

Arginine Metabolism as a Mutualistic Strategy in the Interaction between *Porphyromonas gingivalis* and *Filifactor alocis*

Corresponding Author: Professor Ji Yao Li

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

What are the noteworthy results?

The manuscript presents several findings with varying levels of strength

Weak: Coexistence and copathogenesis of *P. gingivalis* and *F. alocis* in periodontitis: The authors begin by trying to establish that PG and Fa are co-occurring in human periodontitis subjects and are acting synergistically to increase periodontal disease severity. I find this to be the least convincing section of the article for reasons discussed below, and it should be removed. It is not critical to the later findings of the study.

Weak: *F. alocis* reversed the inhibition of *P. gingivalis* growth by arginine. The authors demonstrate that the presence of arginine in the growth media inhibits *P. gingivalis* growth; the inhibitory effect of arginine has been previously published, but that work is not cited or discussed. Further, the authors overstate the result as Fa only partially rescues the effect of arginine. The experiments in this section are not well described, and the figures are difficult to understand. The authors seem to be excessively focused on the Fa – Pg relationship, I think refocusing on Pg as the primary organism of interest and using Fa as “an example” of a food-web partner that removes arginine may improve the manuscript.

The following three sections are stronger, and use metabolomics and gene knockouts to establish potential mechanisms. I would refocus the text on mechanisms in PG, and then discuss the Fa effect afterwards.

F. alocis restored *P. gingivalis* amino acid metabolism disrupted by arginine. : Rename “Arginine disrupts *P. gingivalis* amino acid metabolism.”

F. alocis restored *P. gingivalis* glutamate metabolism

F. alocis ArcA hydrolyzed arginine to relieve its effects on *P. gingivalis*.

Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.

The mechanisms by which arginine influences periodontal pathogens is of interest, and the data demonstrating changes in Pg metabolites in the presence of arginine is beneficial. Some of the work done here is similar with work published elsewhere, and those should be cited.

Li, Yu-Yang, et al. "Effects of D-arginine on *Porphyromonas gingivalis* biofilm." *Journal of Oral Science* 62.1 (2020): 57-61.
Cugini, Carla, et al. "Arginine deiminase inhibits *Porphyromonas gingivalis* surface attachment." *Microbiology* 159.Pt_2 (2013): 275-285.

Chow, Yoke Chan, et al. "Implications of *Porphyromonas gingivalis* peptidyl arginine deiminase and gingipain R in human health and diseases." *Frontiers in Cellular and Infection Microbiology* 12 (2022): 987683.

Does the work support the conclusions and claims, or is additional evidence needed?

There are several areas where conclusions are overstated, which will be listed in the lin comments.

Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

The first two sections of the results are weak, the first can be removed and the second rewritten.

Is the methodology sound? Does the work meet the expected standards in your field? The methodology for metabolites and gene expression, and mouse models are sound.

Is there enough detail provided in the methods for the work to be reproduced? Much of the bacterial culture detail lacks detail, the use of microaerobic growth conditions is not described at all.

Specific comments:

Line 1 The title should be more specific to the study, and include the names of the microorganisms studied.
(666265_0_merged_1762880455, p.1)

Line 31 The cooperation of Pg with Fa is not strongly established in this article, I would rework the abstract to focus on the strengths of the omics work.
(666265_0_merged_1762880455, p.2)

Line 57: The relative amounts of arginine in perio and health should be mentioned, what is an elevated level?
(666265_0_merged_1762880455, p.2)

Line 63: You have not cited or discussed this article, and you should. Li, Y. Y., Li, B. S., Liu, W. W., Cai, Q., Wang, H. Y., Liu, Y. Q., ... & Meng, W. Y. (2020). Effects of D-arginine on Porphyromonas gingivalis biofilm. Journal of Oral Science, 62(1), 57-61.
(666265_0_merged_1762880455, p.3)

Line 87-88 and 95: Collectively the authors overstate the correlation between Pg and Fa as causation of periodontal disease severity, based on data from a small set of human subjects.
(666265_0_merged_1762880455, p.4)

Comment: Only 27 subjects, 14 with periodontitis, and only 7 have elevated levels of Pg. The positive correlation may be due to some disease factor or other external factor that is not accounted for.

Line 117 Scatter plot analysis revealed a significant positive correlation between the 118 relative abundance of P. gingivalis (X-axis) and F. alocis (Y-axis) in clinical saliva
(666265_0_merged_1762880455, p.4) While there is a positive correlation, the figures imply that the relative amounts are the same, and in fact there is a much higher amount of Pg relative to Fa. This should be noted, or the figure y axis adjusted.

Line 124: Network analysis of which data set, the saliva or the dental plaque. Either way, 7 subjects or 14 subjects is not robust for this analysis.
(666265_0_merged_1762880455, p.5)

Line 128 overstated
(666265_0_merged_1762880455, p.5)

Line 130: Since we have no clinical data on the human subjects in the study, we don't know that the co-occurrence of PG-FA creates a more severe disease state.
(666265_0_merged_1762880455, p.5)

Line 135: "In an animal model of periodontitis"
(666265_0_merged_1762880455, p.5)

Line 135: Does it have to be synergistic? Perhaps they each contribute to disease by different mechanisms and there is an additive effect?
(666265_0_merged_1762880455, p.5)

Line 137: Fa. Does not completely reverse this inhibition, this header needs to be more nuanced.
(666265_0_merged_1762880455, p.5)

Line 139: This statement does not make sense when considering the figure, as zero FA supernatant results in zero PG growth. Is this in the presence of arginine? After reviewing the methods, the Pg is grown in microaerobic conditions, with no description of the actual % oxygen, or rationale. This implies FA helps with oxygen stress, not metabolite levels. This data should be removed.
(666265_0_merged_1762880455, p.5)

Line 157: Is Fa reversing periodontitis? This is a misstatement.
(666265_0_merged_1762880455, p.6)

Line 162 How much arginine is naturally present in mouse GCF or saliva? Considering the number of times mentioned in the introduction, I would assume the value in humans and mice is already known. This should be mentioned somewhere as part of your reason for the study design.
(666265_0_merged_1762880455, p.6)

Line 314 How do these levels compare to your mouse model?

(666265_0_merged_1762880455, p.11)

Line 460 Growth of a strict anaerobe in microaerobic conditions? Why? How? Without further explanation this is a huge problem.

Line 633 This figure 1d appears to be duplicated in the Figure 1 and Extended data figure 1
(666265_0_merged_1762880455, p.33)

Line 633 Figure 1 abc. I would like to highlight that you have 14 subjects with periodontitis, but for most of these subjects the levels are very low or absent. How do you justify the synergism with Fa if both are present but the levels are very low?

Line 652 Fig 2a, Ornithine creates the same pattern of growth inhibition as arginine, why is this also not included in the study?

Line 652 Figure 2b this would be better drawn as a bar chart, the line chart makes it look like a growth curve instead of a comparison.

Line 943 I only see three samples from healthy subjects in the PCA plot.
(666265_0_merged_1762880455, p.34)

Line 959 Is the scale meant to represent the final OD of a bacterial culture, and white represents 0.9? This figure needs more explanation.
(666265_0_merged_1762880455, p.35)

Line 959 Growth of PG in arginine with or without Fa supernatant? The legend needs more detail. Why grown microaerobically?
(666265_0_merged_1762880455, p.35)

Reviewer #2

(Remarks to the Author)

The manuscript investigates the metabolic interaction between *Porphyromonas gingivalis* and *Filifactor alocis*, focusing on their adaptation to elevated arginine levels in the periodontal pockets of periodontitis. The authors reported that high arginine accumulates ammonia and inhibits *P. gingivalis* growth by disrupting its intracellular amino acid metabolism, specifically repressing GDH activity and α ketoglutarate production. This metabolic stress activates PorX/PorY-regulated T9SS to increase small peptide acquisition. They further propose that *F. alocis* relieves arginine stress via ArcA mediated arginine consumption, thereby relieving the growth suppression on *P. gingivalis* and exacerbating periodontitis progression in a mouse model. This topic is of interest to researchers in the field of oral microbiology, while there are several concerns that the authors need to clarify.

Major concerns

- Results, L109, 119-129: Salivary metagenome analysis of a public dataset reveals higher relative abundance of *P. gingivalis* and *F. alocis* in periodontitis cases compared to healthy subjects. However, it should be noted that "periodontitis" encompasses a range of clinical states, and authors should provide the data of clinical status (e.g., stage and grade) or key periodontal parameters (e.g., pocket depth, CAL, % of BOP (+) sites) of the subjects. In addition, metagenomic data from subgingival plaque rather than saliva is necessary to support the strong clinical relationship between *P. gingivalis* and *F. alocis* in periodontitis. However, the number of samples used for subgingival plaque validation in this study was very small (five for healthy, two for periodontitis). The authors should reanalyze their results using a larger, publicly available subgingival metagenomic dataset.

- Results, L137: The authors state that the culture supernatant of *F. alocis* promoted the growth of *P. gingivalis* (Extended Data Fig. 2a), but its composition was not defined. To investigate and quantify the specific components enriched in the *F. alocis* culture supernatant that promote *P. gingivalis* growth, targeted metabolomics must be performed by the authors. This is a critical step in validating the proposed symbiosis.

- Results, L137: The authors should clarify in their discussion whether the arginine concentrations used in this study (0.0125~0.2%) are within the physiologically relevant range observed in the GCF of patients with severe periodontitis. If they are not within the physiological range, any conclusions regarding the adaptive strategies of *P. gingivalis* may be artifacts resulting from the unnatural environment.

- Results, L137: This study used *P. gingivalis* W83 strain (L414). Previous study has reported that different *P. gingivalis* strains result in different growth rates of bacteria when co-cultured with *F. alocis*. Did the authors conduct the same experiments using other strains in a pilot study? If not, the authors should mention in the Discussion that the observed

phenomenon might be strain-specific because metabolic responses can vary between different *P. gingivalis* strains. In addition, the coculture ratio used in this study was 10:1 (*P. gingivalis* : *F. alocis*). However, reported relative abundance in saliva and plaque from patients with severe periodontitis (Figure 1a, 1b, 1d) indicate that this difference is much smaller. The authors should justify the 10:1 ratio or demonstrate that synergy is maintained at more clinically relevant ratios (e.g., 5:1 or 1:1) in key in vitro assay.

- Results, L137: When *P. gingivalis* and *F. alocis* were co-cultured in vitro with arginine at concentrations greater than 0.4%, *P. gingivalis* could no longer be detected by qPCR (Figure 2b). However, in mouse model study, significant alveolar bone resorption was observed in the Pg+Fa+0.4% Arg and Pg+Fa+2% Arg groups (Figure 2g, 2h). The authors should discuss the mechanism of bone resorption in the present mouse model at high arginine levels. Is it possible that *P. gingivalis* remains viable/pathogenic in vivo even at growth-inhibitory concentrations of arginine?

- Results, Figure 2 and 4: The authors propose that the growth inhibitory and metabolic disturbance mechanisms of arginine are the result of intracellular ammonia accumulation, which disrupts GDH. However, enzymatic degradation of arginine into ammonia would also cause the medium to become more alkaline. Similarly, adding ammonia to the culture medium would increase pH. Therefore, the authors should provide data on how the pH of the culture medium changes immediately after adding arginine or ammonia, and throughout the culture. While the authors mainly focus on intracellular ammonia toxicity, but if the alkalinization of the medium is observed, then pH stress may also be involved, and the authors must verify its effect.

- Results, L253: The authors mentioned that arginine mediated metabolic disturbance (glutamate pathway) activates PorX/PorY and T9SS to enhance Kgp secretion. However, it remains unclear whether PorX/PorY activation is due to specific arginine/ammonia chemical stress or resultant general nutrient limitation (e.g., glutamate/ α -ketoglutarate depletion).

- Results, L259: The authors emphasize that the ArcA pathway of *F. alocis* is an important mitigation mechanism for *P. gingivalis*. However, several oral commensal bacteria possess the arginine deiminase pathway and could perform the same arginine-consuming function. Therefore, the authors must acknowledge that this function is not unique to *F. alocis*. The current conclusion regarding the unique mutualism between *P. gingivalis* and *F. alocis* is overstated. They must then justify their focus on the *P. gingivalis*/*F. alocis* pair by citing factors that confer a functional advantage to this partnership, such as strong co-localization or correlation between the two bacteria or demonstrated cooperative virulence (e.g., synergistic biofilm formation or host cell invasion). Furthermore, the growth promotion of *P. gingivalis* by *F. alocis* culture supernatant (Extended Data Fig. 2a) suggests the presence of secreted growth-promoting factors secreted by *F. alocis*. The authors should perform targeted LC-MS/MS on *F. alocis* spent media ($\pm$ arginine), quantify changes, and test purified fractions to identify the growth-promoting components.

- Discussion: The Discussion must be revised to explicitly address the major limitations raised above

Specific points

- Abstract, L39-40: Given the results of this study, the statement might be considered somewhat exaggerated; "To overcome stress induced by high arginine levels, *P. gingivalis* strongly cooperated with *F. alocis* ..."

- Introduction, L55-58: As mentioned above, the authors should provide specific clinical ranges for arginine concentrations in saliva and GCF in periodontitis and in healthy subjects. This will clarify the biological relevance of the concentrations used by the authors in this study.

- Introduction, L64: Change the phrasing to improve flow: "Unlike free amino acids, *P. gingivalis* preferentially utilizes peptides as primary energy and carbon sources, particularly glutamate- and aspartate-containing peptides..." → "Unlike many bacteria that utilize free amino acids, *P. gingivalis* preferentially utilizes peptides, particularly glutamate- and aspartate-containing peptides, as primary energy and carbon sources ...".

- M&M, L459: To ensure reproducible metabolite concentrations, please specify the growth stage (or time) of the *F. alocis* culture before collecting the spent medium.

- Figure 3, L677: "Coagulation activity" → "coaggregation activity"

Reviewer #3

(Remarks to the Author)

This is an interesting and well-written paper demonstrating that arginine inhibits *P. gingivalis* growth by disrupting amino acid metabolism by blocking glutamate dehydrogenase activity. This leads to the activation of the PorX/PorY two-component system, which then suppresses the expression of T9SS components and reduces Kgp secretion and Pg virulence in the LIP model. Cross-feeding symbiosis with Filifactor alocis (Fa), which consumes arginine, eliminates the inhibitory effects on Pg amino acid metabolism and virulence. The novelty of the data is diminished by numerous published studies that address the individual findings presented in this report. In addition, I have several comments on the experimental design and data presentation.

General comments:

1. Ligature-induced periodontitis (LIP) model. It is important to note that alveolar bone loss in the LIP model occurs

regardless of exogenous pathobiont infection, driven by dysbiosis in the native oral microbiome of mice. This will undoubtedly happen in mice with a ligature for 4 weeks. This is evident in Fig. 1f, which shows no difference in bone loss between animals infected with Pg alone and non-infected animals. In all other LIP experiments, there are no controls of non-infected ligature and with arginine alone. Therefore, the interpretation that Tf restores Pg pathogenicity in the presence of Arg is highly questionable.

2. Additionally, controls are missing in several experiments, which diminishes the reliability of the conclusions. For example: 2a. The control for the experiment shown in Figure 2d should be the growth curve of Pg/Fa cocultures with varying arginine concentrations. This is displayed in panels b and c at an unspecified time point.

2b. In hemagglutination experiments (Fig. 3e, k, Ext Data Fig. 6), the essential control, such as the effect of arginine and Tf alone, on hemagglutination, is missing. By the way, in several places in the manuscript, hemagglutination is referred to as coagulation or coaggregation.

3. Gingipain activity assay is flawed. The activity should be determined as the initial velocity of the reaction (V_0) expressed as mOD/min from the linear part of the time-recorded increase of absorbance. The results shown in Fig. 3f,g, Ext data Fig. 6b, could be misleading.

4. Importance of the PorX/PorY two-component system in response to arginine and its relation to T9SS system functionality, which is overinterpreted based on incomplete data.

Selected specific comments:

1) Many figures, including 1d, 3a-i, Extended Data Fig. 1a,b, and Extended Data Fig. 3, are unreadable even after zooming in; please enlarge the panels. Because of this, it was difficult to analyze them.

2) It is mentioned in line 319 that arginine formulation may target bacterial viability. As an initial experiment, the survival of Pg at different arginine concentrations should be assessed. The decrease in various parameters may not result from intracellular changes in Pg but rather from arginine-induced cell death.

3) Line 120: Is the number of individuals sufficient to draw such conclusions with statistical significance?

4) Line 180: Why were these specific peptides selected from the many possible sequences? What justifies the assumption that they originate from the breakdown of proteins taken up from the environment? They could equally come from, for instance, the degradation of intracellular proteins by quality control systems. In this context, lines 185–188 appear to overinterpret the data.

5) Lines 193–199: Due to the poor readability of the plot, the reader may be unable to evaluate the conclusions that have been drawn from it.

6) Lines 208–211: Deleting PorX might have multiple effects. Literature shows that removing PorX causes downregulation of T9SS genes and reduces substrate secretion. Since the untreated Δ porX strain shows no hemagglutination, adding any compound to the medium probably won't change this outcome. Therefore, Fig. 3j is not supported by the data presented. The PorY mutant behaves almost exactly like the wild type, so claiming that PorY is activated by arginine is not justified. Likewise, concluding that activated PorY, in turn, activates PorX, is unjustified. To support such claims, an experiment using radioisotope-labeled ATP should be performed to demonstrate that recombinant PorY, in the presence of arginine, activates (phosphorylates) recombinant PorX.

7) Lines 253–257: For the reasons outlined above, this constitutes a significant overinterpretation of the data.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Thank you for revising the paper.

You have successfully addressed the previous concerns by conducting the necessary additional experiments and making appropriate revisions throughout the manuscript. Although the revised study does not fully elucidate the entire mutualistic relationship between *P. gingivalis* and *F. alocis* (given that their interaction may also be influenced by other microbial species and the surrounding microenvironment) the authors have convincingly demonstrated the high probability that these two bacteria maintain a unique, cooperative relationship. The presented data offer significant insights that are highly valuable for understanding the pathogenicity associated with periodontitis.

Reviewer #3

(Remarks to the Author)

I would like to thank the authors for responding constructively to all my comments and critiques, which required conducting several experiments. The revised data fully addressed my critique and made the manuscript acceptable for publication.

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Response to Reviewers

Manuscript title: Arginine Metabolism as a Mutualistic Strategy in the Interaction between *Porphyromonas gingivalis* and *Filifactor alocis*

Former title: Arginine Metabolism as a Mutualistic Strategy in Microbial Communities

Manuscript ID: NCOMMS-25-85197-T

We sincerely thank the reviewers for their careful assessment of our manuscript and for their insightful and constructive comments. We have carefully considered all of the comments and have revised the manuscript accordingly. We believe that these revisions have improved the clarity, rigor, and overall presentation of the study. In this document, we provide a detailed point-by-point response to each comment. For clarity, the reviewers' comments are reproduced first, followed by our responses. All corresponding changes have been incorporated into the revised manuscript and are clearly indicated in **red** in the marked version. We also substantially revised the description of the Results to avoid overstatement and improve precision, and expanded the Discussion to better contextualize the findings.

We hope that the revisions and responses adequately address the reviewers' concerns, and we are grateful for the opportunity to revise and improve our work.

