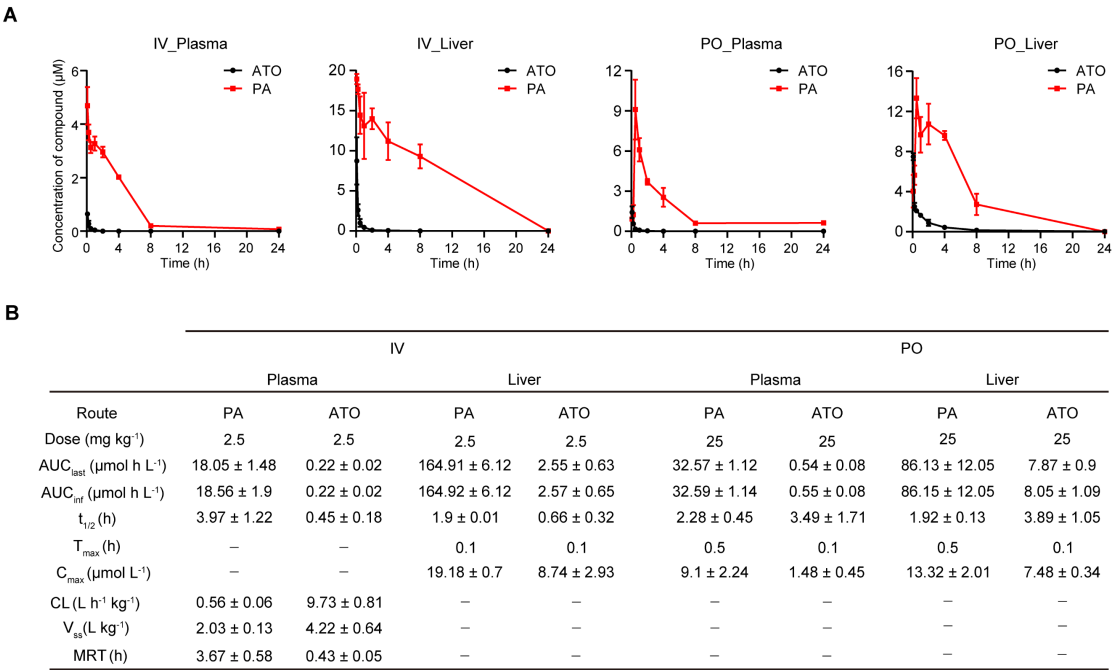
2. It also remains unclear how long *B. uniformis* persists in the host following oral gavage. The authors are encouraged to provide data, or cite existing literature, on the strain's *in vivo* stability, half-life, and colonization dynamics, including its distribution within host tissues. In addition, information on the circulating levels and pharmacokinetics of PA would be critical to understanding the duration of its therapeutic action and whether sustained exposure is necessary for efficacy.

Response: We appreciate your thoughtful questions. To address first question, our colonization-tracking experiments showed that a single oral gavage boosted the level of *B. uniformis* by 10-fold, and the level did not drop significantly until Day 4. The half-life of this strain is around 3.2 days (Extended data Fig. 1K in the revised manuscript, which also shown as below). We have added a sentence and a supplementary figure panel in the *Result* section (Please see Line 96-98 in the revised manuscript).



Extended data Fig. 1K | Absolute quantification of *B. uniformis* abundance in mouse feces collected at multiple time points a single oral gavage, determined by TaqMan qPCR based on the copy numbers of the 16S rRNA-encoding gene (n = 6). Data shown are mean ± SEM.

Next, we characterized the pharmacokinetic profile of PA in mice. The pharmacokinetic parameters indicate that PA exhibits extensive tissue distribution ($V_{ss} = 2.03 \text{ L/kg}$), moderate systemic clearance ($CL = 0.56\text{L/h/kg}$), and an intermediate terminal half-life (Plasma $t_{1/2} = 3.97 \text{ h}$; Liver $t_{1/2} = 1.90 \text{ h}$) (Supplementary Fig. 4 in the revised manuscript, which also shown as below). Together, these parameters describe a balanced pharmacokinetic profile without evidence of markedly impaired elimination. We have added this to *Discussion* (Please see Line 524-532 in the revised manuscript).

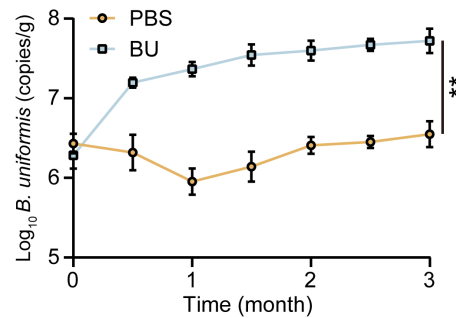


Supplementary Fig. 4 | Murine pharmacokinetic (PK) parameters for PA and atorvastatin. **A.** Comparison of total plasma and liver concentration profiles of PA and ATO administered as IV (2.5 mg/kg), or PO (25 mg/kg) (n = 3). **B.** Male C57BL/6 mice were dosed for the IV and PO arms alternating sampling per time point was performed to limit the number of times blood was collected per mice, therefore we were unable to calculate error for these treatments. AUC: area under the curve; $t_{1/2}$: half-life; T_{max} = time of peak blood concentration; CL: clearance; C_{max} = maximum concentration reached; MRT: Mean residence time; V_{ss} : volume of distribution; Data shown are mean ± SD (A and B).

3. The current experimental design includes measurement of fecal bacterial load at only one time point (Day 4 of Week 12, Extended Data Fig. 1G), without evaluation of baseline levels or longitudinal colonization kinetics. Including multiple time points would provide more detailed insights into bacterial colonization, persistence, and uptake efficiency. The

rationale for measuring at a single time point should be clearly explained.

Response: We appreciate your valuable suggestion. We performed longitudinal colonization tracking, which showed that *B. uniformis* stably colonized the intestinal tract in the treatment group throughout the study (Extended data Fig. 1E in the revised manuscript, which also shown as below). We have added a sentence and a figure panel in *Result* section (Please see Line 100-101 in the revised manuscript).



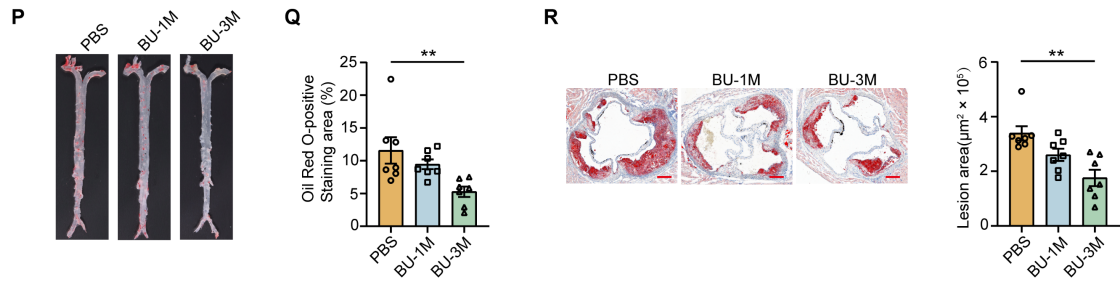
Extended data Fig. 1E | Absolute quantification of *B. uniformis* abundance in mouse feces collected at multiple time points during PBS or BU treatment, determined by TaqMan qPCR based on the copy numbers of the 16S rRNA-encoding gene (n = 6). HFD-fed *Apoe*^{-/-} mice were gavaged with PBS and BU three times per week for 12 weeks. Data are presented as mean ± SEM. Statistical significance was determined using two-tailed t-tests with Welch's correction: ***p* < 0.01

4. The authors are also encouraged to expand the discussion on the long-term therapeutic potential of *B. uniformis*. Given that gut microbial communities and host physiology can change over time, it is essential to address whether the beneficial effects are transient or durable. The manuscript should consider whether short-term administration is sufficient or if sustained or repeated dosing is required to maintain efficacy. Additionally, the influence of host environment on bacterial colonization and function over time should be discussed.