Reviewer #1 (Remarks to the Author):

Comment: *What are the noteworthy results?*

The manuscript presents several findings with varying levels of strength

*Weak: Coexistence and copathogenesis of *P. gingivalis* and *F. alocis* in periodontitis: The authors begin by trying to establish that PG and Fa are co-occurring in human periodontitis subjects and are acting synergistically to increase periodontal disease severity. I find this to be the least convincing section of the article for reasons discussed below, and it should be removed. It is not critical to the later findings of the study.*

*Weak: *F. alocis* reversed the inhibition of *P. gingivalis* growth by arginine. The authors demonstrate that the presence of arginine in the growth media inhibits *P. gingivalis* growth; the inhibitory effect of arginine has been previously published, but that work is not cited or discussed. Further, the authors overstate the result as Fa only partially rescues the effect of arginine. The experiments in this section are not well described, and the figures are difficult to understand. The authors seem to be excessively focused on the Fa – Pg relationship, I think refocusing on Pg as the primary organism of interest and using Fa as “an example” of a food-web partner that removes arginine may improve the manuscript.*

The following three sections are stronger, and use metabolomics and gene knockouts to establish potential mechanisms. I would refocus the text on mechanisms in PG, and then discuss the Fa effect afterwards.

**F. alocis* restored *P. gingivalis* amino acid metabolism disrupted by arginine. : Rename_ “Arginine disrupts *P. gingivalis* amino acid metabolism. “*

**F. alocis* restored *P. gingivalis* glutamate metabolism*

**F. alocis* ArcA hydrolyzed arginine to relieve its effects on *P. gingivalis*.*

Response: We thank the reviewer for this helpful overall assessment. We have revised the manuscript accordingly.

For the first section, we re-integrated multiple public subgingival plaque metagenomic datasets and re-analysed the association between *P. gingivalis* and *F. alocis* in a larger cohort, thereby strengthening the ecological evidence for their

co-occurrence in the periodontitis-associated microbiome. At the same time, we substantially toned down the description of their copathogenesis. Because the public datasets do not provide sufficiently complete clinical periodontal parameters, such as bleeding on probing, probing depth, or clinical attachment loss, we cannot directly infer from these human datasets that the two organisms synergistically aggravate periodontal destruction. Instead, this section is now presented primarily as ecological background to establish that *P. gingivalis* and *F. alocis* coexist and are positively associated in the periodontal niche. Evidence for enhanced pathogenicity was restricted to our animal model, in which co-infection with *P. gingivalis* and *F. alocis* resulted in more severe periodontitis than infection with either species alone.

For the second section, we have added the relevant previous studies on arginine-mediated inhibition of *P. gingivalis* to the Introduction, as suggested by the reviewer. We also rewrote this section to place *P. gingivalis* as the primary organism of interest. Specifically, we first describe the effects of arginine on *P. gingivalis*, and then introduce *F. alocis* as an example of a food-web partner that removes arginine and thereby partially relieves arginine-associated stress. In addition, we revised the figures and figure legends to improve clarity.

The subsequent three sections have also been revised in both title and content in accordance with the reviewer's suggestion, with a stronger focus on the mechanistic responses in *P. gingivalis* and a clearer separation between the core *P. gingivalis* findings and the modulatory effect of *F. alocis*.

Comment: *Will the work be of significance to the field and related fields? How does it compare to the established literature? If the work is not original, please provide relevant references.*

The mechanisms by which arginine influences periodontal pathogens is of interest, and the data demonstrating changes in Pg metabolites in the presence of arginine is beneficial. Some of the work done here is similar with work published elsewhere, and those should be cited.

Li, Yu-Yang, et al. "Effects of D-arginine on Porphyromonas gingivalis biofilm."

Journal of Oral Science 62.1 (2020): 57-61.

Cugini, Carla, et al. "Arginine deiminase inhibits *Porphyromonas gingivalis* surface attachment." *Microbiology* 159.Pt_2 (2013): 275-285.

Chow, Yoke Chan, et al. "Implications of *Porphyromonas gingivalis* peptidyl arginine deiminase and gingipain R in human health and diseases." *Frontiers in Cellular and Infection Microbiology* 12 (2022): 987683.

Response: We thank the reviewer for this suggestion. The three relevant studies recommended by the reviewer have now been incorporated into the revised manuscript as references 17, 18 and 65, and the relevant text has been revised accordingly.

Comment: *Does the work support the conclusions and claims, or is additional evidence needed?*

There are several areas where conclusions are overstated, which will be listed in the lin comments.

Response: We thank the reviewer for this important comment. We agree that several conclusions in the original manuscript were overstated. In the revised version, we have carefully tempered the relevant claims throughout the manuscript and provide point-by-point responses to the specific issues raised below.

Comment: *Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision? The first two sections of the results are weak, the first can be removed and the second rewritten.*

Response: We thank the reviewer for this constructive suggestion. In response, we substantially revised the first two Results sections. Specifically, the original first section was removed in its entirety, and all related analyses were re-performed using an integrated analysis of multiple public subgingival plaque metagenomic datasets. This section was completely rewritten and is now presented as ecological background, with the overstatement regarding copathogenesis removed. The second section was also rewritten to place *P. gingivalis* as the primary organism of interest, with *F. alocis*

presented as an example of an arginine-removing food-web partner. We also revised the relevant figures and figure legends to improve clarity.

Comment: *Is the methodology sound? Does the work meet the expected standards in your field? The methodology for metabolites and gene expression, and mouse models are sound.*

Is there enough detail provided in the methods for the work to be reproduced? Much of the bacterial culture detail lacks detail, the use of microaerobic growth conditions is not described at all.

Response: We thank the reviewer for this comment. We have revised the Methods to provide additional detail for the bacterial experiments. We also clarify that the use of “microaerobic” in the original manuscript was a typographical error; all bacterial cultures in this study were grown under anaerobic conditions (80% N₂, 10% CO₂, and 10% H₂), and this has been corrected throughout the revised manuscript.

Specific comments:

Comment: *Line 1 The title should be more specific to the study, and include the names of the microorganisms studied.*

(666265_0_merged_1762880455, p.1)

Response: We thank the reviewer for this helpful suggestion. To improve the specificity of the title and clearly indicate the microorganisms investigated in this study, we have revised the title of the manuscript from “Arginine Metabolism as a Mutualistic Strategy in Microbial Communities” to “Arginine Metabolism as a Mutualistic Strategy in the Interaction between *Porphyromonas gingivalis* and *Filifactor alocis*” This change more precisely reflects the scope of the study and the microbial interaction examined in this work. These changes have been made in **Lines 1–2** of the revised manuscript.

Comment: *Line 31 The cooperation of Pg with Fa is not strongly established in this article, I would rework the abstract to focus on the strengths of the omics work.*

(666265_0_merged_1762880455, p.2)

Response: We thank the reviewer for this important suggestion. Accordingly, we rewrote the Abstract to better highlight the main focus of the study. The revised Abstract now focuses primarily on the mechanistic effects of elevated arginine on *P. gingivalis*, including disruption of amino acid metabolism, reduced glutamate dehydrogenase activity and α -ketoglutarate production, growth inhibition, and the associated PorX/PorY–T9SS response. At the same time, we toned down the cooperation narrative and removed the previous overstatement that *P. gingivalis* “strongly cooperated” with *F. alocis* in periodontitis patients. In the revised version, *F. alocis* is presented more cautiously as a periodontitis-associated partner that partially relieves arginine-associated growth inhibition through ArcA-dependent arginine degradation. These changes have been made in **Lines 22–35** of the revised manuscript.

Comment: Line 57: The relative amounts of arginine in perio and health should be mentioned, what is an elevated level?

(666265_0_merged_1762880455, p.2)

Response: We thank the reviewer for this important comment. In response, we have now specified the reported arginine levels associated with periodontal health and disease in the revised Introduction. Specifically, we added that arginine concentrations in whole saliva increase from 26.4 (12.4–34.7) mg/dL [0.0264% (0.0124–0.0347%)] in health to 64.6 (39.4–70.8) mg/dL [0.0646% (0.0394–0.0708%)] in chronic periodontitis (*Balkan et al., BMC Oral Health, 2025*). We also revised the Discussion to better contextualize the experimental concentrations used in this study. The revised text now clarifies that our in vitro concentrations are higher than the micromolar levels reported in gingival crevicular fluid, but overlap with, or moderately exceed, the millimolar range measured in whole saliva. In addition, we note that some oral-care formulations contain 1.5% arginine, and certain products contain up to 8%, which can generate transient local exposures above endogenous physiological levels (*Wolff and Schenkel, Adv Dent Res, 2018*) (*Carda-Diéguez et al., Microbiome, 2022*) (*Nanako Kuriki et al., J Dent, 2024*). These revisions were made to clarify what we

refer to as an “elevated” arginine level and to distinguish physiological from pharmacological exposure conditions. These changes have been made in **Introduction, Lines 46–51 and Discussion, Lines 393–406** of the revised manuscript.

***Comment:** Line 63: You have not cited or discussed this article, and you should. Li, Y. Y., Li, B. S., Liu, W. W., Cai, Q., Wang, H. Y., Liu, Y. Q., ... & Meng, W. Y. (2020). Effects of D-arginine on Porphyromonas gingivalis biofilm. Journal of Oral Science, 62(1), 57-61.*

(666265_0_merged_1762880455, p.3)

Response: We thank the reviewer for pointing out this relevant study. In response, we have now cited this article in the revised Introduction as reference 17 and added the following statement: “For example, D-arginine has been reported to inhibit the formation of *P. gingivalis* biofilms and promote their dispersal.” This citation has been included to better acknowledge the existing literature on arginine-mediated inhibition of *P. gingivalis*. These changes have been made in **Lines 52–54** of the revised manuscript.

***Comment:** Line 87-88 and 95: Collectively the authors overstate the correlation between Pg and Fa as causation of periodontal disease severity, based on data from a small set of human subjects.*

(666265_0_merged_1762880455, p.4)

***Comment:** Only 27 subjects, 14 with periodontitis, and only 7 have elevated levels of Pg. The positive correlation may be due to some disease factor or other external factor that is not accounted for.*

Response: We thank the reviewer for this important comment and agree that the correlation between *P. gingivalis* and *F. alocis* should not be overstated as evidence of causation or enhanced periodontal disease severity, particularly on the basis of a small human cohort. In the revised manuscript, we have therefore tempered the relevant statements in the Introduction to make clear that these previous observations reflect

reported associations rather than causal evidence (**Introduction, Lines 82–88**).

In addition, to address the reviewer’s concern regarding the limited sample size in our original analysis, we removed the salivary metagenomic analysis and the small subgingival plaque dataset generated in our laboratory. Instead, we performed a new integrative analysis using five publicly available subgingival plaque metagenomic datasets, including two cohorts from China (PRJNA230363 and PRJNA932553), two from the United States (PRJNA255922 and PRJNA508385), and one from Japan (PRJDB11203). The revised analysis includes 102 samples in total, comprising 44 periodontally healthy individuals and 58 patients with chronic periodontitis (**Fig. 1a**). Using this expanded cohort, we found that both *P. gingivalis* and *F. alocis* were significantly enriched in periodontitis samples relative to healthy controls, and correlation analysis supported a strong ecological association between the two species within the subgingival microbiome. We have revised the text accordingly to present these findings as ecological background rather than evidence of causation, and the updated results are now shown in **Fig. 1a–e** with corresponding revisions to the Results (**Lines 108–146**) and Methods (**Lines 457–483**) sections.

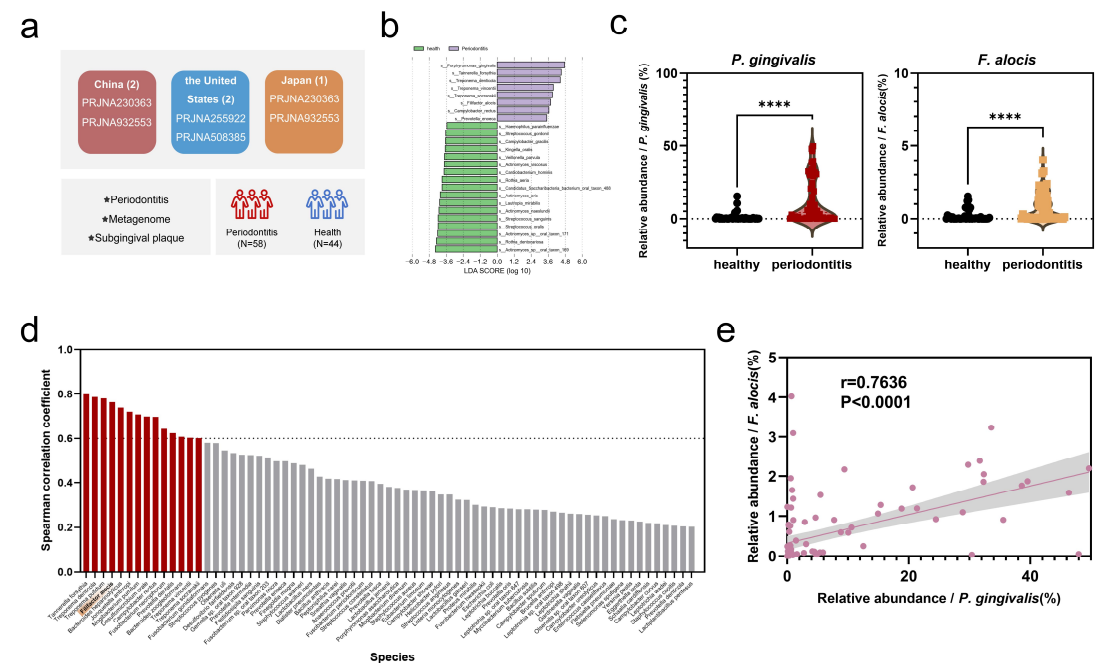


Fig. 1 | a, Overview of the integration of five independent public subgingival metagenomic datasets. **b**, Indicator species in the subgingival microbiota of periodontally healthy subjects and patients with periodontitis identified by LEfSe

analysis. **c**, Relative abundances of *P. gingivalis* and *F. alocis* in subgingival plaque samples from periodontally healthy subjects and patients with periodontitis. **d**, Spearman correlation coefficients of bacterial taxa co-occurring with *P. gingivalis* in the subgingival microbiota, with *F. alocis* highlighted in orange on the x axis and taxa with correlation coefficients >0.6 shown as red bars. **e**, Correlations between the relative abundance of *P. gingivalis* and *F. alocis* in the subgingival microbiota.

Comment: Line 117 Scatter plot analysis revealed a significant positive correlation between the 118 relative abundance of *P. gingivalis* (X-axis) and *F. alocis* (Y-axis) in clinical saliva

(666265_0_merged_1762880455, p.4) While there is a positive correlation, the figures imply that the relative amounts are the same, and in fact there is a much higher amount of Pg relative to Fa. This should be noted, or the figure y axis adjusted.

Response: We thank the reviewer for this helpful comment. We agree that the original scatter plot could give the visual impression that the relative abundances of *P. gingivalis* and *F. alocis* were on a similar scale, whereas *P. gingivalis* was in fact present at a higher relative abundance. To address this concern, we substantially revised this part of the manuscript. Specifically, we removed the original salivary metagenomic analysis and the small laboratory-generated subgingival dataset. These were replaced by a new integrative analysis based on five publicly available subgingival plaque metagenomic datasets, comprising 102 samples in total (44 healthy and 58 periodontitis). In the revised manuscript, the ecological association between *P. gingivalis* and *F. alocis* is now presented in **Fig. 1e** using the new dataset. The axis scaling has been adjusted to more accurately reflect the higher relative abundance of *P. gingivalis* relative to *F. alocis*, while retaining the significant positive correlation between the two species within the subgingival microbiome. These revised results are now presented in **Fig. 1e** and the corresponding Results (**Lines 108–146**).

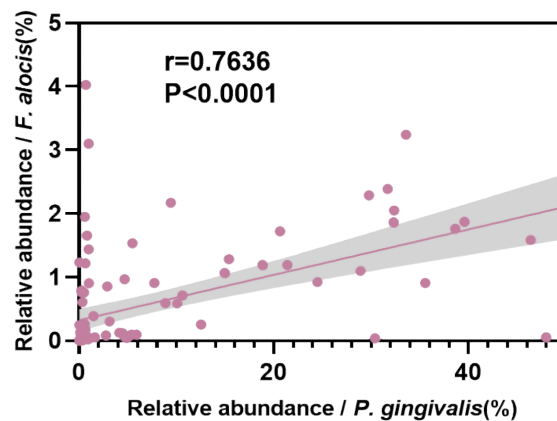


Fig. 1 | e, Correlations between the relative abundance of *P. gingivalis* and *F. alocis* in the subgingival microbiota.

Comment: Line 124: Network analysis of which data set, the saliva or the dental plaque. Either way, 7 subjects or 14 subjects is not robust for this analysis.

(666265_0_merged_1762880455, p.5)

Response: We thank the reviewer for this important comment. In the original manuscript, the network analysis was based on our small subgingival plaque dataset. We agree that this sample size was insufficient to support a robust network analysis.

To address this concern, we removed both the original salivary metagenomic analysis and the small laboratory-generated subgingival plaque dataset from the manuscript. These were replaced by a new integrative analysis using five publicly available subgingival plaque metagenomic datasets, including two cohorts from China (PRJNA230363 and PRJNA932553), two from the United States (PRJNA255922 and PRJNA508385), and one from Japan (PRJDB11203). The revised analysis comprises 102 samples in total, including 44 periodontally healthy controls and 58 patients with chronic periodontitis (**Fig. 1a**). Using this expanded cohort, we performed Spearman correlation analysis between *P. gingivalis* and annotated microbial taxa in the subgingival microbiome. Species with significant correlations ($P < 0.05$) are shown in **Fig. 1d**, and taxa with correlation coefficients >0.6 were retained for construction of the core co-occurrence network associated with *P. gingivalis*. Within this revised network, *F. alocis* remained one of the species most strongly associated with *P. gingivalis*, ranking after *Tannerella forsythia*, *Treponema denticola*, and *Treponema*

putidum. The Results (**Lines 122–131**) sections have been revised accordingly.

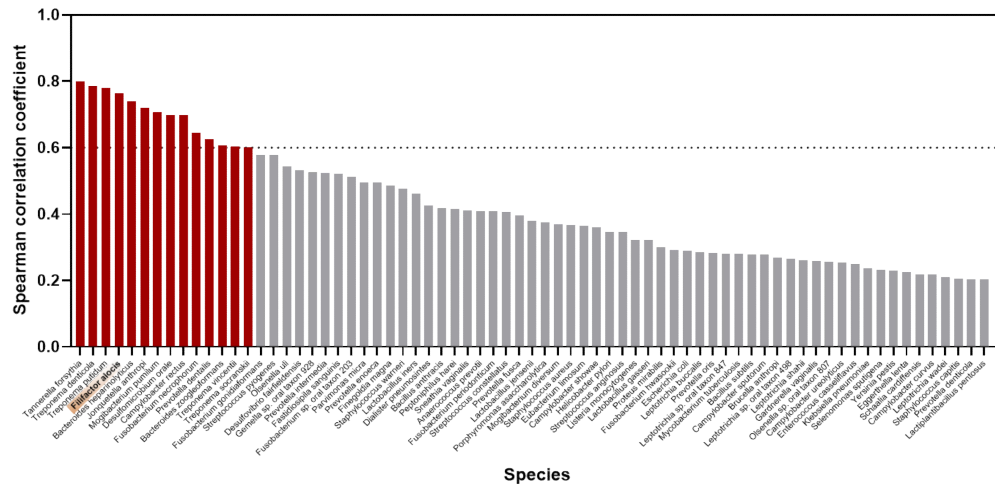


Fig. 1 | d, Spearman correlation coefficients of bacterial taxa co-occurring with *P. gingivalis* in the subgingival microbiota, with *F. alocis* highlighted in orange on the x axis and taxa with correlation coefficients >0.6 shown as red bars.

Comment: Line 128 overstated

(666265_0_merged_1762880455, p.5)

Response: We thank the reviewer for this important comment and agree that the original statement was overstated. The original wording has been removed, and this section has been substantially rewritten. Specifically, we removed the original salivary analysis and the small laboratory-generated subgingival dataset, and replaced them with a new integrative analysis of five publicly available subgingival plaque metagenomic datasets comprising 102 samples in total (44 healthy and 58 periodontitis). We also revised the section title from “Coexistence and copathogenesis of *P. gingivalis* and *F. alocis* in periodontitis” to “Coexistence of *P. gingivalis* and *F. alocis* in periodontitis”. In the revised text, we no longer describe the relationship as evidence of “strong coexistence” or imply causation from the human data. Instead, we now present *P. gingivalis* and *F. alocis* more cautiously as species that are both enriched in periodontitis and positively associated within the subgingival microbiome. We believe this revised wording more accurately reflects the strength of the human metagenomic data. These changes have been made in **Lines 126–134** of the revised

manuscript.

Comment: Line 130: Since we have no clinical data on the human subjects in the study, we don't know that the co-occurrence of PG-FA creates a more severe disease state.

(666265_0_merged_1762880455, p.5)

Response: We thank the reviewer for this important comment and agree that the human metagenomic data do not allow us to conclude that co-occurrence of *P. gingivalis* and *F. alocis* is associated with greater clinical disease severity. In the revised manuscript, we have therefore limited the human data to co-enrichment and ecological association and removed wording that could imply increased disease severity in human subjects. To evaluate functional interaction in vivo, we also repeated the murine ligature-induced periodontitis experiment with an increased sample size (n = 10 per group). In this independent experiment, coinfection with *P. gingivalis* and *F. alocis* resulted in greater alveolar bone destruction than either monoinfection. Accordingly, the revised manuscript now presents the enhanced pathogenic effect only in the murine model, not as an inference from the human data. These changes have been made in **Lines 108–146** of the revised manuscript.

Comment: Line 135: “In an animal model of periodontitis”

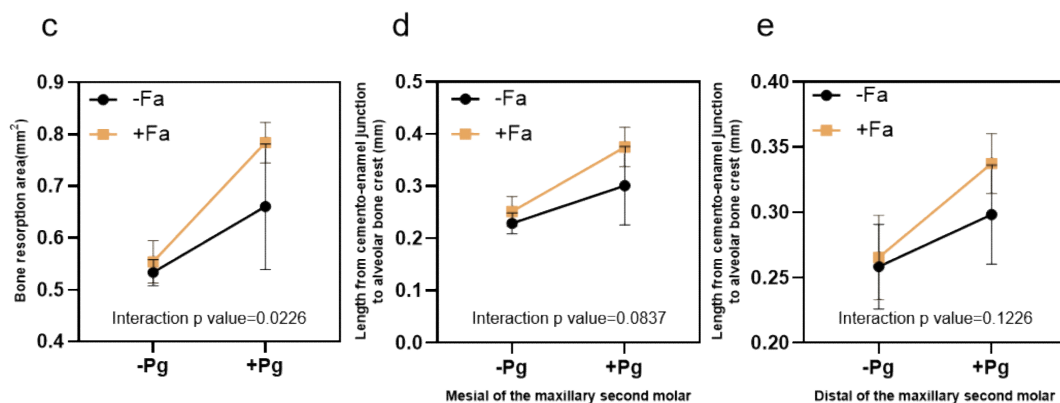
(666265_0_merged_1762880455, p.5)

Response: We thank the reviewer for this comment. In response, we revised the original wording to avoid overgeneralization. Specifically, the previous statement implying “synergistic copathogenesis in periodontitis” has been replaced with the more cautious wording: “These findings indicated that the presence of both species enhanced periodontal bone destruction in this murine model.” This revision is intended to make clear that the conclusion is restricted to the animal experiment and does not imply direct evidence for human disease pathogenesis. These changes have been made in **Lines 140–141** of the revised manuscript.

Comment: Line 135: Does it have to be synergistic? Perhaps they each contribute to disease by different mechanisms and there is an additive effect?

(666265_0_merged_1762880455, p.5)

Response: We thank the reviewer for raising this important point. We agree that the greater pathogenic effect observed during coinfection could, in principle, reflect either additive contributions of the two species or a biological interaction between them. To address this more rigorously, we reanalyzed the animal experiment using a two-factor model including *P. gingivalis* (Pg), *F. alocis* (Fa), and the Pg × Fa interaction term. Two-way ANOVA revealed a significant Pg × Fa interaction effect on alveolar bone loss area, indicating that the combined effect of coinfection exceeded the additive expectation from the two monoinfections (**Extended Data Fig. 1c**, Interaction p value=0.0226). For the CEJ–ABC distance measurement, a similar trend was observed, although the interaction term did not reach statistical significance (**Extended Data Fig. 1d,e**, Interaction p value=0.0837, 0.1226). Based on these analyses, we revised the text to avoid overstatement and no longer use the original wording implying “synergistic copathogenesis.” Instead, we now describe the phenotype more cautiously as an enhanced pathogenic effect in the murine ligature-induced periodontitis model. These changes have been made in **Extended Data Fig. 1c, Lines 141–146** of the revised manuscript.



Extended Data Fig. 1 | (c), mesial CEJ–ABC distance (d), and distal CEJ–ABC distance (e) in the murine ligature-induced periodontitis model. Interaction P values are shown at the bottom of each panel, and $P < 0.05$ was considered statistically significant.

Comment: Line 137: *Fa.* Does not completely reverse this inhibition, this header needs to be more nuanced.

(666265_0_merged_1762880455, p.5)

Response: We thank the reviewer for this helpful suggestion. We agree that the original section header (“*F. alocis* reversed the inhibition of *P. gingivalis* growth by arginine”) could be interpreted as implying a complete reversal of the inhibitory effect. To provide a more accurate description of the experimental observations, we have revised the header to: “Arginine inhibited *P. gingivalis* growth, which was partially alleviated by *F. alocis*.” This revised wording more precisely reflects that *F. alocis* mitigates, but does not fully reverse, the inhibitory effect of arginine on *P. gingivalis* growth. These changes have been made in **Lines 148** of the revised manuscript.

Comment: Line 139: This statement does not make sense when considering the figure, as zero FA supernatant results in zero PG growth. Is this in the presence of arginine? After reviewing the methods, the Pg is grown in microaerobic conditions, with no description of the actual % oxygen, or rationale. This implies FA helps with oxygen stress, not metabolite levels. This data should be removed.

(666265_0_merged_1762880455, p.5)

Response: We thank the reviewer for this careful evaluation and for raising these important concerns regarding the supernatant transfer experiment and the culture conditions. First, to clarify the experimental context and address the reviewer’s concern about the interpretation of the original data, we replaced the original experiment with a revised supernatant transfer assay incorporating clearer controls. Specifically, *F. alocis* was cultured overnight in BB medium either supplemented with 0.2% arginine (A+) or without arginine (A–). Culture supernatants were collected by centrifugation and filtration, and then serially twofold diluted with the corresponding fresh medium (A+ or A–) to generate final supernatant proportions of 50%, 25%, 12.5%, 6.25%, and 3.125% in 96-well plates. *P. gingivalis* was then inoculated, and growth was quantified after 24 h by OD measurement. In the revised experiment,

supernatants derived from *F. alocis* cultured in arginine-supplemented medium (A+) partially rescued *P. gingivalis* growth under arginine stress, whereas supernatants from *F. alocis* cultured without arginine supplementation (A–) did not show this effect. These results indicate that the growth-rescuing activity depends on the metabolic state of *F. alocis* under arginine-supplemented conditions, rather than on the mere presence of culture supernatant. The figure legend and Methods section have been revised accordingly, and this experiment is now presented as **Fig. 2h** in the revised manuscript. These changes have been made in **Fig. 2h; Results, Lines 181–185; Methods, Lines 552–562** of the revised manuscript.

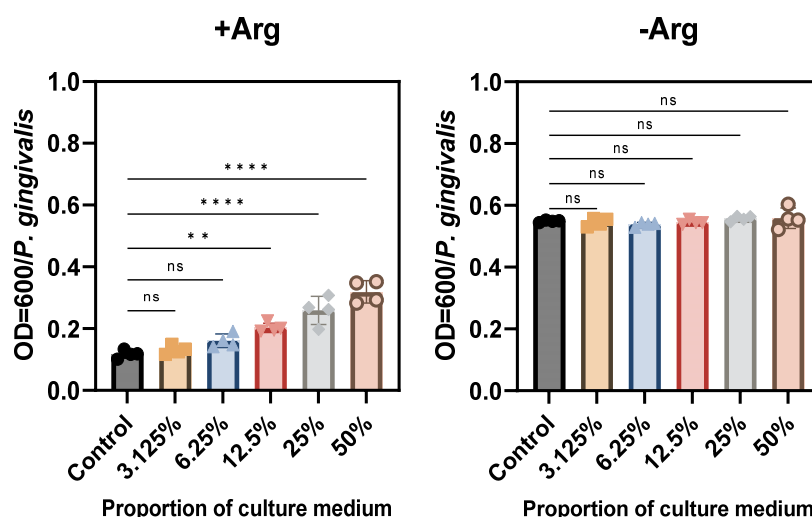


Fig. 2 | h, Effect of 24h *F. alocis* culture supernatants generated with or without 0.2% (w/v) arginine on the growth of *P. gingivalis* (n = 4; ordinary one-way ANOVA). The data are presented as the mean±s.d. Only selected comparisons are shown, ns, not significant, **p<0.01 and ****p<0.0001.

Second, we apologize for the error in the original Methods section, in which the growth conditions were mistakenly described as “microaerobic”. This was a typographical error. All bacterial cultures in this study were performed under strict **anaerobic conditions (80% N₂, 10% CO₂, and 10% H₂)**, and this has now been corrected throughout the revised manuscript. These changes have been made in **Methods, Lines 555 and 564–565** of the revised manuscript.

Comment: Line 157: Is *Fa* reversing periodontitis? This is a misstatement.

(666265_0_merged_1762880455, p.6)

Response: We thank the reviewer for pointing out this misstatement. We agree that the original wording (“*F. alocis* reversed periodontitis in vivo”) was inaccurate and could be misleading. In the revised manuscript, we have rewritten this part of the Results section to more precisely reflect the experimental findings. The revised description now reads: “Consistently, in vivo, *F. alocis* partially attenuated the inhibitory effect of arginine on *P. gingivalis*–associated periodontal bone loss.” This description more accurately reflects that *F. alocis* does not reverse periodontitis itself, but rather attenuates the arginine-mediated suppression of *P. gingivalis* pathogenicity. These changes have been made in **Results, Lines 188–190** of the revised manuscript.

Comment: Line 162 How much arginine is naturally present in mouse GCF or saliva? Considering the number of times mentioned in the introduction, I would assume the value in humans and mice is already known. This should be mentioned somewhere as part of your reason for the study design.

(666265_0_merged_1762880455, p.6)

Response: We thank the reviewer for raising this important point regarding the physiological levels of arginine in oral fluids. To our knowledge, direct measurements of arginine concentrations in mouse gingival crevicular fluid or saliva have not been reported. Only limited data are available from rodent studies; for example, one study reported that the concentration of free arginine in whole saliva of rats is approximately 92.0 ± 29.0 nmol/ml (Enwonwu *et al.*, *Caries Research*, 1994). Due to the scarcity of murine measurements, we therefore referenced available data from human oral fluids to provide a physiological context for the study. Previous studies have reported that arginine concentrations increase in periodontitis, rising in whole saliva from 26.4 (12.4–34.7) mg/dL [0.0264% (0.0124–0.0347%)] in health to 64.6 (39.4–70.8) mg/dL [0.0646% (0.0394–0.0708%)] in chronic periodontitis (Balkan *et al.*, *BMC Oral Health*, 2025), and in gingival crevicular fluid from $2.40\text{--}2.96 \times 10^{-6}$ M to $6.40\text{--}10.00 \times 10^{-6}$ M (Téllez *et al.*, *Brazilian Dental Journal*, 2008). Although

clinical measurements remain limited and vary across analytical methods, these findings suggest disease-associated elevation of arginine within the periodontal niche. In addition, some oral-care formulations contain 1.5% arginine, and certain products contain up to 8%, which may generate transient local concentrations exceeding physiological levels (*Wolff and Schenkel, Adv Dent Res, 2018*) (*Carda-Diéguez et al., Microbiome, 2022*) (*Nanako Kuriki et al., J Dent, 2024*).

Accordingly, we have revised the manuscript in two places. In the Introduction, we now specify the reported arginine concentrations in human oral fluids to clarify what we mean by an “elevated” arginine level (**Introduction, Lines 46–51 and Discussion, Lines 393–406**). In the Discussion, we further clarify that the arginine concentrations used in our in vitro experiments overlap with, or moderately exceed, the range reported in whole saliva, whereas the 0.4% and 2% in vivo regimens should be regarded as exogenous, pharmacological exposure conditions used to probe the effect of elevated arginine on disease outcome, rather than as physiological surrogates for known endogenous arginine concentrations in mouse oral fluids.

Comment: Line 314 How do these levels compare to your mouse model?

(666265_0_merged_1762880455, p.11)

Response: We thank the reviewer for this helpful question regarding how the arginine levels reported in human oral fluids relate to those used in our experimental models. Previous studies have reported that arginine concentrations increase in periodontitis, rising in whole saliva from approximately 26.4 (12.4–34.7) mg/dL [0.0264% (0.0124–0.0347%)] in healthy individuals to 64.6 (39.4–70.8) mg/dL [0.0646% (0.0394–0.0708%)] in patients with chronic periodontitis (*Balkan et al., BMC Oral Health, 2025*), while concentrations in gingival crevicular fluid (GCF) range from $2.40\text{--}2.96 \times 10^{-6}$ M in health to $6.40\text{--}10.00 \times 10^{-6}$ M during disease (*Téllez et al., Brazilian Dental Journal, 2008*). Although these measurements vary across studies and analytical methods, they consistently indicate disease-associated elevation of arginine within the periodontal niche. In our in vitro experiments, arginine concentrations ranged from 0.0125% to 0.8% (approximately 0.7–45 mM). In our in

vitro experiments, the lower arginine concentrations fall within, or close to, the salivary range, whereas the higher concentrations were used to probe the response of *P. gingivalis* to elevated arginine exposure. For the in vivo experiments, arginine was administered at 0.4% and 2%. We now clarify in the revised Discussion that these concentrations should be regarded as exogenous, pharmacological exposure conditions rather than as physiological surrogates. Their selection was informed by the fact that some commercially available oral-care formulations contain 1.5% arginine, and in some cases up to 8%, and can generate transient local exposures above endogenous levels (Wolff and Schenkel, *Adv Dent Res*, 2018) (Carda-Diéguez *et al.*, *Microbiome*, 2022) (Nanako Kuriki *et al.*, *J Dent*, 2024). Thus, the mouse model was designed to test the effect of elevated arginine exposure on disease outcome, rather than to mimic a known physiological arginine concentration in mouse oral fluids.

Following the reviewer's suggestion, we have expanded the Discussion section to clarify the physiological context of arginine concentrations and to explain the rationale for the concentrations used in our in vitro and in vivo models. These changes have been made in **Discussion, Lines 393–406** of the revised manuscript.

Comment: Line 460 Growth of a strict anaerobe in microaerobic conditions? Why? How? Without further explanation this is a huge problem.

Response: We thank the reviewer for pointing out this issue and apologize for the confusion caused by this error. The description of “microaerobic conditions” was a typographical mistake. All bacterial cultures in this study were performed under strictly anaerobic conditions. Specifically, *P. gingivalis*, *F. alocis*, and their mutant strains were cultured in an anaerobic chamber with a gas composition of 80% N₂, 10% CO₂, and 10% H₂. We have carefully reviewed the manuscript and corrected all instances where “microaerobic” was mistakenly used. These changes have been made in **Methods, Lines 555 and 564–565** of the revised manuscript. This correction does not affect the experimental design, results, or conclusions of the study.

Comment: Line 633 This figure 1d appears to be duplicated in the Figure 1 and Extended data figure 1

(666265_0_merged_1762880455, p.33)

Response: We thank the reviewer for pointing this out. In the original manuscript, Fig. 1d and Extended Data Fig. 1 were not duplicate panels. Fig. 1d showed the relative abundances of bacteria in subgingival plaque samples, whereas Extended Data Fig. 1 presented the corresponding analysis in saliva samples. However, in response to the reviewer's broader concerns regarding the robustness of these datasets, we have substantially revised this part of the manuscript. Specifically, we removed the original salivary metagenomic analysis and the small laboratory-generated subgingival dataset, and replaced them with a new integrative analysis based on five publicly available subgingival plaque metagenomic datasets. The revised analysis includes 102 samples in total, comprising 44 periodontally healthy individuals and 58 patients with chronic periodontitis. Accordingly, the original Fig. 1d and the related Extended Data panels have been replaced in the revised manuscript. The updated results are now presented in the revised **Fig. 1**, and the corresponding **Results (Lines 108–146)** and **Methods (Lines 457–483)** sections have been modified accordingly.

Comment: Line 633 Figure 1 abc. I would like to highlight that you have 14 subjects with periodontitis, but for most of these subjects the levels are very low or absent. How do you justify the synergism with Fa if both are present but the levels are very low?

Response: We thank the reviewer for this important observation. We agree that, in the original small cohort, the relative abundances of *P. gingivalis* and *F. alocis* were low or variable in a number of subjects, and that this dataset was not sufficient to support a strong inference regarding their interaction.

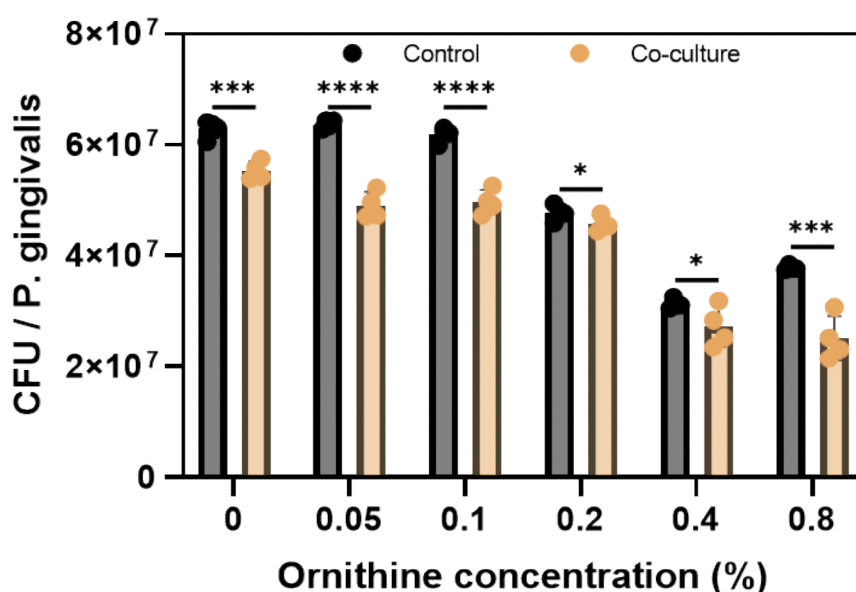
To address this concern, we removed the original small cohort analysis and replaced it with a new integrative analysis based on five publicly available subgingival plaque metagenomic datasets, comprising 102 samples in total (44 healthy and 58 periodontitis). In this expanded cohort, both *P. gingivalis* and *F. alocis*

were significantly enriched in periodontitis compared with healthy controls, and their positive association was more robustly supported. Importantly, in the revised manuscript we no longer use the human metagenomic data to infer synergism. Instead, these data are now presented as evidence of co-enrichment and ecological association only. The functional interaction between *P. gingivalis* and *F. alocis* is addressed separately in the experimental sections. To avoid overinterpretation, we have revised the wording throughout this part of the manuscript to use more cautious terms such as “coexistence”, “co-occurrence”, and “ecological association”, rather than implying strong synergism from the clinical metagenomic data alone. These changes have been made in **Results, Lines 108–146** of the revised manuscript.