Response: We appreciate your valuable suggestion. To directly address the durability of effects, we compared the outcomes of different treatment durations (3 months vs 1 month). We were able to reproduce the significant atheroprotective effect in mice gavaged for 3 months. In contrast, mice gavaged for 1 month did not yield a significant atheroprotective effect (Extended data Fig. 1P-R in the revised manuscript, which also shown as below). These findings indicate short-term administration is not sufficient and repeated dosing is required to maintain efficacy in mice. We have added a few sentences and figure panels in *Result* section (Please see Line 105-107 in the revised manuscript).

As for the influence of the host environment, we acknowledge that colonization and function of *B. uniformis* are likely influenced by dynamic host factors, including diet and the gut microbiome. Investigating host environmental factors that facilitate *B. uniformis*

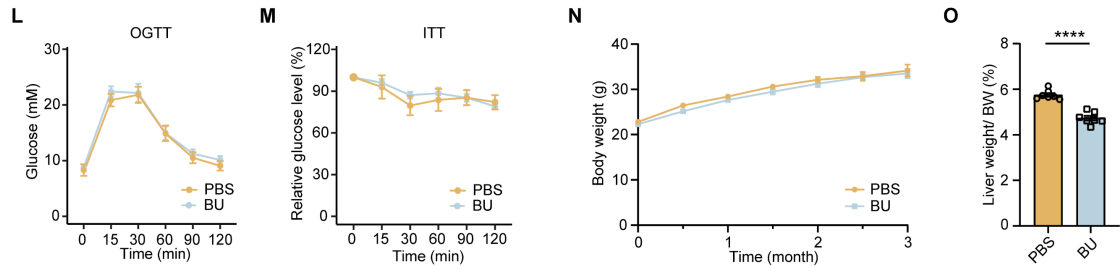
colonization and function could further enhance its atheroprotective effects. We have included this to *Discussion* in the revised manuscript (Please see Line 541-544).



Extended data Fig. 1P-R | P and Q. Representative images of Oil Red O-stained plaques in whole aorta with quantifications. **R.** Representative images of Oil Red O-stained plaques in the sections of aortic roots with quantifications. Scale bar: 200 μm. **P-Q.** HFD-fed *Apoe*^{-/-} mice were gavaged with PBS or *B. uniformis* (BU) three times per week for 1 month (BU-1M group) or 3 months (BU-3M group), respectively. Data are presented as mean ± SEM (Q and R). Statistical significance was determined using one-way ANOVA with Dunnett's test (Q), or Kruskal-Wallis test followed by Dunn's post hoc test (R): ***p* < 0.01.

5. Given the reported reduction in intracellular cholesterol, plasma LDL-C levels, and atherosclerotic plaque formation, it would be useful to assess whether *B. uniformis* treatment also affects broader systemic metabolic parameters such as body weight, liver weight, or insulin sensitivity.

Response: We appreciate your thoughtful questions. To address this, we assessed body weight, liver weight, and insulin sensitivity in HFD-fed *Apoe*^{-/-} mice following 12 weeks of *B. uniformis* (BU) treatment. BU treatment did not affect body weight, and insulin sensitivity, as determined by oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) (Extended data Fig. 1L-N in the revised manuscript, which also shown as below). However, it significantly reduced liver weight (Extended data Fig. 1O in the revised manuscript), a finding consistent with a previous report (e.g., Nie et al. Cell. 2024¹¹). We have added these results and a corresponding figure panel to the *Results* section (Please see Line 111-113 in the revised manuscript).

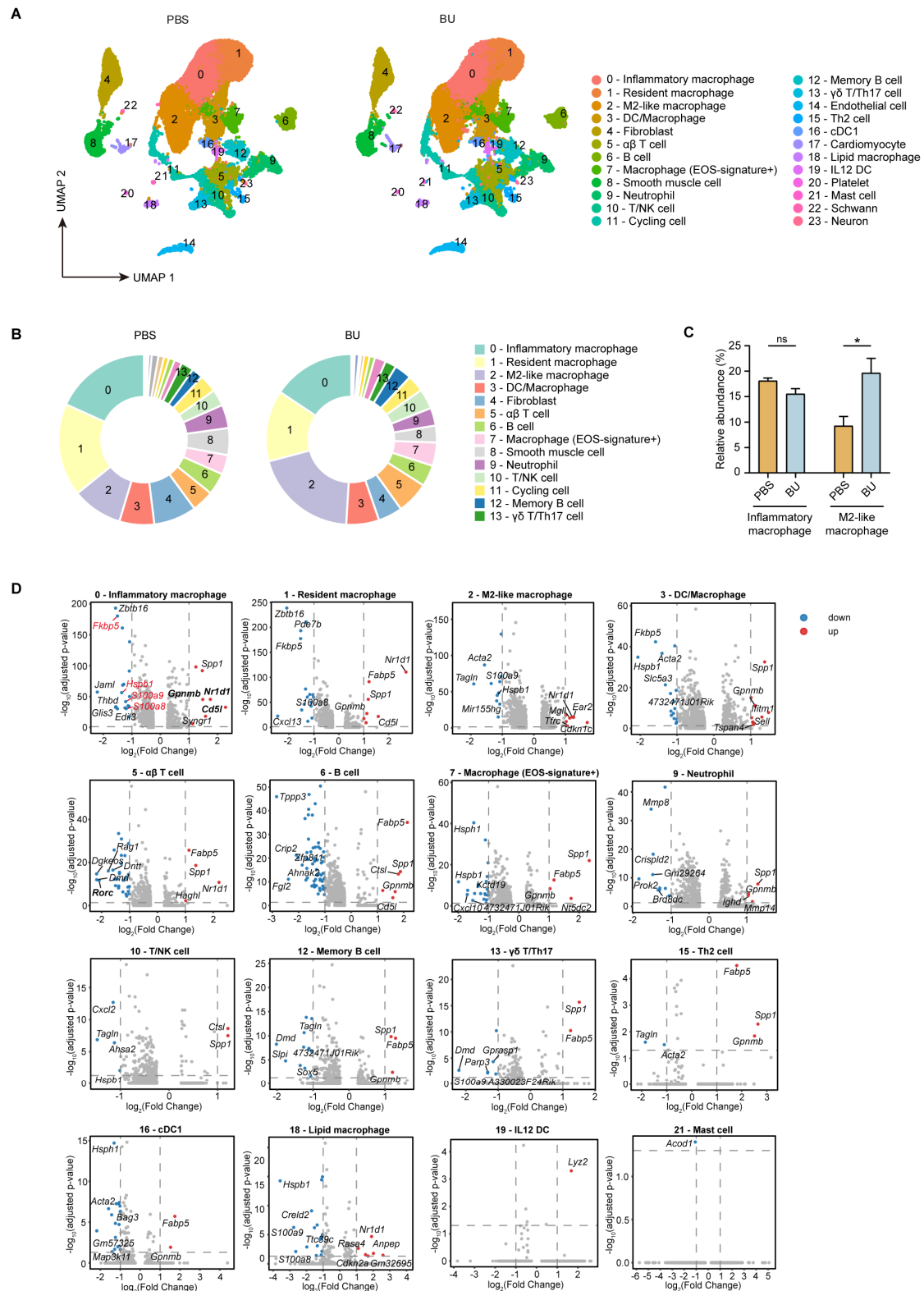


Extended data Fig. 1L-O | L and M. OGTT and ITT indexes in HFD-fed *Apoe*^{-/-} mice following PBS and BU treatment for 12 weeks (n = 7 per group). **N.** Body weight changes in HFD-fed *Apoe*^{-/-} mice following PBS and BU treatment for 12 weeks (n = 7

per group). **O.** Ratios of liver mass to body mass. Data are presented as mean $\pm$ SEM (L-O). Statistical significance was determined using two-tailed Student's t test (L-O): **** $p < 0.0001$.

6. While the study includes data on monocyte/macrophage involvement in atherosclerosis and inflammation, it lacks a broader characterization of immune cell populations in the mouse model. The authors are encouraged to also explore whether *B. uniformis* and PA modulate other immune pathways, particularly T regulatory cells or atherogenic Th1/Th17 subsets. A more comprehensive immunophenotypic analysis would enhance understanding of the immune-modulatory effects of *B. uniformis* / PA.