Comment: Line 652 Fig 2a, Ornithine creates the same pattern of growth inhibition as arginine, why is this also not included in the study?

Response: We thank the reviewer for this insightful comment. We agree that ornithine showed a growth-inhibitory pattern broadly similar to that of arginine in the initial amino acid screen and therefore merited further examination. In response, we added a concentration-gradient coculture experiment for ornithine and included the results in **Extended Data Fig. 2g**. These new data showed that ornithine behaved differently from arginine in coculture: whereas the inhibitory effect of arginine on *P. gingivalis* was partially alleviated by *F. alocis*, ornithine-mediated inhibition was further enhanced. This difference is important to explain our subsequent mechanistic focus. The aim of this study was to determine whether a specific metabolite could reveal a biologically meaningful metabolic interaction between *P. gingivalis* and *F. alocis*. Arginine was prioritized because its inhibitory effect could be partially relieved in coculture, indicating that it is involved in an interspecies metabolic process that can be modified by *F. alocis*. This is consistent with the central mechanism investigated here, namely that *F. alocis* promotes *P. gingivalis* survival under arginine stress by consuming excess environmental arginine. By contrast, ornithine did not show a rescue pattern in coculture; instead, its inhibitory effect became stronger. This suggests that ornithine is likely to act through a distinct process that does not support

the arginine-dependent mutualistic interaction examined in the present study. In addition, as a downstream metabolite of arginine catabolism, ornithine may reflect a biologically different layer of metabolic regulation from the primary arginine-triggered interaction that forms the focus of this work. For this reason, although ornithine was also inhibitory, mechanistic investigation of ornithine would have led the study into a separate question beyond the scope of the present manuscript. Accordingly, we revised the Results section to explicitly state that arginine and ornithine showed the most marked inhibitory effects in the amino acid screen, but diverged in coculture: In coculture, *F. alocis* partially relieved arginine-mediated growth inhibition of *P. gingivalis*, whereas ornithine further enhanced inhibition, suggesting a distinct mode of action. These revisions clarify why the subsequent mechanistic analyses focused on arginine rather than ornithine. These changes have been made in **Extended Data Fig. 2g; Results, Line 174; and Methods, Line 495** of the revised manuscript.



Extended Data Fig. 2 | g, Effect of exogenous ornithine supplementation (0–0.8% w/v) on *P. gingivalis* growth in a *P. gingivalis*-*F. alocis* coculture system (n=4; unpaired multiple t test). The data are presented as the mean±s.d. Only selected comparisons are shown, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Comment: Line 652 Figure 2b this would be better drawn as a bar chart, the line

chart makes it look like a growth curve instead of a comparison.

Response: We thank the reviewer for this helpful suggestion. We agree that a bar chart is more appropriate for this panel, as the original line graph could be misread as a growth curve rather than a direct comparison across conditions. In response, we have redrawn **Fig. 2b** as a bar chart

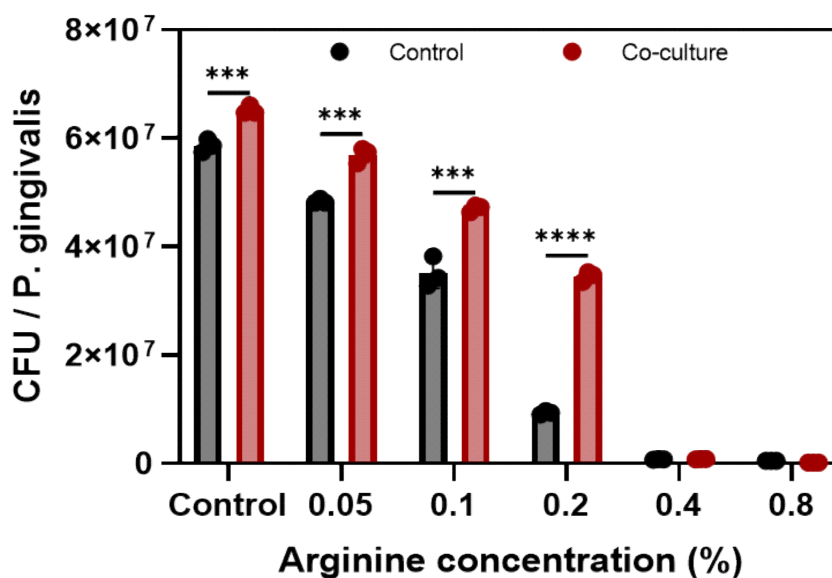


Fig. 2 | b, Effect of exogenous arginine supplementation (0–0.8% w/v) on *P. gingivalis* growth in a *P. gingivalis*-*F. alocis* coculture system (n=3; unpaired multiple t test). The data are presented as the mean±s.d. Only selected comparisons are shown, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Comment: Line 943 I only see three samples from healthy subjects in the PCA plot. (666265_0_merged_1762880455, p.34)

Response: We thank the reviewer for pointing this out. In the original PCA plot, several samples were obscured by overlapping labels, which may have created the impression that only three healthy samples were present. In the revised manuscript, we regenerated the plots in this section to ensure that all samples are clearly visible and properly labeled. In addition, this entire analysis was replaced with a new integrative dataset with an expanded sample size, as described in the revised **Results section (Lines 108–146)**. These revisions resolve the visualization issue and provide a more robust representation of the data.

Comment: Line 959 Is the scale meant to represent the final OD of a bacterial culture, and white represents 0.9? This figure needs more explanation.

(666265_0_merged_1762880455, p.35)

Response: We thank the reviewer for pointing out the lack of clarity in this figure and apologize for the confusion caused. The color scale in the heatmap does not represent absolute OD values. Instead, it indicates the fold change in *P. gingivalis* growth relative to the control condition (exogenous amino acid concentration=0). Specifically, red denotes increased growth (fold change>1), blue denotes decreased growth (fold change<1), and white indicates values close to no change (fold change $\approx$ 1). To avoid further misunderstanding, we have revised the figure legend to explicitly define the normalization method and the meaning of the color scale. These changes have been made in **Lines 1087–1094** of the revised manuscript.

Comment: Line 959 Growth of PG in arginine with or without Fa supernatant? The legend needs more detail. Why grown microaerobically?

(666265_0_merged_1762880455, p.35)

Response: We thank the reviewer for raising this point. In response, we have clarified both the experimental design and the culture conditions in the revised manuscript. To more rigorously evaluate the effect of *F. alocis* supernatant on *P. gingivalis* growth, we repeated the experiment under both arginine-supplemented and arginine-free conditions. Briefly, *F. alocis* was cultured overnight in BB medium either supplemented with 0.2% arginine (A+) or without arginine (A–). Culture supernatants were collected by centrifugation and filtration, and then serially twofold diluted with the corresponding fresh medium (A+ or A–) to generate final supernatant proportions of 50%, 25%, 12.5%, 6.25%, and 3.125%. *P. gingivalis* was then inoculated, and growth was quantified after 24 h by OD measurement. In the revised experiment, supernatants from *F. alocis* cultured in arginine-supplemented medium (A+) partially rescued *P. gingivalis* growth under arginine stress, whereas supernatants from cultures without arginine supplementation (A–) did not show this effect. The **figure legend**

(Lines 785–787) and Methods section (Lines 552–562) have been revised accordingly to make these experimental details explicit.

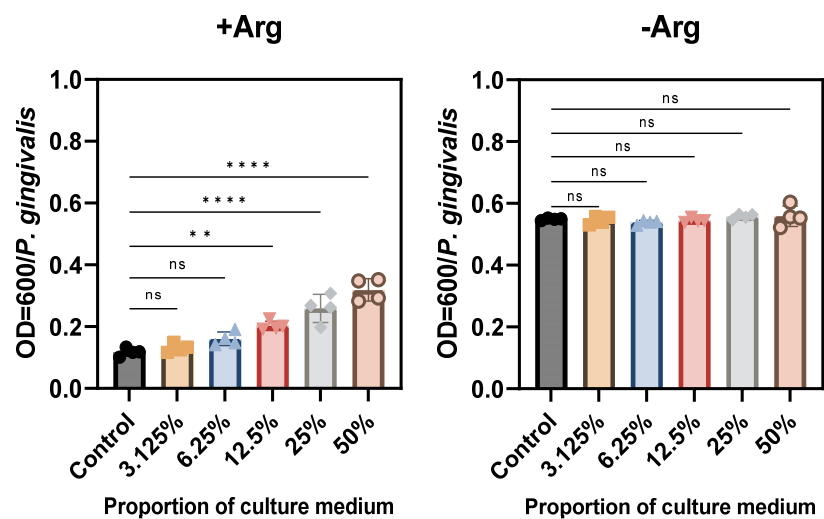


Fig. 2 | h, Effect of 24h *F. alocis* culture supernatants generated with or without 0.2% (w/v) arginine on the growth of *P. gingivalis* (n = 4; ordinary one-way ANOVA). The data are presented as the mean±s.d. Only selected comparisons are shown, ns, not significant, **p<0.01 and ****p<0.0001.

Second, we apologize for the incorrect use of the term “microaerobic.” All bacterial cultures in this study were performed under strict anaerobic conditions (80% N₂, 10% CO₂, 10% H₂). These changes have been made in **Methods, Lines 555 and 564–565** of the revised manuscript.

Reviewer #2 (Remarks to the Author):

The manuscript investigates the metabolic interaction between Porphyromonas gingivalis and Filifactor alocis, focusing on their adaptation to elevated arginine levels in the periodontal pockets of periodontitis. The authors reported that high arginine accumulates ammonia and inhibits P. gingivalis growth by disrupting its intracellular amino acid metabolism, specifically repressing GDH activity and ketoglutarate production. This metabolic stress activates PorX/PorY-regulated T9SS to increase small peptide acquisition. They further propose that F. alocis relieves arginine stress via ArcA mediated arginine consumption, thereby relieving the growth suppression on P. gingivalis and exacerbating periodontitis progression in a mouse model. This topic is of interest to researchers in the field of oral microbiology, while there are several concerns that the authors need to clarify.

Major concerns

Comment: - Results, L109, 119-129: Salivary metagenome analysis of a public dataset reveals higher relative abundance of P. gingivalis and F. alocis in periodontitis cases compared to healthy subjects. However, it should be noted that "periodontitis" encompasses a range of clinical states, and authors should provide the data of clinical status (e.g., stage and grade) or key periodontal parameters (e.g., pocket depth, CAL, % of BOP (+) sites) of the subjects. In addition, metagenomic data from subgingival plaque rather than saliva is necessary to support the strong clinical relationship between P. gingivalis and F. alocis in periodontitis. However, the number of samples used for subgingival plaque validation in this study was very small (five for healthy, two for periodontitis). The authors should reanalyze their results using a larger, publicly available subgingival metagenomic dataset.

Response: We thank the reviewer for this important comment and agree that salivary metagenomic data provide limited resolution for evaluating microbial associations relevant to periodontal disease. We also agree that subgingival plaque metagenomes are more appropriate for assessing the ecological relationship between P. gingivalis and F. alocis in periodontitis. Following the reviewer's suggestion, we substantially

revised this section of the manuscript. Specifically, we removed the original salivary metagenomic analysis and the small laboratory-generated subgingival plaque dataset. In their place, we performed a new integrative analysis using five publicly available subgingival plaque metagenomic datasets, including two cohorts from China (PRJNA230363 and PRJNA932553), two from the United States (PRJNA255922 and PRJNA508385), and one from Japan (PRJDB11203). The revised analysis comprises 102 samples in total, including 44 periodontally healthy individuals and 58 patients with chronic periodontitis (**Fig. 1**). Using this expanded cohort, we found that both *P. gingivalis* and *F. alocis* were significantly enriched in periodontitis compared with healthy controls, and correlation analysis supported a strong ecological association between the two species in the subgingival microbiome. At the same time, because these public datasets do not contain sufficiently complete and harmonized clinical metadata across studies, we cannot use them to determine whether co-occurrence of *P. gingivalis* and *F. alocis* is directly linked to greater periodontal destruction in patients. We have therefore revised the manuscript to present these human metagenomic data as evidence of co-enrichment and ecological association only, rather than as evidence of clinical severity or copathogenesis. This point is now also explicitly acknowledged in the Discussion as a study limitation (**Discussion, Lines 428–432**). These revised results are now presented in **Fig. 1 a–e and Extended Data Fig. 1 a–b**, and the corresponding **Results (Lines 108–146)**, **Methods (Lines 457–483)**, and **Discussion (Lines 428–432)** sections have been updated accordingly.

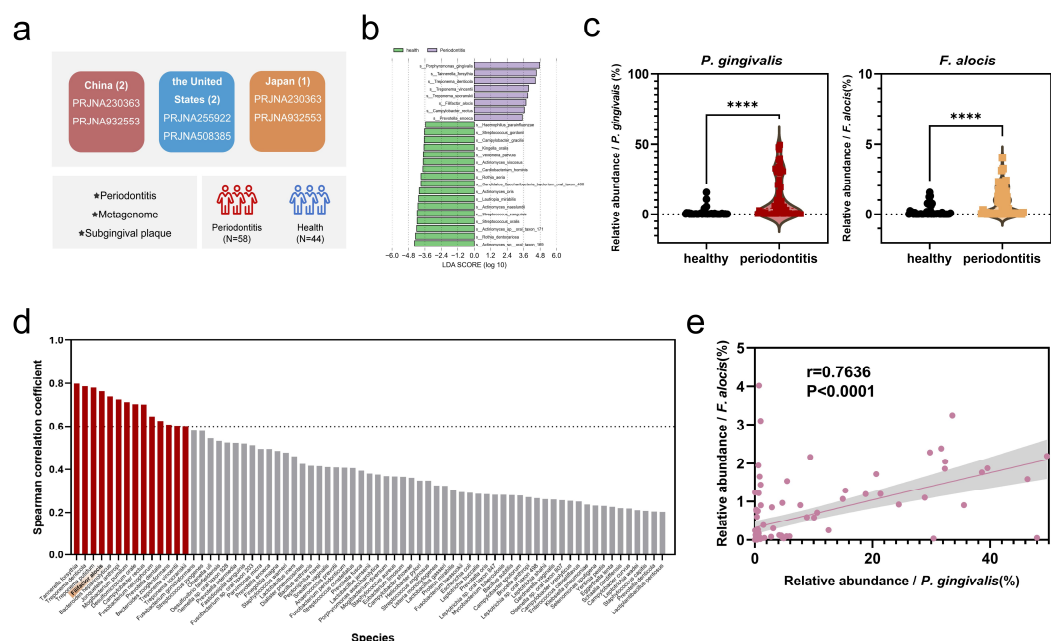
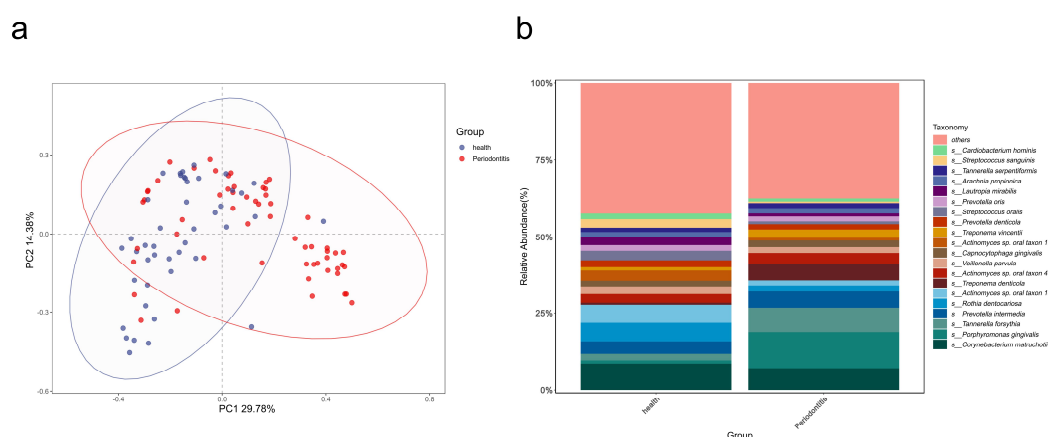


Fig. 1 | **a**, Overview of the integration of five independent public subgingival metagenomic datasets. **b**, Indicator species in the subgingival microbiota of periodontally healthy subjects and patients with periodontitis identified by LEfSe analysis. **c**, Relative abundances of *P. gingivalis* and *F. alocis* in subgingival plaque samples from periodontally healthy subjects and patients with periodontitis. **d**, Spearman correlation coefficients of bacterial taxa co-occurring with *P. gingivalis* in the subgingival microbiota, with *F. alocis* highlighted in orange on the x axis and taxa with correlation coefficients >0.6 shown as red bars. **e**, Correlations between the relative abundance of *P. gingivalis* and *F. alocis* in the subgingival microbiota.



Extended Data Fig. 1 | **a**, Principal coordinate analysis of the subgingival microbiota in periodontally healthy subjects and patients with periodontitis. **b**, Bar plot showing the relative abundances of the top 20 most abundant species in the subgingival microbiota of periodontally healthy subjects and patients with periodontitis.

Comment: - Results, L137: The authors state that the culture supernatant of *F. alocis* promoted the growth of *P. gingivalis* (Extended Data Fig. 2a), but its composition was not defined. To investigate and quantify the specific components enriched in the *F. alocis* culture supernatant that promote *P. gingivalis* growth, targeted metabolomics must be performed by the authors. This is a critical step in validating the proposed symbiosis.

Response: We thank the reviewer for this important suggestion. In response, we performed additional experiments to define the composition of the *F. alocis* culture supernatant and to test whether specific enriched metabolites could account for its effect on *P. gingivalis*.

First, we repeated the supernatant transfer experiment under both arginine-supplemented and arginine-free conditions. *F. alocis* was cultured overnight in BB medium with or without 0.2% arginine, and the filtered supernatants were serially diluted with the corresponding fresh medium before inoculation of *P. gingivalis*. In the revised experiment, supernatants from *F. alocis* cultured in arginine-supplemented medium partially rescued *P. gingivalis* growth under arginine stress, whereas supernatants from cultures without arginine supplementation did not show this effect. These revised data are now presented in **Fig. 2h**.

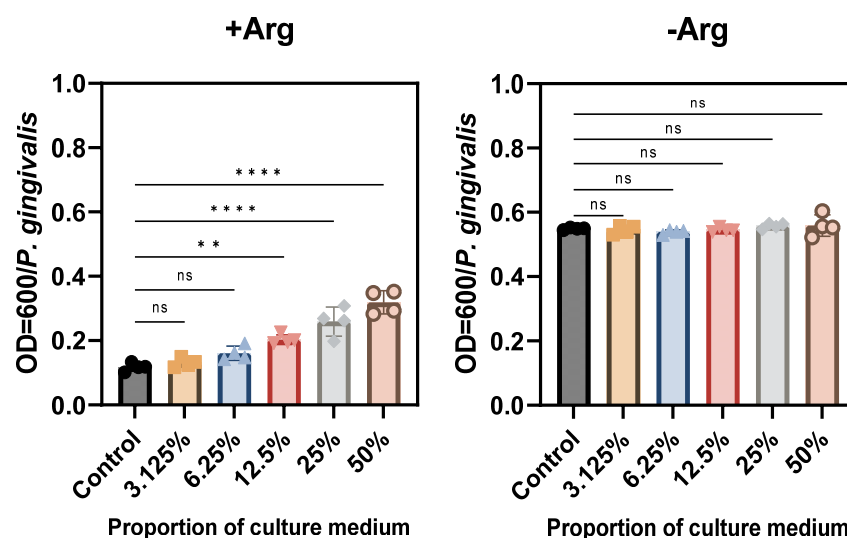
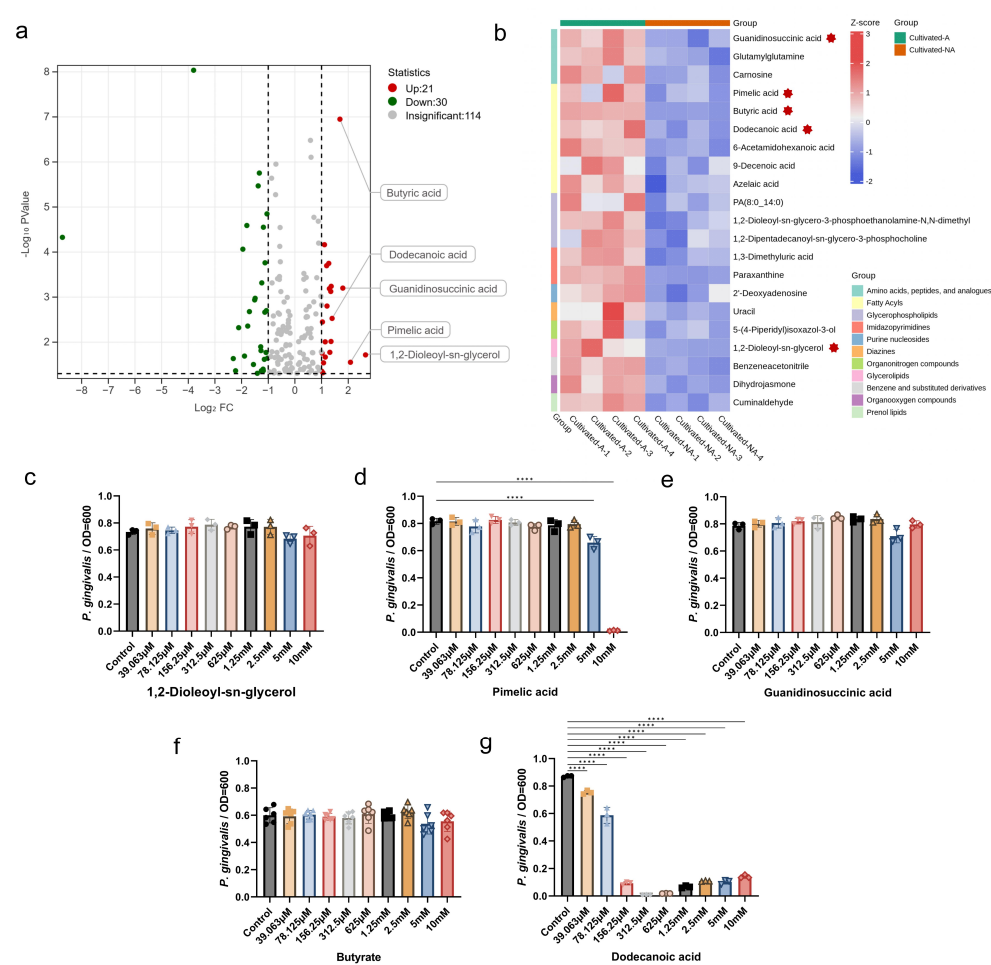


Fig. 2 | h, Effect of 24h *F. alocis* culture supernatants generated with or without 0.2%

(w/v) arginine on the growth of *P. gingivalis* (n = 4; ordinary one-way ANOVA). The data are presented as the mean±s.d. Only selected comparisons are shown, ns, not significant, **p<0.01 and ****p<0.0001.

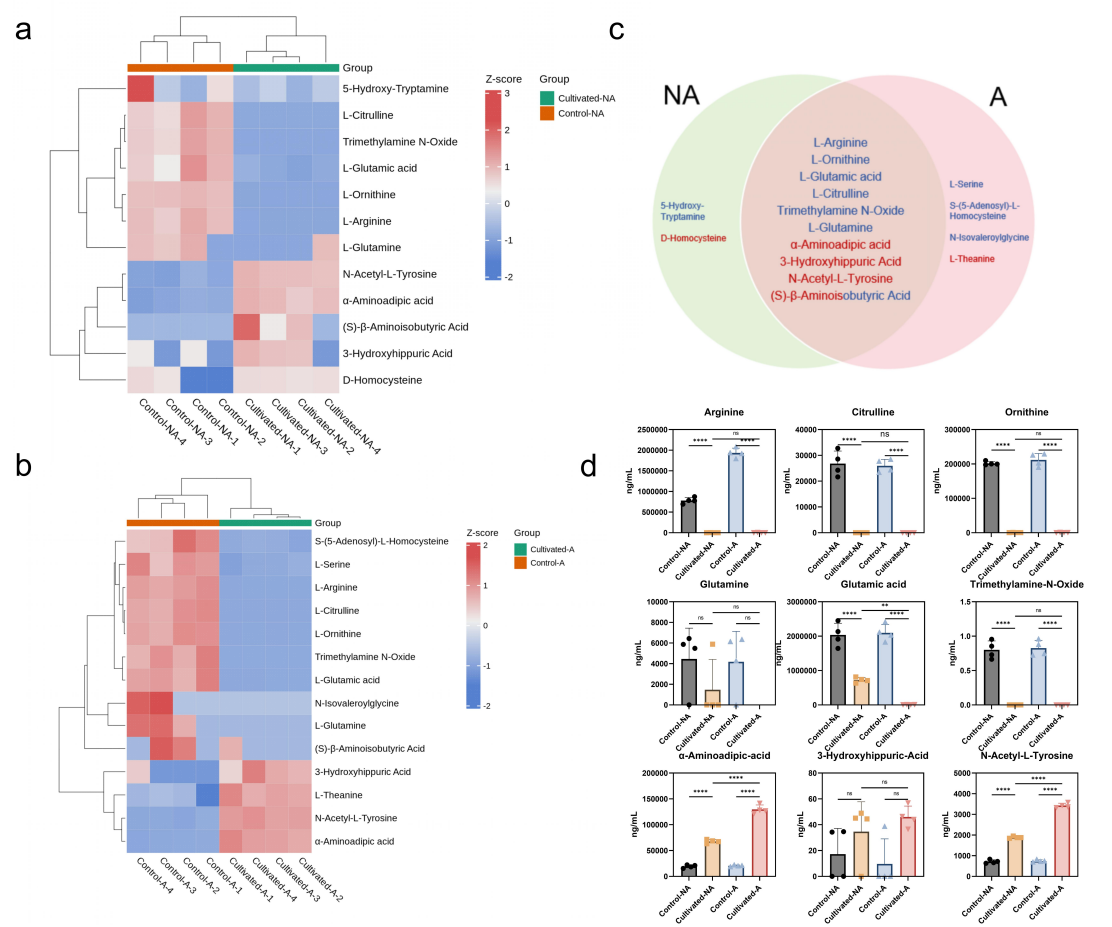
Second, as suggested by the reviewer, we performed untargeted metabolomic profiling of *F. alocis* culture supernatants grown with or without 0.2% arginine. This analysis identified several metabolites enriched after *F. alocis* growth under arginine-supplemented conditions, including 1,2-dioleoyl-sn-glycerol, pimelic acid, guanidinosuccinic acid, butyric acid, and dodecanoic acid. We then tested these top 5 candidate metabolites individually for their effects on *P. gingivalis* growth, but none showed a detectable growth-promoting effect in our validation assays. These results are now presented in **Extended Data Fig. 7**.



Extended Data Fig. 7 | **a**, Volcano plot of differentially abundant metabolites in the culture supernatants of *F. alocis* grown in medium with or without 0.2% arginine. **b**,

Top 20 differentially abundant metabolites in the culture supernatants, ranked by fold change, between the Fa and Fa+0.2% arginine groups. Red asterisks denote the five metabolites with the largest between-group differences. Effects of different concentrations of 1,2-dioleoyl-sn-glycerol (c), pimelic acid (d), guanidinosuccinic acid (e), butyric acid (f), and dodecanoic acid (g) on the growth of *P. gingivalis* (n=3/6, one-way ANOVA). Only selected comparisons are shown, ***p<0.0001. All other comparisons were not significant.

Third, we performed targeted amino acid metabolomics on culture media before and after *F. alocis* growth in the presence or absence of arginine. These analyses showed marked depletion of arginine, citrulline, and ornithine, together with changes in other amino acid-related metabolites, indicating substantial remodeling of the extracellular metabolic environment by *F. alocis*. These data are presented in **Extended Data Fig. 8**.



Extended Data Fig. 8 | a,b, Hierarchical clustering of differential metabolites in culture medium before and after *F. alocis* cultivation in the absence (a) or presence (b) of 0.2% exogenous arginine. **c,** Venn diagram of altered amino acid-related metabolites in medium before and after *F. alocis* cultivation under no-arginine (NA) or 0.2% arginine-supplemented (A) conditions. Red indicates increased metabolites and blue indicates decreased metabolites. (S)- β -Aminoisobutyric acid was increased in the NA group but decreased in the A group. **d,** Absolute abundances of selected metabolites in the four groups. Control-NA, uncultivated medium without exogenous arginine; Cultivated-NA, supernatant after *F. alocis* cultivation without exogenous arginine; Control-A, uncultivated medium supplemented with 0.2% arginine; Cultivated-A, supernatant after *F. alocis* cultivation with 0.2% arginine. The data are presented as the mean $\pm$ s.d. Only selected comparisons are shown, ns, not significant, ****p<0.0001.

Taken together, these new experiments show that we could not identify a single dominant secreted metabolite responsible for the growth-rescuing effect, while the results support the interpretation that the effect of *F. alocis* is primarily linked to remodeling of the extracellular metabolic environment, with relief of arginine stress / arginine depletion emerging as the major mechanism under our experimental conditions. The corresponding **Results (Lines 333–347), Discussion (Lines 437–442) and Methods (Lines 582–593, Lines 620–629)** sections have been revised accordingly.

Comment: - Results, L137: The authors should clarify in their discussion whether the arginine concentrations used in this study (0.0125~0.2%) are within the physiologically relevant range observed in the GCF of patients with severe periodontitis. If they are not within the physiological range, any conclusions regarding the adaptive strategies of *P. gingivalis* may be artifacts resulting from the unnatural environment.

Response: We thank the reviewer for raising this important point. We agree that the physiological relevance of the arginine concentrations used in this study should be clearly discussed. Available clinical measurements of arginine in oral fluids remain

limited and vary across analytical methods. Nevertheless, published studies consistently indicate that arginine levels are elevated in periodontal disease. Reported concentrations increase in whole saliva from 26.4 (12.4–34.7) mg/dL [0.0264% (0.0124–0.0347%)] in health to 64.6 (39.4–70.8) mg/dL [0.0646% (0.0394–0.0708%)] in chronic periodontitis (*Balkan et al., BMC Oral Health, 2025*), whereas reported gingival crevicular fluid (GCF) concentrations remain in the low micromolar range (*Téllez et al., Brazilian Dental Journal, 2008*). These observations indicate disease-associated elevation of arginine in the oral environment, while also highlighting differences between oral fluid compartments. In this context, the arginine concentrations used in our in vitro experiments (0.0125–0.2%) span a range in which the lower concentrations overlap with those reported in saliva, whereas the higher concentrations moderately to substantially exceed salivary levels. As now clarified in the revised Discussion, our experimental design models a spectrum ranging from physiologically relevant salivary exposure to elevated, pharmacological exposure conditions. This is also relevant because some commercially available oral-care formulations contain 1.5% arginine, and in some cases up to 8%, and can generate transient local exposures above endogenous levels (*Wolff and Schenkel, Adv Dent Res, 2018*) (*Carda-Diéguez et al., Microbiome, 2022*) (*Nanako Kuriki et al., J Dent, 2024*). Accordingly, we have revised the manuscript to make this point explicit. In the Introduction, we now specify the reported arginine levels in human oral fluids (**Lines 46–51**). In the Discussion, we now clarify that the in vitro concentrations exceed reported GCF levels, but overlap with, or moderately exceed, the range measured in whole saliva, and that the in vivo regimens should be regarded as pharmacological exposures rather than physiological surrogates (**Lines 393–406**). We believe these revisions more accurately define the physiological context of the study and avoid overinterpreting the adaptive responses of *P. gingivalis* as direct reflections of endogenous GCF conditions.

Comment: - Results, L137: This study used *P. gingivalis* W83 strain (L414). Previous study has reported that different *P. gingivalis* strains result in different growth rates of

bacteria when co-cultured with F. alocis. Did the authors conduct the same experiments using other strains in a pilot study? If not, the authors should mention in the Discussion that the observed phenomenon might be strain-specific because metabolic responses can vary between different P. gingivalis strains.

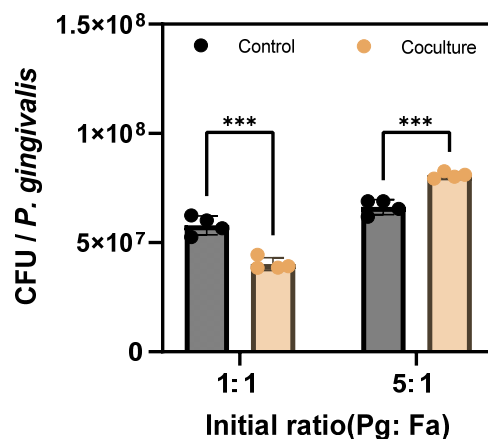
In addition, the coculture ratio used in this study was 10:1 (P. gingivalis : F. alocis). However, reported relative abundance in saliva and plaque from patients with severe periodontitis (Figure 1a, 1b, 1d) indicate that this difference is much smaller. The authors should justify the 10:1 ratio or demonstrate that synergy is maintained at more clinically relevant ratios (e.g., 5:1 or 1:1) in key in vitro assay.

Response: We thank the reviewer for these helpful comments. First, we agree that the present study was performed mainly in the *P. gingivalis* W83 background and that metabolic responses may vary among *P. gingivalis* strains. We did not perform systematic comparisons across multiple strains in this study. Following the reviewer's suggestion, we have now added a statement in the Discussion section to explicitly acknowledge that the observed interaction between *P. gingivalis* and *F. alocis* may be strain-dependent and that strain-level variation may limit the generality of these findings. These changes have been made in **Discussion, Lines 435–437** of the revised manuscript.

Second, regarding the coculture ratio, we further analyzed the clinical metagenomic dataset of subgingival plaque samples used in this study. Among the samples in which both *P. gingivalis* and *F. alocis* were detected, the distribution of the Pg/Fa abundance ratio showed a median value of approximately **12**, with the detailed data provided in **Supplementary Table 6**. On this basis, we selected 10:1 (Pg:Fa) as the primary coculture ratio and have now clarified this rationale in the **Methods section (Lines 496–499)**.

To further address the reviewer's concern, we additionally examined the interaction between the two species at 5:1 and 1:1 ratios (**Extended Data Fig. 2h**). In the revised manuscript, we now show that *F. alocis* still increased *P. gingivalis* after 24 h at a 5:1 ratio, whereas at 1:1 the effect was lost and coculture instead further reduced *P. gingivalis* growth. These findings indicate that the interaction between the

two species is abundance-dependent, consistent with the revised Discussion. The relevant results and explanations have been added to the revised **Results (Lines 179–181), Discussion (Lines 432–433) and Methods (Lines 496–499)** sections.



Extended Data Fig. 2 | h, Effect of *F. alocis* on the growth of *P. gingivalis* in the presence of 0.2% arginine. *P. gingivalis* and *F. alocis* are cocultured for 24 h at initial Pg: Fa ratios of 1:1 and 5:1 (n=4; unpaired multiple t test). The data are presented as the mean±s.d. ***p<0.001.

Comment: - Results, L137: When *P. gingivalis* and *F. alocis* were co-cultured in vitro with arginine at concentrations greater than 0.4%, *P. gingivalis* could no longer be detected by qPCR (Figure 2b). However, in mouse model study, significant alveolar bone resorption was observed in the Pg+Fa+0.4% Arg and Pg+Fa+2% Arg groups (Figure 2g, 2h). The authors should discuss the mechanism of bone resorption in the present mouse model at high arginine levels. Is it possible that *P. gingivalis* remains viable/pathogenic in vivo even at growth-inhibitory concentrations of arginine?

Response: We thank the reviewer for raising this important point. We agree that the discrepancy between the in vitro growth inhibition data and the in vivo bone loss phenotype requires clarification. First, the arginine concentrations used in the mouse experiments should be regarded as exogenous, pharmacological exposure conditions rather than direct surrogates for the local concentrations experienced by *P. gingivalis* in vitro. Notably, arginine is also used in oral care products at relatively high concentrations, commonly around 1.5%, with some formulations reported to contain

up to 8% (Wolff and Schenkel, *Adv Dent Res*, 2018) (Carda-Diéguez *et al.*, *Microbiome*, 2022) (Nanako Kuriki *et al.*, *J Dent*, 2024). In vivo, arginine was delivered through drinking water, and the effective concentration at the subgingival site is likely influenced by dilution, clearance, microbial consumption, and host tissue interactions. Thus, the local arginine exposure encountered by *P. gingivalis* in the periodontal pocket is unlikely to be equivalent to the static concentrations used in vitro.

Second, the ligature-induced periodontitis model used here is a widely established model in periodontal research and includes both mechanical and microbial components of tissue damage. Therefore, even when arginine attenuates the *P. gingivalis*-driven pathogenic component, it would not be expected to abolish bone resorption completely. This interpretation is consistent with our additional control groups, in which arginine reduced bone loss in the ligature + *P. gingivalis* group but did not significantly affect the ligature-only control, indicating that arginine primarily modulates the *P. gingivalis*-dependent component of pathology rather than ligature-induced damage alone.

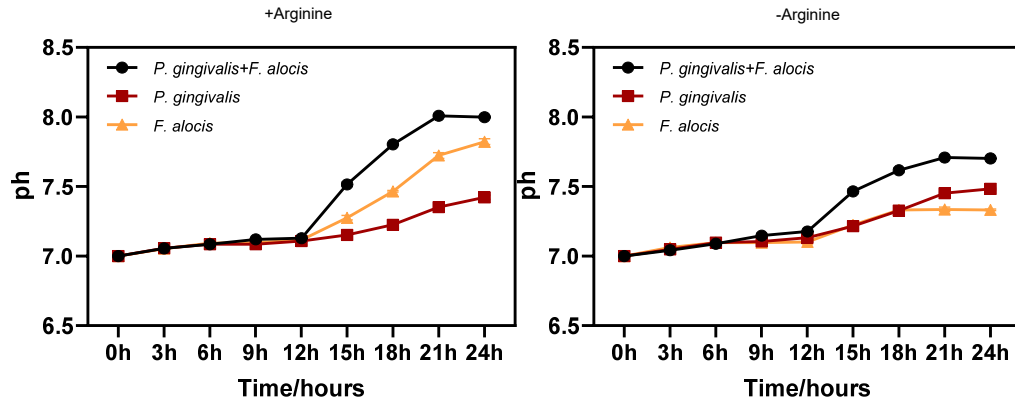
Third, the in vivo findings are consistent with our overall conclusion that arginine exerts inhibitory pressure on *P. gingivalis*, but that this effect can be partially attenuated in the presence of *F. alocis*. In other words, our data do not suggest that *P. gingivalis* is fully suppressed under all in vivo high-arginine conditions, but rather that its pathogenic contribution is reduced and can be partially restored in the polymicrobial context.