Response: We appreciate your valuable suggestion. To conduct a more comprehensive analysis, we carried out single-cell RNA sequencing within CD45⁺ cells dissociated from the whole aorta from both groups, respectively. Our panoramic analysis annotated 24 distinct cell clusters including macrophage subtypes, lymphocytes, other immune cells, and some non-immune cells (Supplementary Fig. 1A in the revised manuscript, which also shown as below). In both groups, inflammatory, M2-like and residential macrophages consist a substantial portion (~45%) of all CD45⁺ cells (Supplementary Fig. 1B in the revised manuscript, which also shown as below). The relative abundance of M2-like macrophage in BU-gavaged mice increased significantly compared to PBS-gavaged mice (Supplementary Fig 1C in the revised manuscript, which also shown as below; the revised Supplementary Table 2A). Further, we identified differentially expressed genes (DEGs) by each of the annotated 16 immune cell clusters between the two groups. A total number of 67 DEGs were upregulated and 238 were down-regulated in the BU group ($|\text{Fold Change}| \geq 2$; adjusted $p < 0.05$; Supplementary Fig 1D in the revised manuscript, which also shown as below; the revised Supplementary Table 2B). Notably, although the relative abundance of inflammatory macrophages did not differ significantly between the two groups, the inflammatory macrophages from the BU group exhibited a less inflammatory transcriptional profile, characterized by the downregulation of inflammation-associated genes (*S100a8*, *S100a9*, *Fkbp5*, and *Hspb1*) and the upregulation of genes previously reported to exert anti-inflammatory or inflammation-resolving functions (*Gpnmb*, *Nr1d1*, and *Cd5l*). Interestingly, $\alpha\beta$ T cells from the BU group exhibited reduced expression of *Rorc*, suggesting a diminished propensity toward Th17 differentiation. Together, these shifts indicate that *B. uniformis* remodels the plaque immune microenvironment toward a less inflammatory state.



Supplementary Fig. 1 | Single-cell RNA sequencing of CD45⁺ cells dissociated from the whole aorta of PBS- and BU-gavaged mice. A. Uniform Manifold Approximation and Projection (UMAP) visualization of 24 annotated cell types. B. Relative abundance of each of the 24 cell types in CD45⁺ cells. C. Relative abundance of M2 macrophages. Data are the mean $\pm$ SEM of 3 samples. * $p < 0.05$ (two sided Welch's two-sample t-

test). D. Differentially expressed genes by designated cell type of the BU group compared with the PBS group (cut off line: |fold change| ≥ 2 ; adjusted $p < 0.05$). In each volcano plot, the 5 most down-regulated genes and 5 most up-regulated genes are labeled in black. Additional genes of interest are labeled in red.

7. The manuscript notes that PA biosynthesis involves acetate and propionate as precursors (lines 318–320). Since propionate has known atheroprotective and cholesterol-lowering effects, the authors should consider whether the observed outcomes may be influenced by increased levels of these precursors. It would be informative to assess whether acetate or propionate concentrations change following *B. uniformis* administration, potentially contributing to the observed atheroprotection.

Response: We appreciate your thoughtful question. To address this, we measured acetate and propionate levels in feces, liver tissues, and serum from PBS- and BU-treated HFD-fed *Apoe*^{-/-} mice. We observed similar levels of acetate (Please see Panel A-C below) and elevated levels of propionate (Please see Panel D-F below) in feces, liver tissue and serum samples from BU-treated mice compared to the PBS group. Notably, propionate level observed in our model is substantially lower (approximately 10-fold) than the concentrations required to reduce plasma cholesterol in prior interventional studies where propionate was directly fed to mice¹². Therefore, it is unlikely that the modest increase in propionate alone accounts for the significant atheroprotective and cholesterol-lowering effects observed following *B. uniformis* administration.

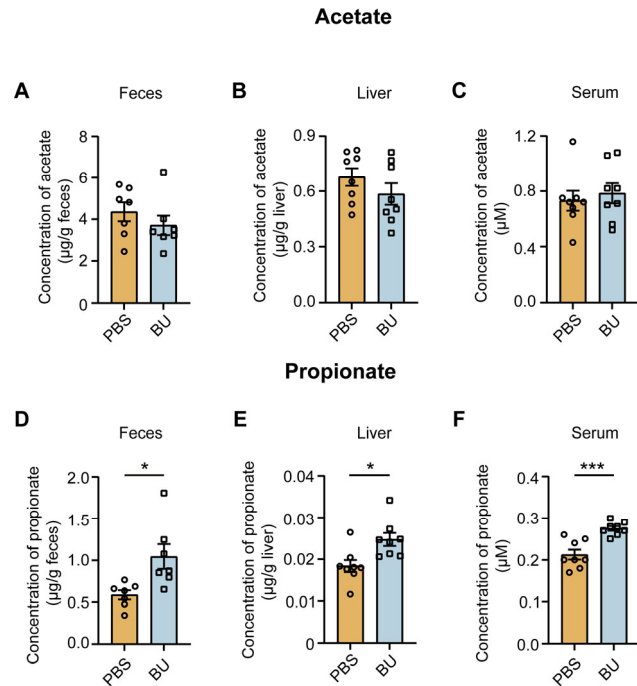


Figure legend | A-F. Precursor levels of PA in feces (A and C), liver tissues (B and E), and serum (C and F) of HFD-fed *Apoe*^{-/-} mice after a 12-week gavage with PBS (n = 7), BU (n = 7). Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-tailed Student's t test (A-C, E and F), or two-tailed t-tests with

Welch's correction (D): * $p < 0.05$; *** $p < 0.001$.

Minor points:

1. To improve the clinical interpretation of the findings, the manuscript should incorporate a detailed table of cohort characteristics, including age, sex, presence of hypertension, hyperlipidemia, diabetes, and other relevant factors. These host variables are known to influence both metabolism and gut microbiota composition, and could help determine whether the effects of B. uniformis are consistent across diverse patient profiles or specific to certain risk groups.

Response: We appreciate your valuable suggestion. we have now included a detailed table of cohort characteristics (e.g., age, sex, hypertension, hyperlipidemia, diabetes) in the revised manuscript. Please see the revised Supplementary Table 7.

2. Quantitative analysis and statistical evaluation of the Western blot results should be included to support the conclusions and enhance the methodological rigor of the study.

Response: Thank you for this constructive suggestion. To strengthen our conclusions, we have conducted quantitative analysis for every Western blot, normalizing band intensities to loading controls and subjecting the data to statistical testing. These quantitative results are now presented as graphs alongside the original blot images in the revised figures.

3. In the figure legends, the precise concentration of PA used in each experiment should be explicitly stated. The authors should also provide a rationale for selecting this concentration and discuss whether it reflects physiologically relevant levels found in the human bloodstream, especially in the context of healthy individuals versus patients with ASCVD.

Response: We thank you for this suggestion to clarify the dosing rationale and physiological relevance. The concentration of PA used in each cellular experiment is now explicitly stated in the corresponding figure legends. This concentration was selected with reference to the effective in vivo doses of lipid-lowering agents (e.g., statins) reported in prior animal studies^{13,14}. To assess physiological relevance, our pharmacokinetic data show that an oral dose of 25 mg/kg PA in mice yields a maximum blood concentration of ~2.3 µg/mL. This level is comparable to the endogenous PA concentration (~2 µg/mL) measured in healthy humans, indicating that the exposure achieved in our study approaches the physiological range.

Reference

1156 1 Morselli, E. *et al.* The effects of oestrogens and their receptors on cardiometabolic health. *Nat*
1157 *Rev Endocrinol* **13**, 352-364, doi:10.1038/nrendo.2017.12 (2017).

1158 2 Xing, B., Wang, R., Kou, T., Liu, W. & Zhu, Z. J. Unraveling tissue-specific fatty acid biosynthesis
1159 and inter-tissue crosstalk in mice through stable-isotope tracing metabolomics. *Adv Sci (Weinh)*
1160 **12**, e03662, doi:10.1002/advs.202503662 (2025).

1161 3 Xiao, H. *et al.* Gentiopicroside targets PAQR3 to activate the PI3K/AKT signaling pathway and
1162 ameliorate disordered glucose and lipid metabolism. *Acta Pharm Sin B* **12**, 2887-2904,
1163 doi:10.1016/j.apsb.2021.12.023 (2022).

1164 4 He, Y. *et al.* Palmitic acid accelerates endothelial cell injury and cardiovascular dysfunction via
1165 palmitoylation of PKM2. *Adv Sci (Weinh)* **12**, e2412895, doi:10.1002/advs.202412895 (2025).

1166 5 Trieu, K. *et al.* Biomarkers of dairy fat intake, incident cardiovascular disease, and all-cause
1167 mortality: a cohort study, systematic review, and meta-analysis. *PLoS Med* **18**, e1003763,
1168 doi:10.1371/journal.pmed.1003763 (2021).

1169 6 Wang, X. *et al.* *Candida albicans* accelerates atherosclerosis by activating intestinal hypoxia-
1170 inducible factor2alpha signaling. *Cell Host Microbe*, doi:10.1016/j.chom.2024.04.017 (2024).

1171 7 Qiao, S. *et al.* Gut *Parabacteroides merdae* protects against cardiovascular damage by
1172 enhancing branched-chain amino acid catabolism. *Nat Metab* **4**, 1271-1286,
1173 doi:10.1038/s42255-022-00649-y (2022).

1174 8 Haghighi, A. *et al.* Propionate attenuates atherosclerosis by immune-dependent regulation of
1175 intestinal cholesterol metabolism. *Eur Heart J*, doi:10.1093/eurheartj/ehab644 (2021).

1176 9 Zhang, S. *et al.* Sensing ceramides by CYSLTR2 and P2RY6 to aggravate atherosclerosis.
1177 *Nature* **641**, 476-485, doi:10.1038/s41586-025-08792-8 (2025).

1178 10 Morita, H. *et al.* *Bacteroides uniformis* and its preferred substrate, α -cyclodextrin, enhance
1179 endurance exercise performance in mice and human males. *Sci Adv* **9**, eadd2120,
1180 doi:10.1126/sciadv.add2120 (2023).

1181 11 Nie, Q. *et al.* Gut symbionts alleviate MASH through a secondary bile acid biosynthetic pathway.
1182 *Cell*, doi:10.1016/j.cell.2024.03.034 (2024).

1183 12 Brutting, C., Lara Bisch, M., Brandsch, C., Hirche, F. & Stangl, G. I. Impact of dietary propionate
1184 on fructose-induced changes in lipid metabolism, gut microbiota and short-chain fatty acids in
1185 mice. *Int J Food Sci Nutr* **72**, 160-173, doi:10.1080/09637486.2020.1773415 (2021).

1186 13 She, J. *et al.* Statins aggravate insulin resistance through reduced blood glucagon-like peptide-
1187 1 levels in a microbiota-dependent manner. *Cell Metab* **36**, 408-421 e405,
1188 doi:10.1016/j.cmet.2023.12.027 (2024).

1189 14 Hu, X. *et al.* A gut-derived hormone regulates cholesterol metabolism. *Cell*,
1190 doi:10.1016/j.cell.2024.02.024 (2024).

Referee #1 (Remarks to the Author):

The authors have addressed most of my previous comments, some with experimental evidence. I really appreciate the additional experiments in response to reviewer comments, which have strengthened several aspects of the study. However, a few issues remain that, in my opinion, require further clarification or textual revision.

1. Some inconsistencies in PA production suggest that microbe-microbe interactions may contribute to the observed phenotype and should be discussed: while BU supplementation increases PA levels in both germ-free and SPF backgrounds, the magnitude of PA production is dramatically higher in SPF mice. This difference implies that community context substantially amplifies PA production beyond what is achieved by BU alone. In addition, Δ pta/mutA mutant strains, which are proposed to lack the sole pathway for PA production, still exhibit substantially higher PA levels than PBS controls. If this pathway were exclusively responsible for PA synthesis, such elevations would not be expected. Together, these observations suggest that other *Bacteroides* species or community-level interactions may contribute to PA production, potentially through metabolic cross-feeding or indirect modulation of microbial pathways. A formal test of this would be ideal, at a minimum, a discussion of potential microbe-microbe interactions and community effects would provide a more balanced interpretation of the data.

Response:

We thank you for this constructive comment. We fully agree that this point helps to make our interpretation more complete and precise.

First, we agree that the markedly greater increase in PA levels observed in BU-supplemented SPF mice compared with BU-supplemented germ-free mice suggests that community context amplifies PA production beyond the contribution of BU alone. This raises the possibility that other *Bacteroides* species, or broader microbiota-dependent interactions, may contribute to the elevated PA pool. Such effects could plausibly arise through mechanisms including metabolite cross-feeding (for example, via propionate- or succinate-related intermediates) or indirect modulation of pathways influencing PA biosynthesis. We have revised the manuscript to explicitly acknowledge this

possibility (**Lines 583-590**).

Second, we would like to clarify that both the Δpta and $\Delta mutA$ strains are PA-reduced rather than PA-null *in vitro* (**Fig. 5A**). These strains retain residual PA-producing capacity; therefore, the observation that mice gavaged with these mutant strains still show higher PA levels than PBS-treated controls is actually not an inconsistent result. At the same time, we agree that this residual PA pool may also be further shaped *in vivo* by interactions with the surrounding microbial community.

Overall, we agree that our current data do not rule out contributions from other PA-producing taxa or microbe-microbe interactions, and we have revised the text accordingly to provide a more balanced interpretation.

2. A key concern is whether supplementation with PA can be verified within relevant biological compartments. If PA administration is effective, one would expect measurable changes in at least one compartment of the system. While the absence of changes in circulating levels is noted, it remains unclear whether PA accumulates in the gut, liver, or other tissues, or whether it is rapidly cleared or excreted. If rapid excretion occurs, corresponding changes in fecal levels would be expected. Without evidence that PA exposure is detectable in any biological compartment, it is difficult to assess confidence in the interpretation of the supplementation experiment.

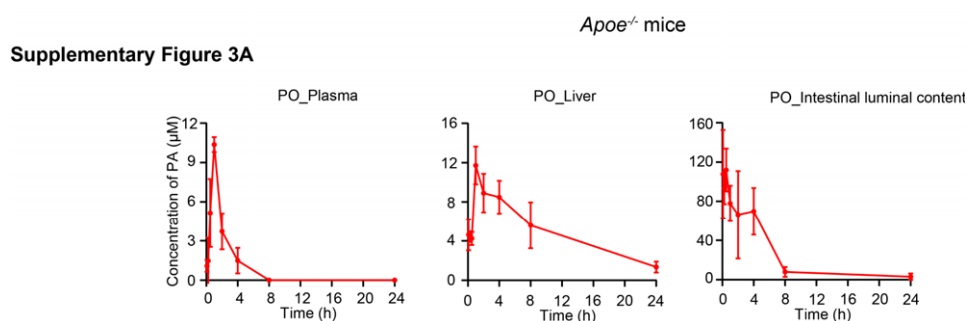
Response:

We thank you for this important comment regarding the biological verification of PA exposure following supplementation. To address this concern directly, we performed additional time-course measurements after oral PA administration and reorganized some pharmacokinetic (PK) data.