Finally, to avoid overinterpretation, we have revised the manuscript to clarify that the in vivo arginine regimens (0.4% and 2%) represent pharmacological exposure conditions used to probe the effect of elevated arginine on disease outcome, rather than physiological surrogates for endogenous oral arginine concentrations. We now interpret the in vivo phenotype more cautiously as being consistent with partial suppression of the *P. gingivalis*-dependent pathogenic component under high-arginine conditions, rather than complete elimination of *P. gingivalis* activity in vivo. These changes have been made in **Discussion, Lines 393–406** of the revised manuscript.

Comment: - Results, Figure 2 and 4: The authors propose that the growth inhibitory and metabolic disturbance mechanisms of arginine are the result of intracellular ammonia accumulation, which disrupts GDH. However, enzymatic degradation of arginine into ammonia would also cause the medium to become more alkaline. Similarly, adding ammonia to the culture medium would increase pH. Therefore, the authors should provide data on how the pH of the culture medium changes immediately after adding arginine or ammonia, and throughout the culture. While the authors mainly focus on intracellular ammonia toxicity, but if the alkalinization of the medium is observed, then pH stress may also be involved, and the authors must verify its effect.

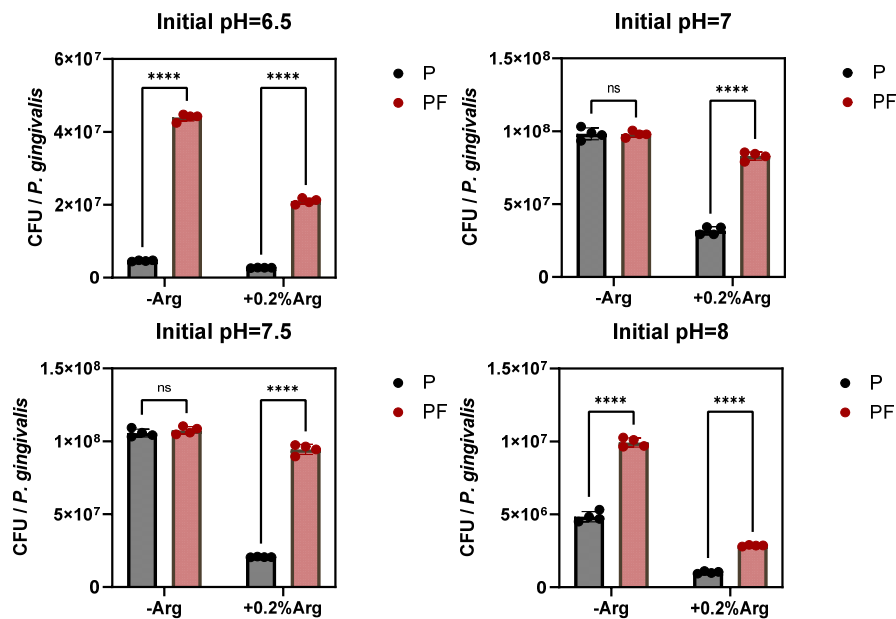
Response: We thank the reviewer for this important and insightful comment regarding the potential contribution of extracellular pH changes. In response, we performed additional experiments to monitor pH dynamics in the culture medium and to test whether pH changes alone could account for the observed phenotypes.

First, we measured pH changes over 24 h in *P. gingivalis* monoculture, *F. alocis* monoculture, and coculture systems in the presence or absence of 0.2% arginine, with the initial pH adjusted to 7.0 in all groups to exclude baseline differences. In the revised experiment, pH increased over time in all conditions, and the extent of alkalinization depended on both arginine and microbial composition. In particular, arginine enhanced medium alkalinization in *F. alocis* monoculture and in coculture, with the highest final pH observed in the coculture condition. These data are now presented in **Extended Data Fig. 2i**.



Extended Data Fig. 2 | i, Dynamic changes in pH over 24 h in *P. gingivalis* monoculture, *F. alocis* monoculture, and coculture systems with (left) or without (right) the treatment of 0.2% arginine.

Second, to determine whether extracellular pH change could explain the growth phenotypes, we performed monoculture and coculture experiments under controlled initial pH conditions (pH 6.5, 7.0, 7.5, and 8.0). Across this tested range, arginine consistently inhibited *P. gingivalis* growth, and *F. alocis* retained its ability to partially alleviate arginine-associated growth inhibition. These data are now presented in **Extended Data Fig. 2j**.



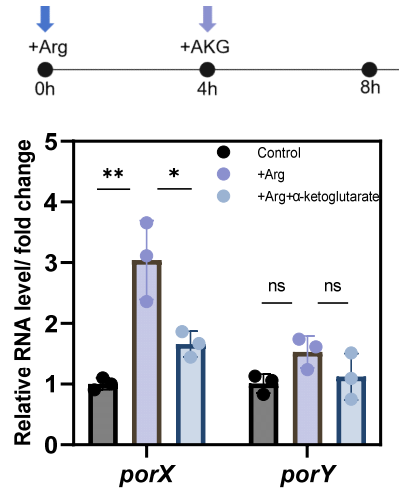
Extended Data Fig. 2 | j, Effects of 0.2% arginine and *F. alocis* on the growth of *P. gingivalis* under different pH conditions (pH=6.5, 7.0, 7.5, 8.0). The data are presented as the mean±s.d. Only selected comparisons are shown, ns, not significant,

****p<0.0001.

Taken together, these results show that although arginine metabolism leads to measurable alkalinization of the medium, the inhibitory effect of arginine on *P. gingivalis* and the growth promotion of *F. alocis* under different pH conditons still exist. We have therefore revised the manuscript to state more clearly that, although arginine metabolism causes measurable extracellular alkalinization, the observed phenotypes are not primarily driven by pH. These changes have been made in **Results, Lines 185–188 and Methods, Lines 506–513, 578–581** of the revised manuscript.

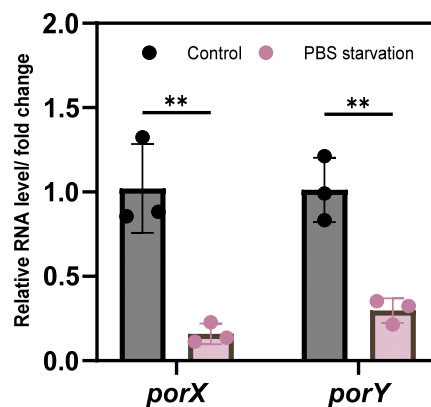
Comment: - Results, L253: The authors mentioned that arginine mediated metabolic disturbance (glutamate pathway) activates PorX/PorY and T9SS to enhance Kgp secretion. However, it remains unclear whether PorX/PorY activation is due to specific arginine/ammonia chemical stress or resultant general nutrient limitation (e.g., glutamate/ α -ketoglutarate depletion).

Response: We thank the reviewer for this insightful comment regarding the mechanism underlying the PorX/PorY response. To distinguish whether the observed PorX/PorY changes were more consistent with direct arginine/ammonia chemical stress or with secondary metabolic consequences, we performed three additional experiments. First, we supplemented α -ketoglutarate (α -KG) in arginine-treated cultures. Under these conditions, α -KG supplementation reversed the arginine-associated increase in *porX* expression (**Extended Data Fig. 5d**), supporting a link between PorX/PorY and perturbation of the glutamate/ α -ketoglutarate axis.



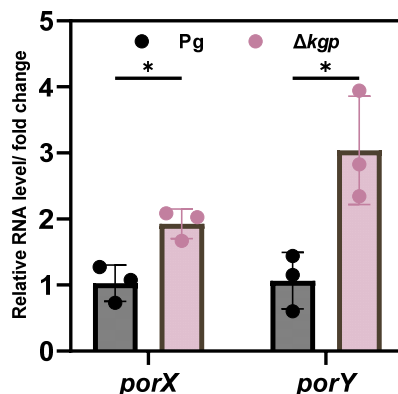
Extended Data Fig. 5 | d, Effect of α -ketoglutarate supplementation on *porX* and *porY* transcription in *P. gingivalis* (n=3, one-way ANOVA). The data are presented as the mean $\pm$ s.d. Only selected comparisons are shown, ns, not significant, *p<0.05, **p<0.01.

Second, to test whether the response could be explained simply by acute non-specific starvation, we transferred overnight-grown *P. gingivalis* cells into PBS and measured *porX* and *porY* expression. Under this stringent starvation condition, both genes were downregulated rather than induced (**Extended Data Fig. 5e**), indicating that the PorX/PorY response observed under arginine treatment is not recapitulated by acute general starvation alone.



Extended Data Fig. 5 | e, Effect of PBS starvation on *porX* and *porY* transcription in *P. gingivalis* (n=3, unpaired t test). The data are presented as the mean $\pm$ s.d. Only selected comparisons are shown, **p<0.01.

Third, because Kgp contributes to peptide acquisition and nitrogen utilization in *P. gingivalis*, we examined a Δkgp mutant as a model of impaired peptide-dependent nutrient acquisition. Compared with the wild-type strain, the Δkgp mutant showed increased expression of *porX* and *porY* (**Extended Data Fig. 5f**), supporting an association between PorX/PorY and intracellular metabolic imbalance related to altered nutrient acquisition.



Extended Data Fig. 5 | f, Changes in *porX* and *porY* transcription in the protein acquisition-restricted mutant Δkgp compared with the Pg WT (n=3, unpaired t test). The data are presented as the mean $\pm$ s.d. Only selected comparisons are shown, *p<0.05.

Taken together, these additional results support a more refined interpretation of our data. Rather than being best explained by a direct effect of arginine or ammonia itself, the PorX/PorY response is more consistent with an adaptive response associated with intracellular metabolic imbalance, particularly perturbation of the glutamate/ α -ketoglutarate pathway. At the same time, the PBS starvation experiment indicates that this response is not simply reproduced by acute non-specific nutrient deprivation. We have revised the manuscript accordingly to clarify this mechanistic interpretation and to avoid overstating PorX/PorY as a direct arginine-responsive signaling system. These changes have been made in **Extended Data Fig. 5d–f** and **Results, Lines 281–293** of the revised manuscript.

Comment: - Results, L259: The authors emphasize that the ArcA pathway of *F. alocis* is an important mitigation mechanism for *P. gingivalis*. However, several oral

commensal bacteria possess the arginine deiminase pathway and could perform the same arginine-consuming function. Therefore, the authors must acknowledge that this function is not unique to F. alocis. The current conclusion regarding the unique mutualism between P. gingivalis and F. alocis is overstated.

They must then justify their focus on the P. gingivalis/F. alocis pair by citing factors that confer a functional advantage to this partnership, such as strong co-localization or correlation between the two bacteria or demonstrated cooperative virulence (e.g., synergistic biofilm formation or host cell invasion).

Furthermore, the growth promotion of P. gingivalis by F. alocis culture supernatant (Extended Data Fig. 2a) suggests the presence of secreted growth-promoting factors secreted by F. alocis. The authors should perform targeted LC-MS/MS on F. alocis spent media ($\pm$ arginine), quantify changes, and test purified fractions to identify the growth-promoting components.

Response: We thank the reviewer for these important comments. We agree that the arginine deiminase system (ADS) is not unique to *F. alocis* and that our original description overstated the exclusivity of the *P. gingivalis*–*F. alocis* interaction. In response, we have revised the manuscript to avoid presenting this mechanism as a species-exclusive mutualism. In the revised Results and Discussion, *F. alocis* is now described as a representative periodontal partner in which ArcA-dependent arginine metabolism alleviates arginine-associated stress in *P. gingivalis*, rather than as a uniquely specialized organism. We also now explicitly acknowledge that other ADS-positive oral bacteria may potentially perform similar arginine-consuming functions. These changes have been made in **Results, Lines 295–296, 309–312, 329–332** and **Discussion, Lines 407–427** of the revised manuscript.

At the same time, we have revised the manuscript to better justify our focus on the *P. gingivalis*–*F. alocis* pair as a biologically relevant model in the periodontal niche. First, our integrative analysis of five independent subgingival metagenomic datasets ($n = 102$) shows that both *P. gingivalis* and *F. alocis* are significantly enriched in periodontitis and positively associated within the subgingival microbiome (**Results, Lines 108–146**). Second, previous studies have reported that these two species exhibit

spatial co-localization in diseased periodontal tissues and positively correlated in situ metabolic activity (**Introduction, Lines 82–88**). Third, prior functional studies have shown that coculture of *P. gingivalis* and *F. alocis* enhances biofilm development and host cell invasion (**Introduction, Lines 88–90**). In addition, in our study, *F. alocis* modulated the response of *P. gingivalis* to arginine stress in vitro and enhanced *P. gingivalis*-associated pathogenic effects in vivo under the tested conditions. Together, these data justify our focus on the *P. gingivalis*–*F. alocis* pair as a biologically relevant and functionally supported model for studying metabolic cooperation in the periodontal niche.

To address the reviewer’s concern regarding the composition of *F. alocis* culture supernatants, we performed a combination of functional assays, untargeted metabolomics, and targeted amino acid metabolomics. First, supernatant transfer experiments showed that only supernatants derived from *F. alocis* cultured in arginine-supplemented medium partially rescued *P. gingivalis* growth under arginine stress, whereas supernatants from cultures without arginine supplementation did not show this effect (**Extended Data Fig. 2 h**). Second, untargeted metabolomics identified several metabolites enriched after *F. alocis* growth under arginine-supplemented conditions. However, functional testing of the top candidate metabolites, including 1,2-dioleoyl-sn-glycerol, pimelic acid, guanidinosuccinic acid, butyric acid, and dodecanoic acid, did not reproduce the growth-rescuing effect (**Extended Data Fig. 7**). Third, targeted amino acid metabolomics showed marked depletion of arginine, citrulline, and ornithine after *F. alocis* growth (**Extended Data Fig. 8**). Taken together, these results indicate that the observed effect is unlikely to be explained by a single dominant secreted metabolite. Instead, our current data support extracellular arginine depletion and relief of arginine-associated stress as the major mechanism under these experimental conditions. We acknowledge that further fractionation and targeted identification of additional bioactive components may provide further insight, and we have revised the **Results (Lines 181–184, 333–347)**, **Discussion (Lines 437–442)**, and **Methods (Lines 552–562, 582–593, 620–629)** sections accordingly.

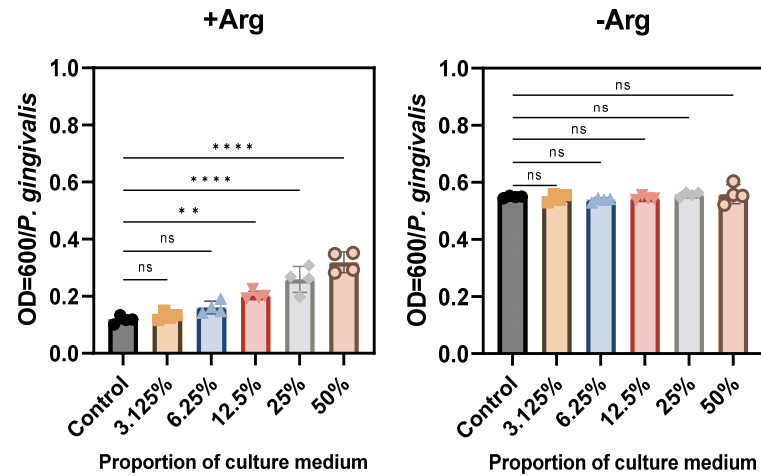
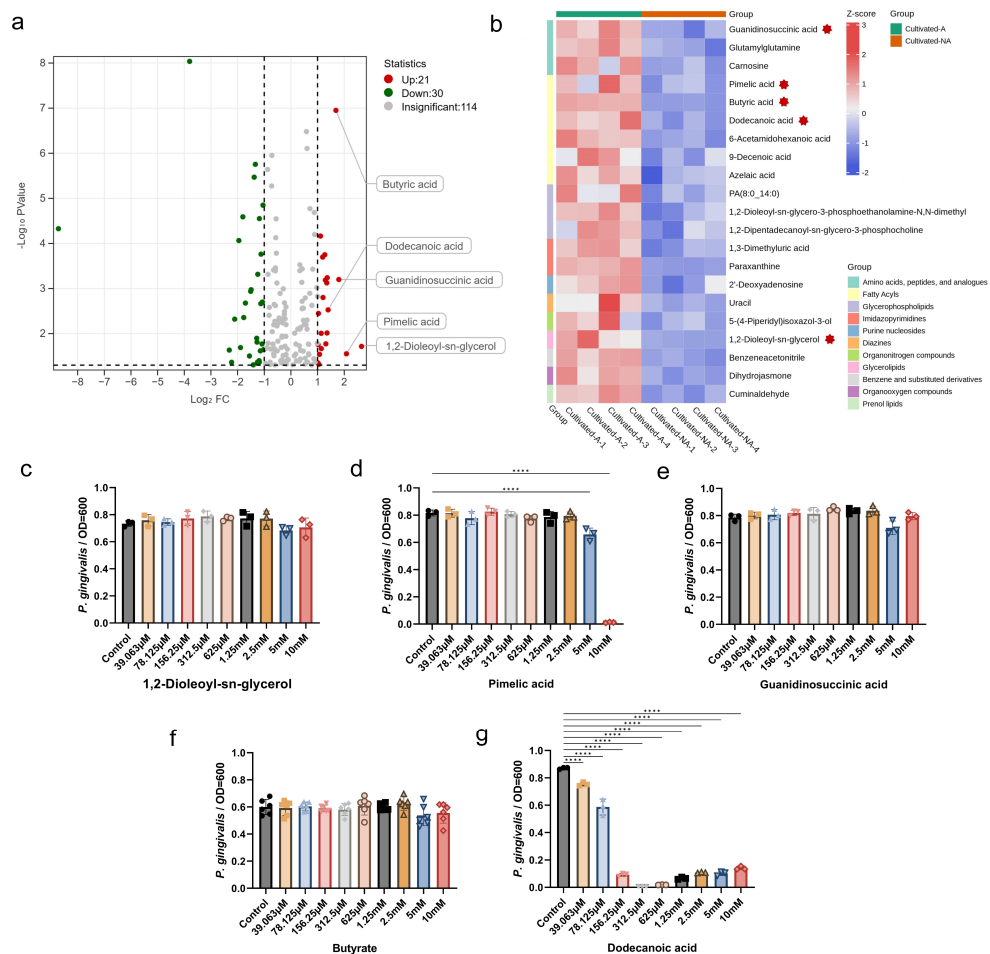
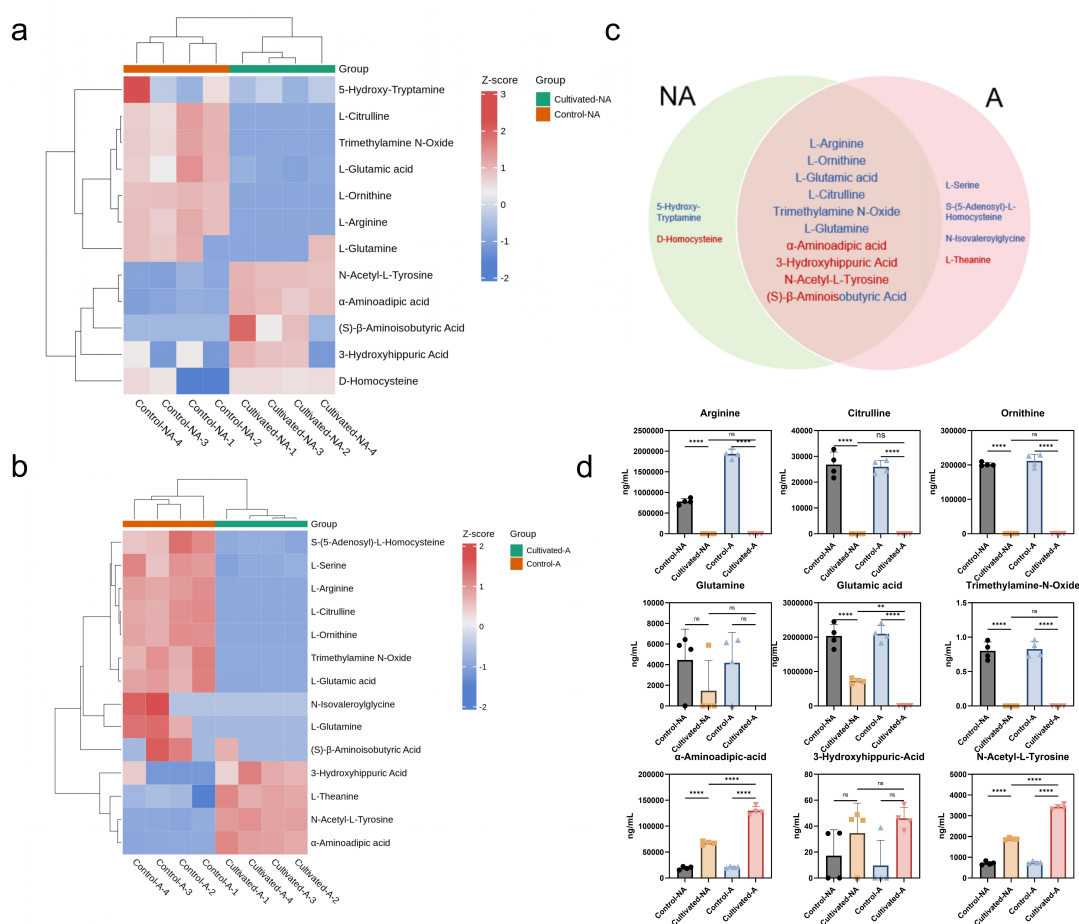


Fig. 2 | h, Effect of 24h *F. alocis* culture supernatants generated with or without 0.2% (w/v) arginine on the growth of *P. gingivalis* (n = 4; ordinary one-way ANOVA). The data are presented as the mean±s.d. Only selected comparisons are shown, ns, not significant, **p<0.01 and ****p<0.0001.



Extended Data Fig. 7 | a, Volcano plot of differentially abundant metabolites in the

culture supernatants of *F. alocis* grown in medium with or without 0.2% arginine. **b**, Top 20 differentially abundant metabolites in the culture supernatants, ranked by fold change, between the Fa and Fa+0.2% arginine groups. Red asterisks denote the five metabolites with the largest between-group differences. Effects of different concentrations of 1,2-dioleoyl-sn-glycerol (**c**), pimelic acid (**d**), guanidinosuccinic acid (**e**), butyric acid (**f**), and dodecanoic acid (**g**) on the growth of *P. gingivalis* (n=3/6, one-way ANOVA). The data are presented as the mean±s.d. Only selected comparisons are shown, ****p<0.0001. All other comparisons were not significant.



Extended Data Fig. 8 | **a,b**, Hierarchical clustering of differential metabolites in culture medium before and after *F. alocis* cultivation in the absence (**a**) or presence (**b**) of 0.2% exogenous arginine. **c**, Venn diagram of altered amino acid-related metabolites in medium before and after *F. alocis* cultivation under no-arginine (NA) or 0.2% arginine-supplemented (A) conditions. Red indicates increased metabolites and blue indicates decreased metabolites. (S)-β-Aminoisobutyric acid was increased in the NA group but decreased in the A group. **d**, Absolute abundances of selected metabolites in the four groups. Control-NA, uncultivated medium without exogenous

arginine; Cultivated-NA, supernatant after *F. alocis* cultivation without exogenous arginine; Control-A, uncultivated medium supplemented with 0.2% arginine; Cultivated-A, supernatant after *F. alocis* cultivation with 0.2% arginine. The data are presented as the mean $\pm$ s.d. Only selected comparisons are shown, ns, not significant, **** $p < 0.0001$.

Comment: - Discussion: The Discussion must be revised to explicitly address the major limitations raised above

Response: We thank the reviewer for this important comment. In response, we have revised the Discussion to explicitly address the major limitations of the present study. Specifically, we now acknowledge that: (1) the public subgingival metagenomic datasets support co-enrichment and ecological association between *P. gingivalis* and *F. alocis*, but do not contain sufficiently complete clinical metadata to determine whether their co-occurrence is directly linked to greater periodontal destruction in patients; (2) the interaction between these organisms is likely to be abundance-dependent; (3) the mechanistic analyses were performed mainly in the *P. gingivalis* W83 background, and strain-level variation may limit the generality of the findings; and (4) although untargeted metabolomics and targeted amino acid profiling were performed on *F. alocis* supernatants, no single dominant growth-rescuing metabolite for *P. gingivalis* was identified. These points are now explicitly discussed in the revised Discussion section (**Lines 428–447**).

Specific points

Comment: - Abstract, L39-40: Given the results of this study, the statement might be considered somewhat exaggerated; “To overcome stress induced by high arginine levels, *P. gingivalis* strongly cooperated with *F. alocis* ...”

Response: We thank the reviewer for this comment. We agree that the original wording in the Abstract was overstated. In response, we revised this sentence to remove the phrase “*P. gingivalis* strongly cooperated with *F. alocis*” and replaced it with more cautious wording indicating that *F. alocis* partially relieved arginine-associated growth inhibition through ArcA-dependent arginine degradation.

This change has been made in the revised **Abstract (Lines 30–34)**.

***Comment:** - Introduction, L55-58: As mentioned above, the authors should provide specific clinical ranges for arginine concentrations in saliva and GCF in periodontitis and in healthy subjects. This will clarify the biological relevance of the concentrations used by the authors in this study.*

Response: We thank the reviewer for this important suggestion. In response, we have revised both the **Introduction (Lines 46–51)** and the **Discussion (Lines 393–406)** to provide specific clinical ranges for arginine concentrations and to better clarify the biological relevance of the concentrations used in this study. In the revised Introduction, we now state that arginine concentrations in whole saliva increase from 26.4 (12.4–34.7) mg/dL [0.0264% (0.0124–0.0347%)] in health to 64.6 (39.4–70.8) mg/dL [0.0646% (0.0394–0.0708%)] in chronic periodontitis (*Balkan et al., BMC Oral Health, 2025*). In the revised Discussion, we further clarify that the in vitro arginine concentrations used in this study (0.0125–0.2%) exceed the reported micromolar range in gingival crevicular fluid, but overlap with, or moderately exceed, the range measured in whole saliva, and that commercially available oral-care formulations containing 1.5% arginine (or 8% arginine) can generate transient local exposures above endogenous levels (*Wolff and Schenkel, Adv Dent Res, 2018*) (*Carda-Diéguez et al., Microbiome, 2022*) (*Nanako Kuriki et al., J Dent, 2024*). We also now state more explicitly that the 0.4% and 2% in vivo regimens should be regarded as pharmacological exposures rather than physiological surrogates. These additions were made to better define the physiological context and rationale for our experimental design.

***Comment:** - Introduction, L64: Change the phrasing to improve flow: "Unlike free amino acids, *P. gingivalis* preferentially utilizes peptides as primary energy and carbon sources, particularly glutamate- and aspartate-containing peptides..." → "Unlike many bacteria that utilize free amino acids, *P. gingivalis* preferentially utilizes peptides, particularly glutamate- and aspartate-containing peptides, as*

primary energy and carbon sources ...".

Response: We thank the reviewer for this helpful suggestion. We have revised the sentence accordingly. This change has been made in the revised **Introduction (Lines 60–62)**

Comment: - M&M, L459: To ensure reproducible metabolite concentrations, please specify the growth stage (or time) of the F. alocis culture before collecting the spent medium.

Response: We thank the reviewer for this helpful suggestion. In response, we redesigned and repeated the spent-medium experiment with improved controls and a more explicit description of the culture conditions to ensure reproducibility. Specifically, we now state that *F. alocis* was cultured in BB broth with or without 0.2% arginine for 24 h at 37 °C under anaerobic conditions before supernatants were collected by centrifugation and filtration. The revised Methods section also now specifies how the spent media were serially diluted with the corresponding fresh media before inoculation of *P. gingivalis*. These revisions have been incorporated into the Methods section (**Lines 552–562**), and the corresponding experimental results have been updated accordingly.

Comment: - Figure 3, L677: “Coagulation activity” → "coaggregation activity"

Response: We thank the reviewer for this helpful suggestion. We agree that the original term “coagulation activity” was not sufficiently precise. Because this assay measured the agglutination of erythrocytes by *P. gingivalis*, we have revised the terminology in the manuscript to “hemagglutination activity”, which more accurately reflects the nature of the assay. This change has been made in the revised **Lines 801**.

Reviewer #3 (Remarks to the Author):

*This is an interesting and well-written paper demonstrating that arginine inhibits *P. gingivalis* growth by disrupting amino acid metabolism by blocking glutamate dehydrogenase activity. This leads to the activation of the PorX/PorY two-component system, which then suppresses the expression of T9SS components and reduces Kgp secretion and Pg virulence in the LIP model. Cross-feeding symbiosis with *Filifactor alocis* (Fa), which consumes arginine, eliminates the inhibitory effects on Pg amino acid metabolism and virulence. The novelty of the data is diminished by numerous published studies that address the individual findings presented in this report. In addition, I have several comments on the experimental design and data presentation.*

General comments:

Comment: 1. Ligature-induced periodontitis (LIP) model. It is important to note that alveolar bone loss in the LIP model occurs regardless of exogenous pathobiont infection, driven by dysbiosis in the native oral microbiome of mice. This will undoubtedly happen in mice with a ligature for 4 weeks. This is evident in Fig. 1f, which shows no difference in bone loss between animals infected with Pg alone and non-infected animals. In all other LIP experiments, there are no controls of non-infected ligature and with arginine alone. Therefore, the interpretation that Tf restores Pg pathogenicity in the presence of Arg is highly questionable.

Response: We thank the reviewer for this important comment regarding interpretation of the ligature-induced periodontitis (LIP) model. We agree that ligature placement alone can induce alveolar bone loss through dysbiosis of the endogenous oral microbiota, and that this must be taken into account when interpreting the effects of exogenous bacterial infection.

First, to address the reviewer's concern regarding the pathogenic contribution of *P. gingivalis* and *F. alocis* in the LIP model, we repeated the experiment with an increased sample size (n = 10 per group) and included the following groups: ligature-only control, ligature + *P. gingivalis*, ligature + *F. alocis*, and ligature + *P. gingivalis* + *F. alocis*. In this independent experiment, *P. gingivalis* infection resulted

in significantly greater alveolar bone loss than the ligature-only control group, whereas *F. alocis* alone did not significantly increase bone loss. Importantly, coinfection with *P. gingivalis* and *F. alocis* led to greater alveolar bone destruction than either monoinfection, supporting an enhanced pathogenic effect during coinfection in this murine model. These changes have been made in **Fig. 1 f–g** and **Results, Lines 135–146** of the revised manuscript.

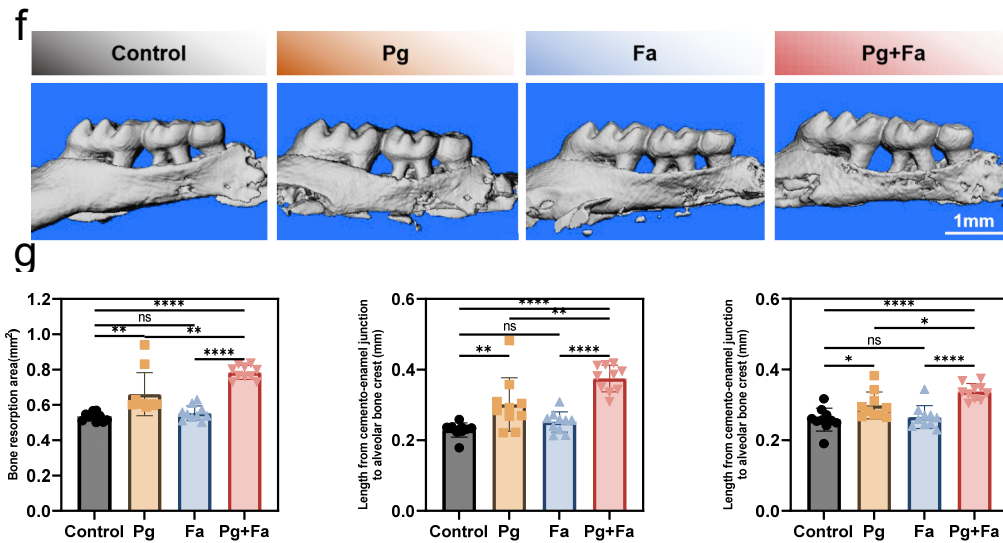
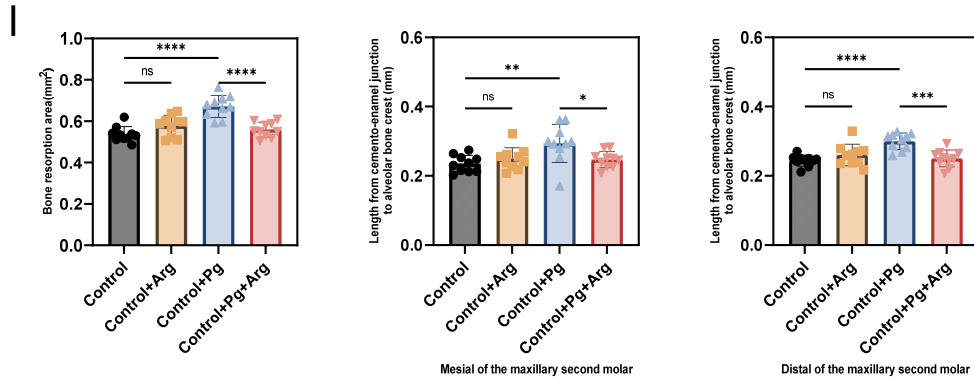
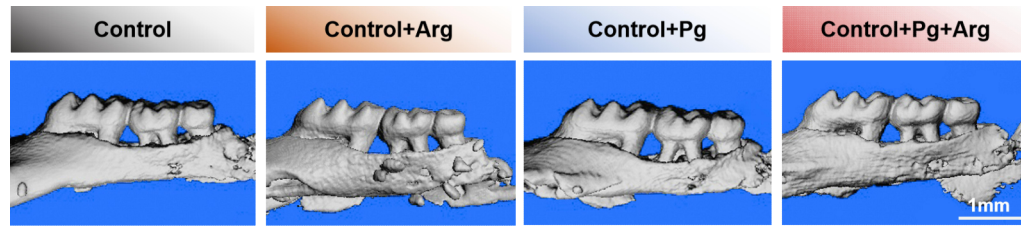


Fig. 1 | f, Representative 3D-reconstructed micro-CT images of maxillae (buccal view) from (1) the noninfected control, (2) Pg monoinfection, (3) Fa monoinfection, and (4) Pg+Fa coinfection groups (n=10). **g**, Bone loss area (left) and CEJ-to-alveolar crest distance were mesiodistally (middle and right) measured across experimental groups (n=10; one-way ANOVA). The data are presented as the mean±s.d. Only selected comparisons are shown, ns, not significant, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Second, to determine whether arginine itself alters the LIP model independently of *P. gingivalis*, we performed an additional control experiment including ligature-only groups with or without arginine supplementation. In this experiment, arginine alone did not significantly affect alveolar bone loss in the ligature-induced periodontitis model. In contrast, when *P. gingivalis* infection was present, arginine supplementation significantly reduced bone destruction. These changes have been made in **Extended Data Fig. 2 k–l** and **Results, Lines 162–167** of the revised manuscript.

k



Extended Data Fig. 2 | k, Representative 3D-reconstructed micro-CT images of maxillae (buccal view) from the (1) Control, (2) Control+Arg, (3) Control+Pg and (4) Control+Pg+Arg groups (n=5). **l**, Bone loss area (left) and CEJ to-alveolar crest distance were mesiodistally (middle and right) measured across experimental groups (n=5; one-way ANOVA). The data are presented as the mean±s.d. Only selected comparisons are shown, ns, not significant, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

In the subsequent mechanistic experiments presented in later sections of the manuscript, the LIP + *P. gingivalis* condition was therefore used as the baseline disease model to specifically examine how arginine and *F. alocis* influence *P. gingivalis*-associated pathogenicity. Because the key control experiments were added here to define the contribution of ligature alone and arginine alone, the ligature-only group was therefore not repeated in every subsequent experiment. The relevant Results and Methods sections have been revised accordingly, and the new control data have been incorporated into the updated figure set.

Comment: 2. Additionally, controls are missing in several experiments, which

diminishes the reliability of the conclusions. For example:

2a. The control for the experiment shown in Figure 2d should be the growth curve of Pg/Fa cocultures with varying arginine concentrations. This is displayed in panels b and c at an unspecified time point.

2b. In hemagglutination experiments (Fig. 3e, k, Ext Data Fig. 6), the essential control, such as the effect of arginine and Tf alone, on hemagglutination, is missing. By the way, in several places in the manuscript, hemagglutination is referred to as coagulation or coaggregation.

Response: We thank the reviewer for this important comment regarding the lack of controls in several experiments. In response, we have added the requested control experiments and revised the manuscript accordingly.

For original Fig. 2d, we agree that the original presentation did not provide a sufficiently clear control for interpreting the coculture growth curve under arginine treatment. In response, we performed additional 24 h growth-curve experiments in which *P. gingivalis* monoculture, *F. alocis* monoculture, and *P. gingivalis*–*F. alocis* coculture were monitored in parallel under both 0% and 0.2% arginine conditions (**Fig. 2d, e**). These added controls are now incorporated into the revised figure and allow direct comparison of mono- and coculture growth dynamics with and without arginine. The corresponding figure (**Fig. 2d, e**) and legend have been revised accordingly, and the **Results (Lines 158–159, 178)** section has also been updated.