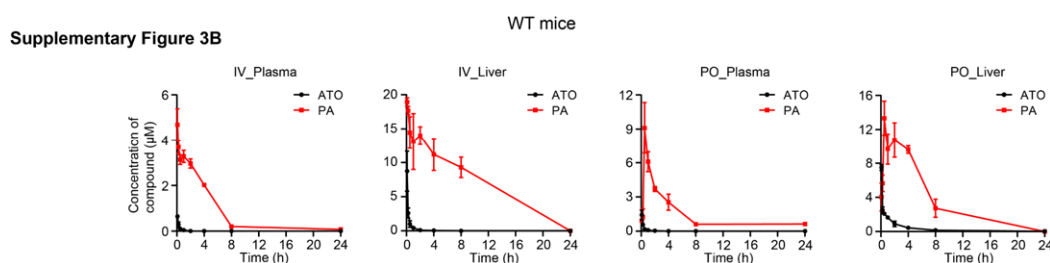
Our results show that supplemented PA is readily detectable in multiple biologically relevant compartments. Following oral gavage, PA was measurable in both plasma and liver in $Apoe^{-/-}$ and WT mice models (**Supplementary Fig. 3A, B**). In both models, PA levels increased rapidly after dosing, peaked at early time points, and then declined over time. In $Apoe^{-/-}$ mice, PA remained detectable in plasma for at least 4 hours, whereas hepatic PA persisted for at least 8 hours (**Supplementary Fig. 3A, B**). In addition, in WT mice, intravenous administration also resulted in more sustained liver exposure than plasma

exposure (**Supplementary Fig. 3B**). Together, these data demonstrate that administered PA reaches the systemic circulation and, importantly, the liver under our experimental conditions. We have revised the manuscript accordingly (**Line 289-292**).

Moreover, in *Apoe*^{-/-} mice, luminal PA was high at early time points after oral gavage but declined rapidly and was largely cleared by 8 h (**Supplementary Fig. 3A**), arguing against persistent accumulation in the gut lumen. Thus, although circulating PA does not remain durably elevated at later time points, the supplementation clearly results in measurable but transient systemic exposure together with more sustained hepatic exposure.



Supplementary Figure 3A: Concentration profiles of PA in plasma, liver and intestinal luminal content following a single oral administration in *Apoe*^{-/-} mice (25 mg/kg; n = 3). Data shown are mean ± SD.



Supplementary Figure 3B: Comparison of plasma and liver concentration profiles of PA and Atorvastatin (ATO) following oral (PO) and intravenous (IV) administration in WT mice (25 mg/kg; n = 3). Data shown are mean ± SD.

3. I'm not fully convinced by the statement that host doesn't produce PA. The current data does not rule out host production, particularly given the high baseline PA detected in germ-free mice. This baseline could reflect a combination of dietary input (as cited) and host synthesis. Have PA levels in the diet been directly measured, and do those levels align with what is detected

in vivo? At least, the authors should modify the text to better reflect the limitations of the current experimental evidence.

Response:

We thank you for this important point. We agree that our current data does not rule out the possibility of host-derived PA production, particularly in light of the baseline PA detected in germ-free mice. Accordingly, we have revised the manuscript to avoid the definitive statement that the host does not produce PA and to present a more balanced interpretation (**Lines 435-438, 441, 443-444, 446, 448-450**).

In addition, we directly quantified PA in the high-fat diet used in this study and detected approximately ~3.3 μg PA per gram of dry diet (**Please see below**). This finding supports dietary intake as a contributor to the baseline PA observed *in vivo*, including in germ-free mice. However, we cannot determine from the current data whether dietary PA fully accounts for this baseline, nor can we rule out a contribution from host synthesis.

Consistent with prior literature, our data support diet as an important source of mammalian C15:0, while not excluding a limited endogenous contribution¹⁻³.

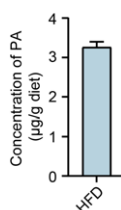


Figure legend | PA level in the high-fat diet. Data are presented as mean $\pm$ SEM.

Reference

1. Jenkins, B. J. *et al.* Odd chain fatty acids; New insights of the relationship between the gut microbiota, dietary intake, biosynthesis and glucose intolerance. *Sci Rep* **7**, 44845, doi:10.1038/srep44845 (2017).
2. Weitkunat, K. *et al.* Odd-chain fatty acids as a biomarker for dietary fiber intake: a novel pathway for endogenous production from propionate. *Am J Clin Nutr* **105**, 1544-1551, doi:10.3945/ajcn.117.152702 (2017).
3. Venn-Watson, S., Lumpkin, R. & Dennis, E. A. Efficacy of dietary odd-chain saturated fatty

acid pentadecanoic acid parallels broad associated health benefits in humans: could it be essential? *Sci Rep* **10**, 8161, doi:10.1038/s41598-020-64960-y (2020).

Minor comments:

1. There are abbreviations that need to be defined, particularly in Figure legends. For example, in Extended data Fig 1, BU-L, BU-H, 1M, 3M etc. are not immediately clear to the reader. In addition, the nature of PBS control requires clarification - whether it corresponds to a 4-week or 12-week time point. Given that atherosclerosis progression is highly time-dependent, the use of appropriately matched longitudinal controls is essential for meaningful comparisons.

Response:

We thank you for this helpful comment. We have revised the figure labels and legends to define the abbreviations more clearly. Specifically, “BU-L” and “BU-H” are now labeled as “BU - low dose” and “BU - high dose”, and “1M” and “3M” are now explicitly defined as 1 month and 3 months of BU treatment, respectively.

We have also clarified the PBS control in the revised figure legend. All groups were analyzed at the same 12-week experimental endpoint. In the “BU - 1 month” group, mice received BU treatment during the first 4 weeks followed by 8 weeks without treatment, whereas the “BU - 3 month” group received continuous treatment throughout the 12-week period. Accordingly, the PBS group shown in this experiment is the matched 12-week control for both comparisons. These clarifications have been added to the revised manuscript (**Lines 622-624**).

2. Lines 87-90: The sentence repeats the same point about *Bacteroides* abundance in two ways. The authors could combine these statements to avoid redundancy.

Response:

We thank you for this helpful suggestion. We have combined the two statements into a single sentence to avoid redundancy and improve clarity (**Lines 87-89**).

3. Line 178: LDL receptor (LDLR) should be defined earlier.

Response:

We thank you for this careful reading. We have now defined LDLR (low-density lipoprotein receptor) as its first occurrence in the main text (**Line 175**).

4. Lines 281-282: Add citation(s) to “Contrary to the traditional view that dietary 282 components like milk fat and ruminant meat are the primary sources of circulating PA.”

Response:

We thank you for this helpful suggestion. We have added the relevant references (20 and 21) to support this statement in the revised manuscript (**Line 284**).

Referee #2:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Response: We sincerely appreciate your insightful and constructive suggestions.

Referee #3 (Remarks to the Author):

I am mostly satisfied with the response to my critiques and suggestions, but still believe that a large question remains about this study that should be addressed. Is the effect from *B. uniformis* and its reduction in atherosclerosis due to PA (15:0) exclusively or is this more about odd-chain fatty acids? The authors describe evidence in their experiments and the literature that C17 also has some effects on improving dyslipidemia, though their data does not indicate that is through HMG-CoA reductase, which is an important result. This new data and the literature is brings up the question whether or not this is an effect of odd chain fatty acids or purely PA 15:0. The authors did not include any of the responses to my questions in their revised manuscript (as far as I can find) which is a significant oversight.

If PA C15:0 is exclusive in its action of inhibiting HMG-CoA reductase, then how is C17:0 also lowering cholesterol in their HepG2 cells? I don't expect the authors to answer the second question, that could be a whole study on its own, but this is a very important question and the matter of odd-chain vs. even chain FAs and the specificity of PA 15:0 must be addressed where appropriate.

Response:

We thank you for highlighting this important conceptual issue. We agree that the extent to which the BU-associated phenotype is specific to C15:0, as compared with C17:0 and other odd-chain fatty acids, should be discussed more explicitly in the manuscript.

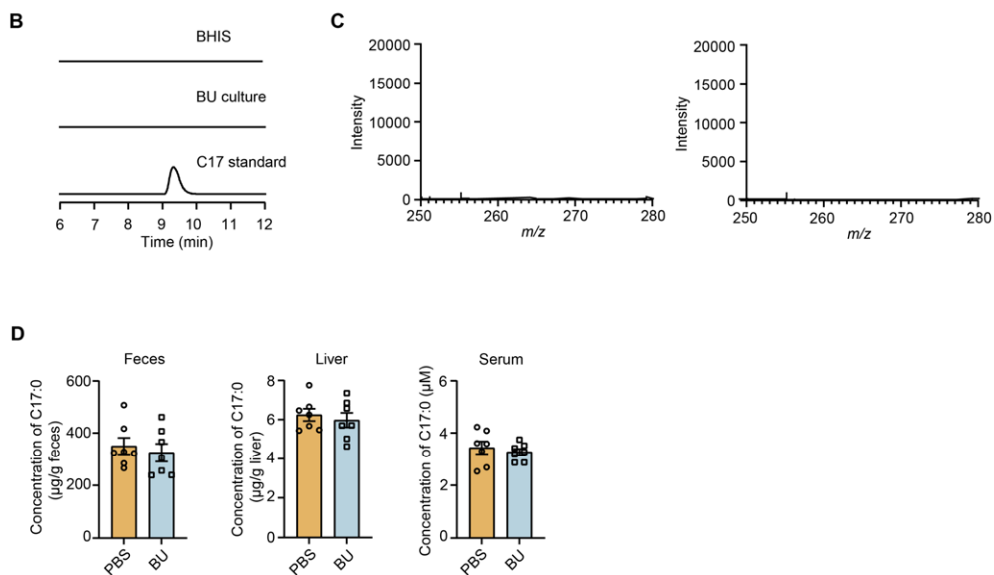
To address this point, we examined C17:0 in BU culture and *in vivo* samples, as C17:0 was specifically raised during the revision and has also been associated in previous studies with beneficial effects on lipid metabolism, making it an especially relevant odd-chain fatty acid for comparison with C15:0. Under our experimental conditions, *B. uniformis* did not produce detectable C17:0, as assessed by HPLC–MS analysis of culture supernatants (**Extended data Fig. 11B**). Moreover, supplementation of the culture medium with ¹³C-labeled acetate or ¹³C-labeled propionate still did not yield detectable C17:0 (**Extended data Fig. 11C**), whereas C15:0 production was readily observed under the same conditions. Consistent with this, in HFD-fed *Apoe*^{-/-} mice, BU gavage markedly increased fecal C15:0, whereas fecal C17:0 was not

significantly altered (**Extended data Fig. 11D**).

Together, these findings support C15:0 as the best-supported BU-associated odd-chain fatty acid linked to the *in vivo* phenotype in this study. At the same time, we agree with you that our data do not exclude the possibility that other odd-chain fatty acids may also contribute to cholesterol-lowering effects or related metabolic phenotypes through mechanisms distinct from HMGCR inhibition. In particular, although our data show that C17:0 can lower cholesterol levels in HepG2 cells, they do not support direct HMGCR inhibition by C17:0. By contrast, palmitate (C16:0), a representative even-chain fatty acid tested in parallel, neither reduced cholesterol synthesis in HepG2 cells nor inhibited HMGCR under our experimental conditions. We therefore interpret the current results as supporting mechanistic specificity of C15:0 with respect to HMGCR inhibition, while recognizing that C17:0 may influence cholesterol metabolism through other pathways. How C17:0 lower cholesterol synthesis in HepG2 cells is an interesting question for future study.

We apologize that our previous responses on this point were not sufficiently incorporated into the prior revised manuscript. We have now added both the new data and the corresponding discussion to address this issue more directly (**Extended data Fig. 11A–F; Lines 529–549**). Specifically, we revised the *Discussion* to make two points more explicit: first, that the BU-associated phenotype in our model is most consistent with C15:0 as the relevant BU-derived odd-chain fatty acid; and second, that broader comparisons among odd-chain and even-chain fatty acids, as well as clarification of how C17:0 lowers cholesterol independently of HMGCR, will require future investigation.

Extended data Fig. 11B-D



Extended data Fig. 11 | B. HPLC-MS analysis of BHIS, BU culture, and C17:0 standards. Extracted ion chromatograms (EICs) were obtained at m/z 269.2461 $\pm$ 0.01 $[\text{M}-\text{H}]^-$ for C17:0. **C.** High-resolution mass spectrometry (HRMS) of C17:0 BU fermentation supplemented with ^{13}C -labeled acetate (Left) and propionate (Right). **D.** C17:0 levels in feces, liver tissues and serum. of HFD-fed *Apoe*^{-/-} mice after 12-week gavage with PBS or BU (n = 7). Data are presented as mean $\pm$ SEM. Statistical significance was determined using two-tailed Student's t test.

Referee #4 (Remarks to the Author):

1. Although the authors have satisfactorily addressed some of the comments, the following concerns remain and should be addressed.

The authors do not accurately quantify atherosclerotic plaques. They analyzed only a single section at the level of the valves, rather than the entire aortic sinus. Even if plaque size is maximal at this level, quantifying plaques from only one section is not technically appropriate. Therefore, this major concern remains unresolved. The authors need to repeat at least the main experiments and section the entire aortic sinus to obtain accurate plaque quantification. This is particularly important because the previously shown images indicate that the sections were not anatomically matched across samples, which makes comparisons difficult and potentially misleading.

Response:

We thank you for this important comment regarding plaque quantification in the aortic root/sinus. To further address this concern, we revisited the remaining frozen heart samples from the previous WT vs. BU experiment and performed additional serial sectioning of the aortic root/sinus, guided by the approach described in the reference you suggested. We were able to obtain and analyze the lower portion of each aortic sinus, including the ~120 μm region above the bottom of each sinus. This additional analysis showed that *B. uniformis* treatment significantly reduced plaque area in HFD-fed *Apoe*^{-/-} mice (**Please see below**), consistent with our original result in **Fig. 1D**.

Specifically, hearts were serially sectioned and observed in an apical-to-basal direction with intervals of 8 μm . When any of the three sinus appears (labeled as **1a, 2a, or 3a** in **Panel A** below), the sections were defined as +0 μm from the bottom of the specific sinus. We further identified the +40 μm (labeled as **1b, 2b, or 3b**), +80 μm (labeled as **1c, 2c, or 3c**), and +120 μm (labeled as **1d, 2d, or 3d**) sections of each sinus, and carried out oil-O red staining. In **Panel B**, lesions of anatomically matched sinuses were quantified and totaled per mouse, and then averaged among mice (n=6 per group). **Panel C** summarizes these data by averaging lesion measurements across the entire 0-120 μm region above sinus bottom (n=6).

These data provide a broader assessment of plaque burden than the

representative level shown in the original figure and confirmed our conclusion that *B. uniformis* significantly reduces plaque area in this model. Furthermore, we have replaced the previously misaligned representative images with anatomically matched sections from the same level of the aortic sinus.

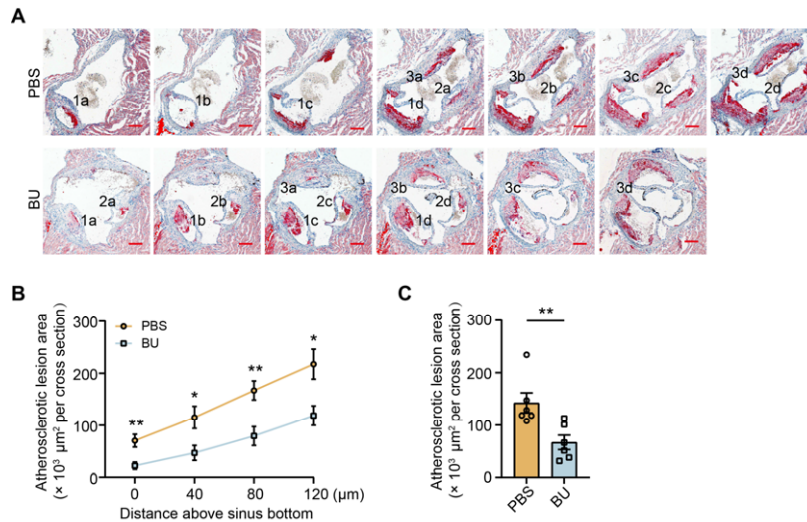


Figure legend | HFD-fed *Apoe*^{-/-} mice were gavaged with PBS or BU three times per week for 12 weeks (n = 6 per group). **A.** Representative images of Oil Red O-stained plaques in the sections of aortic roots. Scale bar: 200 μm . Hearts were serially sectioned and observed in an apical-to-basal direction with intervals of 8 μm . When any of the three sinus appears (labeled as 1a, 2a, or 3a), the sections were defined as +0 μm from the bottom of the specific sinus. We further identified the +40 μm (labeled as 1b, 2b, or 3b), +80 μm (labeled as 1c, 2c, or 3c), and +120 μm (labeled as 1d, 2d, or 3d) sections of each sinus. **B.** The absolute atherosclerotic lesion area plotted as a function of the distance from the sinus bottom. **C.** Average atherosclerotic lesion area derived from the data in (B). Data are presented as mean $\pm$ SEM (B and C). Statistical significance was determined using two-tailed Student's t test (B and C): * $p < 0.05$; ** $p < 0.01$.

2. My point 4 also remains insufficiently addressed. Although the authors estimated total microbiota-derived PA production at approximately 175 $\mu\text{g/g}$ of feces and stated that they screened around 100 *Bacteroides* species capable of producing PA, they did not adequately address my original concern regarding the specific contribution of *Bacteroides uniformis* to systemic PA levels in vivo. At minimum, the authors should estimate the contribution of *Bacteroides*

uniformis to fecal PA levels to provide insight into the relative importance of PA production by this species compared with other bacteria.

Response:

We thank you for this important comment, and we apologize for not adequately addressing the specific contribution of BU to systemic PA levels in our previous revision. In response to this concern, we now provide quantitative estimates of BU-associated PA contribution in both feces and liver, the latter serving as a physiologically relevant tissue readout given that our data support the liver as a key target organ in this study.

Estimated contribution of BU to fecal PA levels.

In mice gavaged with WT BU, fecal PA levels reached approximately 670 $\mu\text{g/g}$ feces (**Fig. 5D**). We estimated the BU-associated contribution in two ways. First, comparing WT BU with PA-reduced BU mutants (Δpta or ΔmutA) yields a difference of approximately 240 $\mu\text{g/g}$ feces, which reflects the PA biosynthesis-dependent contribution of BU. Second, comparing WT BU with PBS controls yields a difference of approximately 440 $\mu\text{g/g}$ feces, which reflects the overall net increase associated with WT BU gavage, while also potentially capturing indirect community effects. These estimates suggest that BU accounts for approximately **36–65%** of total fecal PA in this model (240/670 to 440/670).

Fig. 5D

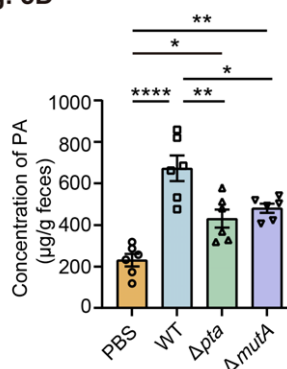


Fig. 5 | D. PA levels in feces of HFD-fed SPF *Apoe*^{-/-} mice after a 12-week gavage with PBS, WT, Δpta and ΔmutA (n = 6). Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test: * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Estimated contribution of BU to hepatic PA levels.

Because our data support the liver as the relevant target organ, we also estimated BU-associated contribution to hepatic PA. In mice gavaged with WT BU, hepatic PA levels were approximately 58 $\mu\text{g/g}$ liver (**Fig. 5E**). The difference between WT BU and the PA-reduced mutant groups was approximately 25–30 $\mu\text{g/g}$ liver, whereas the difference between WT BU and PBS controls was approximately 30 $\mu\text{g/g}$ liver. These values correspond to an estimated BU-associated contribution of approximately **43–52%** of total hepatic PA in this model (25/58 to 30/58). Together, these estimates suggest that BU makes a substantial contribution to both fecal and hepatic PA pools *in vivo*. We have now added text in the revised manuscript to clarify this point (**Lines 451–456**).

Fig. 5E

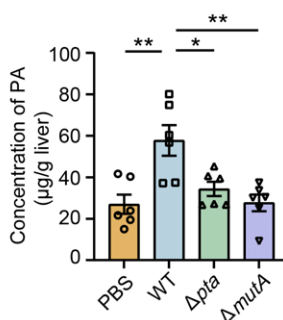


Fig. 5 | E. PA levels in liver of HFD-fed SPF *Apoe*^{-/-} mice after a 12-week gavage with PBS, WT, Δpta and ΔmutA (n = 6). Data are presented as mean $\pm$ SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test: * $p < 0.05$; ** $p < 0.01$.

The contribution of other commensal bacteria can be roughly estimated by the differential amount between the PBS group in SPF mice (27 $\mu\text{g/g}$ liver in **Fig 5E**) and the PBS group in germ-free (GF) mice on the same diet (4 $\mu\text{g/g}$ liver; **please see below**). The differential amount of 23 $\mu\text{g/g}$ suggests other commensal bacteria may have contributed ~40% (23/58).

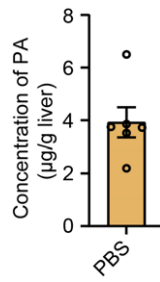


Figure legend |: PA levels in liver of HFD-fed GF *Apoe*^{-/-} mice after a gavage with PBS (n = 6). Data shown are mean ± SEM.

Referee #5 (Remarks to the Author):

The revised manuscript addresses the major scientific and technical concerns raised in my initial review and presents a more coherent mechanistic framework linking *Bacteroides uniformis*–derived pentadecanoic acid (PA) to cholesterol metabolism and atherosclerosis. By integrating microbiota manipulation, lipidomics, pharmacokinetics, scRNA-seq, and murine atherosclerosis models, the authors provide evidence that PA functions as an endogenous inhibitor of HMGCR, leading to reduced cholesterol synthesis and atheroprotection in vivo

Although inverse associations between circulating PA levels and cardiometabolic disease—including LDL-cholesterol–lowering effects in human cohorts—have been reported previously (e.g., Chooi, Y. C. et al., *Am. J. Clin. Nutr.* 119, 788–799 (2024)), the identification of a defined microbial source and the accompanying mechanistic analyses represent a meaningful advance beyond prior correlative studies. The experimental approach is technically sound, the datasets are of high quality, and the statistical analyses appear appropriate, with uncertainty handled in a careful and conservative manner.

The conclusions are largely supported by the data and are appropriately framed within the limitations of the murine models used. The authors also acknowledge important constraints, including sex bias, dietary context, and lesion quantification, which lends credibility to the interpretation. One minor point is that, given circulating PA can also originate from dietary sources, the Discussion would benefit from a brief clarification of how microbial and dietary contributions to the PA pool might be disentangled, particularly in human settings where diet and microbiome composition are highly variable. Addressing this point would further strengthen the translational relevance of the work without necessitating additional experiments. Overall, the revised manuscript is clearly written, the abstract and introduction provide appropriate context, and prior literature is cited in a balanced and comprehensive manner.

Response:

We thank you for this thoughtful and supportive assessment of our work. We particularly appreciate your recognition that identifying a defined microbial source of PA and establishing its mechanistic link to cholesterol metabolism extends prior correlative studies.

We also agree that distinguishing microbial and dietary contributions to the PA pool will be important for translational interpretation, especially in human settings where diet and microbiome composition are highly variable. We have therefore added a brief discussion of this point in the revised manuscript (**Lines 575-582**).

Referee #1 (Remarks to the Author):

The authors have sufficiently addressed my previous comments, mostly with experimental evidence, and the paper reads well now. I appreciate the authors' efforts for additional experiments. I have a few remaining comments/suggestions that I think are aimed at further clarification and improving interpretability.

1. In genetic studies such as this, complementation experiments are particularly important. Gene disruptions can lead to unintended polar effects on downstream genes, especially when they are located within the same operon or share regulatory elements. While I regret not raising this earlier, I would encourage the authors to consider complementation – if not in the current study, then at least as an important point for future validation.

Response: We thank you for this thoughtful suggestion. We fully agree that genetic complementation represents an important validation strategy for confirming mutant phenotypes, particularly when gene disruption may introduce unintended polar effects on downstream genes.

We would like to clarify that both the Δpta and $\Delta mutA$ mutants used in this study were constructed as markerless in-frame deletion mutants using a CRISPR–Cas genome-editing strategy, rather than by insertional mutagenesis. In both mutants, only sequences in open reading frame (ORF) were deleted (Please see Panel A and B below). The depicted sequences were verified by PCR and Sanger sequencing, which confirmed the intergenic region between *mutA* and its downstream *WNG09508.1* (Panel A) and the one between *ptaA* and its downstream *WNG09992.1* (Panel B) are unchanged. We have carefully ensured that no antibiotic-resistance cassette, plasmid sequence, or other exogenous DNA was inserted in the chromosome following genome editing. Consequently, the possibility of classical insertion-associated polar effects is expected to be minimal.

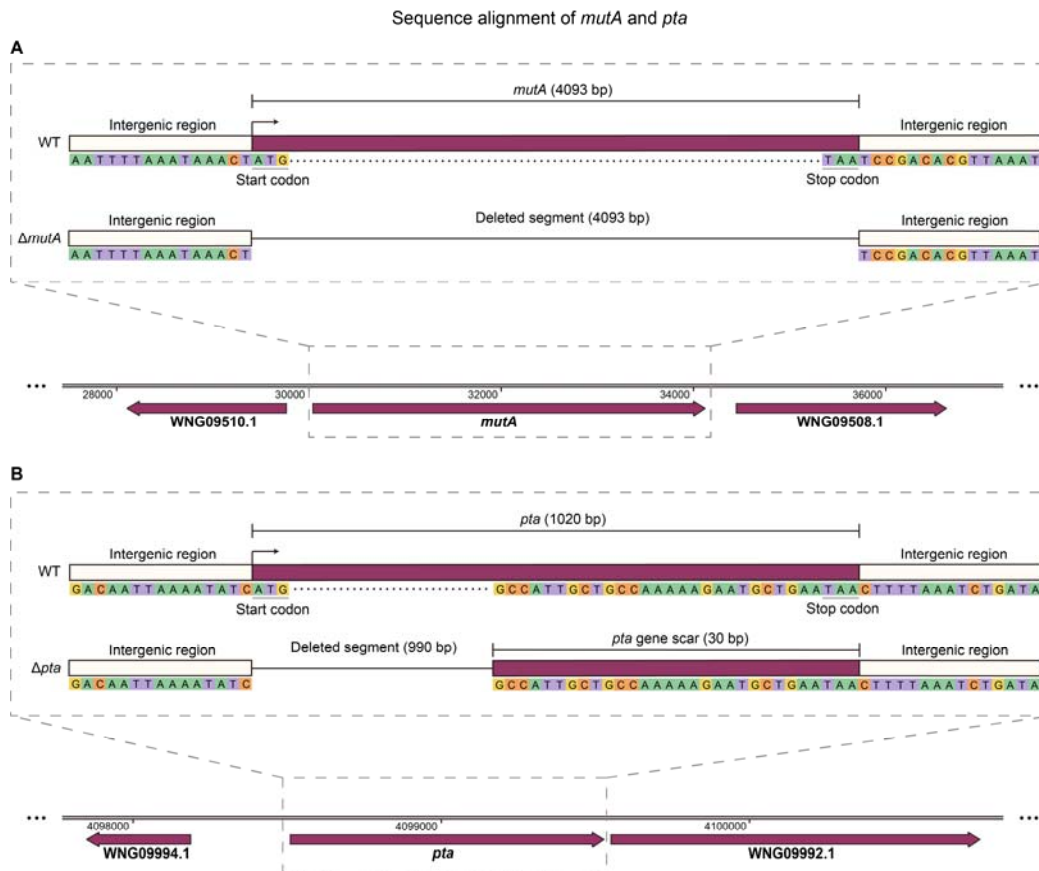


Figure legend | Sanger sequencing validation of the **(A)** *pta* and **(B)** *mutA* deletion mutants.

Importantly, the two independently generated mutants affecting distinct metabolic steps but both exhibited a marked reduction in PA production together with similarly attenuated protective effects *in vivo*. The concordance of these independent genetic perturbations provides additional support that the observed phenotypes are associated with impaired PA biosynthesis rather than unintended consequences of mutant construction.

Although genetic complementation would provide an additional layer of validation, the seamless in-frame deletion strategy, preservation of the native genomic context, sequencing-based verification of the mutant alleles, and the concordant phenotypes observed in two independent mutants collectively support the conclusion that *pta* and *mutA* contribute to PA biosynthesis in *Bacteroides uniformis*. Accordingly, we have clarified the mutant construction strategy in the Methods section (Lines 1173–1175) and have acknowledged genetic complementation as an important direction for future validation (Lines 496–497).

2. Are there differences in the abundance of *Bacteroides uniformis* colonization under germ-free (GF) versus specific pathogen-free (SPF) conditions? I wonder if there are differences that could partly explain PA levels between these settings.

Response: We thank you for this helpful comment. To address this point, we performed absolute quantification of *Bacteroides uniformis* in fecal samples from HFD-fed GF and SPF *Apoe*^{-/-} mice following PBS or BU gavage using a TaqMan qPCR assay targeting the *B. uniformis* 16S rRNA gene. We found that BU colonization was approximately 450-fold higher in GF mice than in SPF mice after BU gavage, with approximately 2.83×10^{10} versus 6.27×10^7 copies per g feces, respectively. This result is consistent with the well-established colonization resistance of the resident microbiota in SPF animals, which limits engraftment of exogenously administered commensal bacteria^{1,2}.

We agree that differences in BU colonization may contribute to differences in fecal PA abundance within a given experimental setting. However, BU abundance alone is unlikely to fully account for PA levels or to permit direct comparisons between GF and SPF mice. PA accumulation *in vivo* is likely influenced not only by bacterial abundance, but also by the metabolic activity of BU, substrate availability, interactions with the resident microbiota, and host physiological conditions. Therefore, the relationship between BU colonization and PA production is not expected to be simply linear across distinct microbial backgrounds. Accordingly, throughout this study, the functional contribution of BU-derived PA was evaluated by comparing WT BU with PA-deficient mutant strains under matched host, diet, and microbiota conditions, thereby minimizing potential confounding effects arising from differences in microbial background.

3. I appreciate the clarification regarding the involvement of alternative (and potentially additive) pathways in generating propionyl-CoA. My understanding now is that *mutA* and *pta* likely contribute through parallel routes, and in that context, a double mutant might be expected to behave like PA-null phenotype?

Response: We thank you for this thoughtful comment. We agree that *mutA* and *pta* likely contribute to PA production through distinct, and potentially parallel or additive, metabolic routes. This interpretation is consistent with our observation that disruption of either gene reduced, but did not abolish, PA production.

Based on available literature and our analyses, we identified two contributing routes involving *mutA* and *pta*. However, we cannot exclude the possibility that a third, as-yet

unidentified parallel pathway also contributes to propionyl-CoA generation. A $\Delta mutA\Delta pta$ double mutant in future studies would help test whether the two identified routes are sufficient to account for propionyl-CoA generation and PA production in *B. uniformis*. We have acknowledged this point in the Discussion (Lines 497–499).

Minor:

Regarding the *in vivo* experiments described (for eg. lines 412-413), it would improve clarity to explicitly state whether these experiments were conducted in ApoE^{-/-} mice under SPF or GF conditions. Since both models appear to be used, clearly specifying this here and throughout the manuscript would help avoid confusion.

Response: We thank you for this helpful suggestion. We have carefully checked the manuscript and revised it throughout to clearly specify whether each *in vivo* experiment was performed under SPF or GF conditions.

References

- 1 Zmora, N. *et al.* Personalized gut mucosal colonization resistance to empiric probiotics is associated with unique host and microbiome features. *Cell* **174**, 1388-1405 e1321, doi:10.1016/j.cell.2018.08.041 (2018).
- 2 Grimm, V., Radulovic, K. & Riedel, C. U. Colonization of C57BL/6 mice by a potential probiotic *Bifidobacterium bifidum* strain under germ-free and specific pathogen-free conditions and during experimental colitis. *PLoS One* **10**, e0139935, doi:10.1371/journal.pone.0139935 (2015).