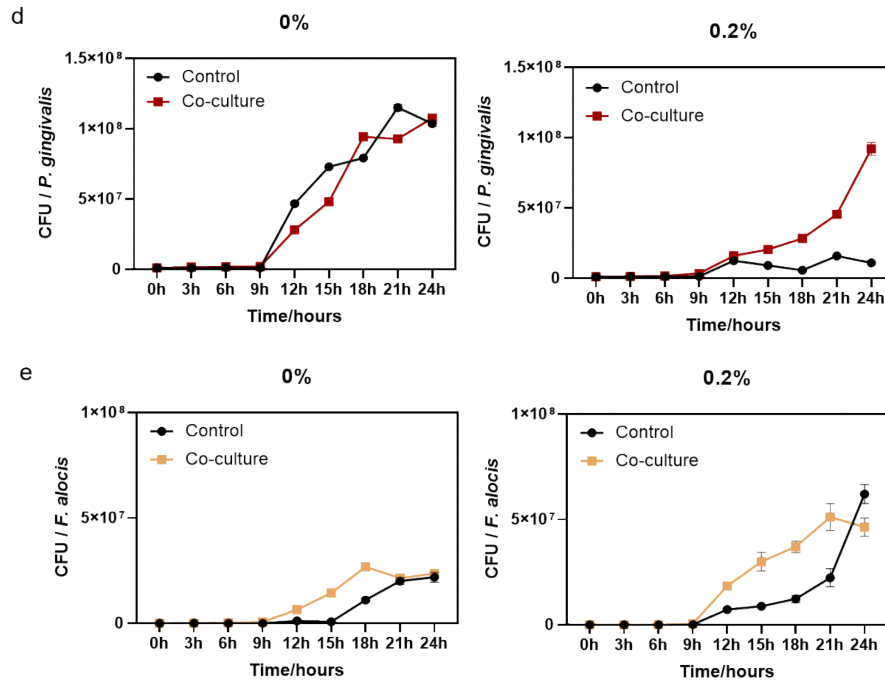
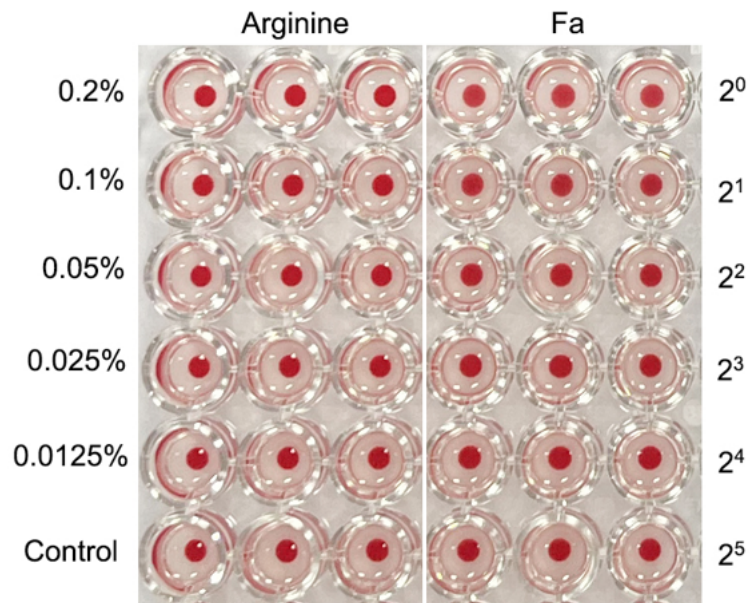


Fig. 2 | d, Growth curves of *P. gingivalis* in monoculture and coculture with or without 0.2% (w/v) arginine over 24 h. **e**, Growth curves of *F. alocis* in monoculture and coculture with or without 0.2%(w/v) arginine over 24 h.

For the hemagglutination assays (original Fig. 3e, k and Extended Data Fig. 6), we agree that direct controls for arginine alone and *F. alocis* alone were required. First, we clarify that *P. gingivalis* cells were collected after arginine treatment and thoroughly washed with PBS before the hemagglutination assay. Therefore, arginine present during bacterial culture was removed before incubation with erythrocytes and did not directly contact red blood cells during the assay. This point has now been clarified in the revised **Methods (Lines 633–635)** section. Second, following the reviewer’s suggestion, we performed additional control experiments in which erythrocytes were incubated with arginine alone (0.125–0.2%) or with *F. alocis* alone (10^8 cells, twofold serial dilution) under the same assay conditions. In the revised results, neither arginine alone nor *F. alocis* alone induced hemagglutination relative to the control group. These findings indicate that the hemagglutination observed in our experiments is attributable to *P. gingivalis* rather than to a direct effect of arginine or *F. alocis* (**Extended Data Fig. 3g**).



Extended Data Fig. 3 | g, Effects of arginine and *F. alocis* on hemagglutination function.

In addition, as noted by the reviewer, we have carefully revised the terminology throughout the manuscript and replaced all inaccurate uses of “coagulation” or “coaggregation” with “hemagglutination” to ensure consistent and accurate description of the assay. These changes have been made in **Results, Lines 211, 225 and Lines 801, 810, 1128, 1139** of the revised manuscript.

Comment: 3. Gingipain activity assay is flawed. The activity should be determined as the initial velocity of the reaction (V_o) expressed as mOD/min from the linear part of the time-recorded increase of absorbance. The results shown in Fig. 3f,g, Ext data Fig. 6b. could be misleading.

Response: Thank you for this helpful comment. We have reanalyzed the gingipain activity data following the reviewer’s suggestion. Specifically, gingipain activity was recalculated as the initial reaction velocity (V_o) derived from the linear portion of the absorbance increase over time. Based on the kinetic measurements, the reaction velocity was calculated from the slope of the absorbance change between 30 and 90 min, and expressed as mOD/min. Accordingly, the data presentation has been revised.

The original plots showing absorbance changes over time have been replaced with bar graphs representing gingipain activity (mOD/min) for each experimental group in **Fig. 3f, g, l, m; Extended Data Fig. 3k, m** and **Extended Data Fig. 4e, g**. The kinetic curves of absorbance versus time are now provided in the **Extended Data Fig. 3h, i, j, l** and **Extended Data Fig. 4a, b, d, f** as supporting information. Importantly, this revised analysis does not alter the conclusions of the study.

***Comment:** 4. Importance of the PorX/PorY two-component system in response to arginine and its relation to T9SS system functionality, which is overinterpreted based on incomplete data.*

Response: We thank the reviewer for this important comment. We agree that the original manuscript overstated the role of the PorX/PorY two-component system in the response to arginine and its relationship to T9SS functionality. In response, we have substantially revised both the Results and the Discussion to make the mechanistic interpretation more precise and better aligned with the scope of the data.

In the revised Results section, we no longer state that arginine directly activates the PorX/PorY system. Instead, we now present the data more cautiously to show that arginine treatment was associated with increased *porX/porY* expression, elevated T9SS-associated transcription, and enhanced Kgp activity, and that these responses required the PorX/PorY regulatory system, with PorX acting as a central regulator and PorY contributing to the arginine-associated response. We also state that direct biochemical evidence for arginine-dependent activation of PorY or PorY-mediated phosphorylation of PorX was not obtained in our study. In addition, the revised glutamate metabolism section now incorporates the new α -ketoglutarate rescue experiment, the PBS starvation experiment, and the Δkgp analysis. Together, these additional data suggest that the PorX/PorY–T9SS response may be linked to intracellular metabolic imbalance associated with glutamate/ α -ketoglutarate perturbation, rather than being explained solely by a direct effect of arginine or ammonia itself (**Results, Lines 233–236, 246–249, 281–293**).

We have also revised the Discussion accordingly (**Discussion, Lines 381–392**).

The revised text now places the PorX/PorY–T9SS response in the broader context of nutrient limitation and metabolic stress, rather than describing PorX/PorY as a direct arginine-responsive signaling system. We further state explicitly that, although our genetic data support the involvement of the PorX/PorY system under these conditions, direct biochemical evidence for arginine-dependent activation of PorY or PorY-mediated phosphorylation of PorX was not obtained in this study. We believe these revisions substantially reduce the previous overinterpretation and more accurately reflect the strength of the evidence presented.

Selected specific comments:

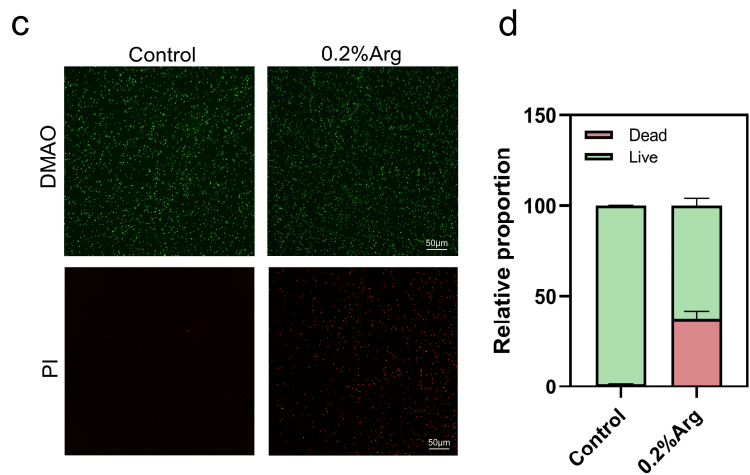
***Comment:** 1) Many figures, including 1d, 3a-i, Extended Data Fig. 1a,b, and Extended Data Fig. 3, are unreadable even after zooming in; please enlarge the panels. Because of this, it was difficult to analyze them.*

Response: We thank the reviewer for this helpful comment and apologize for the poor readability of several figures in the original submission. In response, we have revised all affected figures, including Fig. 1d, Fig. 3a–i, Extended Data Fig. 1a,b, and Extended Data Fig. 3, by enlarging the panels, increasing image resolution, and optimizing font size, labeling, and overall layout. These revised figures are now included **in the updated manuscript**.

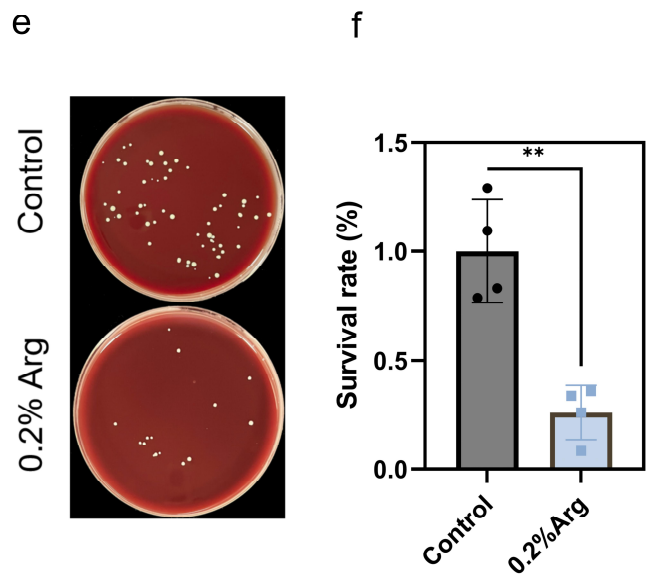
***Comment:** 2) It is mentioned in line 319 that arginine formulation may target bacterial viability. As an initial experiment, the survival of Pg at different arginine concentrations should be assessed. The decrease in various parameters may not result from intracellular changes in Pg but rather from arginine-induced cell death.*

Response: We thank the reviewer for this important suggestion. We agree that it is necessary to determine whether the observed effects of arginine reflect intracellular metabolic perturbation or are secondary to arginine-induced cell death. In response, we performed two additional viability assays for *P. gingivalis* under arginine treatment: live/dead staining (**Extended Data Fig. 2c, d**) and colony-forming unit (CFU) enumeration (**Extended Data Fig. 2e, f**). These experiments showed that 0.2%

arginine significantly reduced, but did not completely abolish, *P. gingivalis* viability under the conditions used for the subsequent mechanistic analyses. These new data have been incorporated into the revised manuscript, and the **Results (Lines 156–158)** and **Methods (Lines 530–541)** section has been updated accordingly.



Extended Data Fig. 2 | c, Representative live/dead staining images showing the effect of arginine treatment on *P. gingivalis*. **d**, Quantification of live/dead staining of *P. gingivalis* following arginine treatment. Stacked bar plots show the relative proportions of live and dead bacteria under the indicated arginine concentrations, with red indicating the percentage of dead bacteria among total bacteria (n=5).



Extended Data Fig. 2 | e, *P. gingivalis* colonies after arginine treatments. **f**, Relevant viability of *P. gingivalis* (n=4) after arginine treatments. The data are presented as the mean±s.d. Only selected comparisons are shown, **p<0.01.

Comment: 3) Line 120: Is the number of individuals sufficient to draw such conclusions with statistical significance?

Response: We thank the reviewer for this important comment. We agree that the original subgingival validation cohort (5 periodontitis vs. 2 healthy individuals) was too small to support robust statistical inference. In response, we removed this analysis from the manuscript and replaced it with a new integrative analysis of five publicly available subgingival plaque metagenomic datasets.

The revised analysis comprises 102 samples in total, including 44 periodontally healthy individuals and 58 patients with chronic periodontitis, from two cohorts from China (PRJNA230363 and PRJNA932553), two from the United States (PRJNA255922 and PRJNA508385), and one from Japan (PRJDB11203) (**Fig. 1a**). In this expanded cohort, both *P. gingivalis* and *F. alocis* were significantly enriched in periodontitis compared with healthy controls (*P. gingivalis*: RH = 0.01057, RP = 0.1171, $p < 0.0001$; *F. alocis*: RH = 0.002129, RP = 0.008562, $p < 0.0001$). In addition, correlation analysis showed that *F. alocis* remained one of the taxa most strongly associated with *P. gingivalis* in the subgingival microbiome (Spearman's $r = 0.7636$, $p < 0.0001$). These revised results are now presented in **Fig. 1** and the corresponding **Results (108–134)** and **Methods (457–483)** sections, and provide a substantially more robust basis for the ecological association described in the revised manuscript.

Comment: 4) Line 180: Why were these specific peptides selected from the many possible sequences? What justifies the assumption that they originate from the breakdown of proteins taken up from the environment? They could equally come from, for instance, the degradation of intracellular proteins by quality control systems. In this context, lines 185–188 appear to overinterpret the data.

Response: We thank the reviewer for this insightful comment. We agree that the origin of the intracellular peptides cannot be unequivocally determined from the metabolomic data alone. Regarding peptide selection, these peptides were not chosen arbitrarily. We first classified all significantly altered metabolites and found that

amino acids and their derivatives, particularly small peptides, represented the major class affected by arginine treatment (**Fig. 3a, b and Extended Data Fig. 3d, e**). The peptides highlighted in the Results section were selected as representative examples because they showed the most pronounced abundance changes within this category. Specifically, Tyr–Asn–Asp, Tyr–Glu–Val–Lys, and Asp–Gly–Asp–Lys–Asn were the top three most increased peptides following arginine treatment, whereas Asp–Asn–Gly–Asn–Phe, Tyr–Pro–Tyr, and Lys–Glu–Asp were among the most altered peptides in the *F. alocis* coculture condition.

We originally interpreted these peptide changes in the context of the well-established proteolytic physiology of *P. gingivalis*, which relies on gingipain-dependent protein degradation for nutrient acquisition. However, as the reviewer correctly points out, the intracellular peptides detected here could also arise from intracellular protein turnover or quality-control processes. We have therefore revised the corresponding text to avoid overinterpretation. The original statement, “The increased abundance of intracellular small peptides in *P. gingivalis* indicated that arginine disturbed amino acid metabolism and activated the predation system through the Kgp/Rgp protease system, which promoted environmental protein degradation and nutrient acquisition,” has been revised to the following more cautious wording: “The increased abundance of intracellular small peptides in *P. gingivalis* suggests that arginine perturbs amino acid metabolism and may enhance proteolytic activity mediated by the Kgp/Rgp system, which could facilitate protein degradation and nutrient acquisition” (**Results Lines 208–211**). This revision is intended to reflect association rather than direct proof of peptide origin.

Comment: 5) Lines 193-199: Due to the poor readability of the plot, the reader may be unable to evaluate the conclusions that have been drawn from it.

Response: Thank you for this helpful comment. We apologize for the poor readability of the original plot. All figures have now been revised to improve its clarity, including increasing the resolution and optimizing the labeling and formatting. The updated version is presented **in the revised manuscript**.

Comment: 6) Lines 208-211: Deleting *PorX* might have multiple effects. Literature shows that removing *PorX* causes downregulation of T9SS genes and reduces substrate secretion. Since the untreated $\Delta porX$ strain shows no hemagglutination, adding any compound to the medium probably won't change this outcome. Therefore, Fig. 3j is not supported by the data presented.

Response: We thank the reviewer for this important comment. We agree that deletion of *porX* is expected to have pleiotropic effects, including reduced T9SS gene expression and impaired substrate secretion. We also agree that hemagglutination in the untreated $\Delta porX$ mutant is already markedly reduced and close to background levels, which limits the dynamic range of this assay for assessing additional arginine responsiveness in the $\Delta porX$ background. We have therefore revised the manuscript to interpret this result more cautiously, taking into account the markedly reduced hemagglutination signal in the $\Delta porX$ background. Importantly, although Kgp activity was strongly reduced in the $\Delta porX$ mutant, a low basal level of activity remained detectable. In contrast to the wild-type strain, arginine failed to enhance Kgp activity in the $\Delta porX$ mutant. In addition, as part of the revision, we performed new transcriptional analyses to further assess the arginine response in the WT, $\Delta porX$, and $\Delta porY$ backgrounds (**Fig. 3n**). These newly added data showed that arginine increased the expression of T9SS-associated genes in the wild-type strain, whereas this induction was markedly reduced in $\Delta porX$ and partially attenuated in $\Delta porY$. Together, these data support the conclusion that the arginine-associated T9SS/Kgp response requires the PorX/PorY regulatory system, with PorX playing a more prominent role.

Accordingly, we have revised the **Results (Lines 224–236)** section to make this interpretation more cautious. In particular, we now state that the data support the involvement of PorX/PorY in the T9SS-associated response of *P. gingivalis* to arginine, rather than using the $\Delta porX$ hemagglutination phenotype alone to infer a stronger mechanistic conclusion.

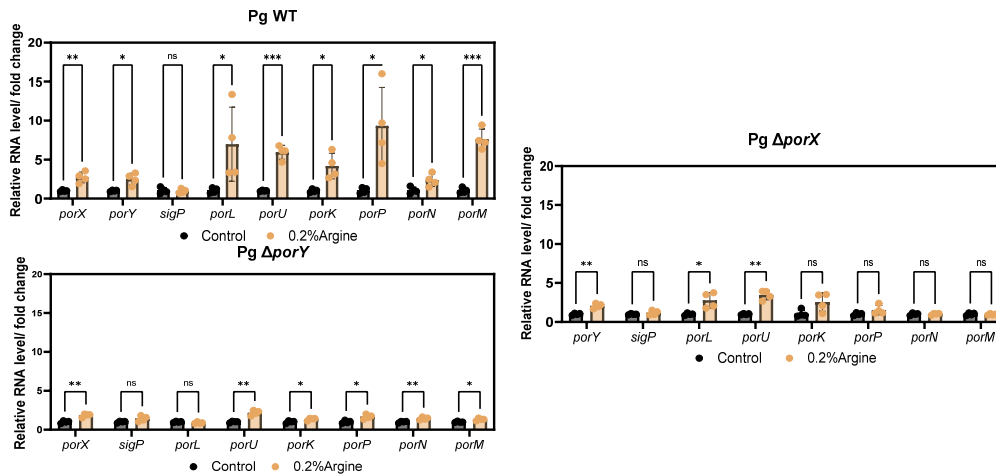


Fig. 3 | n, Effect of arginine on T9SS transcriptional responses in Pg WT, $\Delta porX$, and $\Delta porY$ strains (n=4; unpaired multiple t-tests). The data are presented as the mean $\pm$ s.d. Only selected comparisons are shown, ns, not significant, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

Comment: The *PorY* mutant behaves almost exactly like the wild type, so claiming that *PorY* is activated by arginine is not justified. Likewise, concluding that activated *PorY*, in turn, activates *PorX*, is unjustified. To support such claims, an experiment using radioisotope-labeled ATP should be performed to demonstrate that recombinant *PorY*, in the presence of arginine, activates (phosphorylates) recombinant *PorX*.

Response: We thank the reviewer for this important comment. We agree that the phenotype of the $\Delta porY$ mutant does not justify the conclusion that arginine directly activates *PorY*, nor does it support the claim that activated *PorY*, in turn, activates *PorX*. We also agree that direct demonstration of *PorY*-dependent phosphorylation of *PorX* would require dedicated biochemical assays, such as the radioisotope-labeled ATP experiment suggested by the reviewer.

In the revised manuscript, we have therefore removed statements implying direct activation of *PorY* by arginine or direct *PorY*-to-*PorX* signaling. Instead, we now interpret our data more cautiously. Although the $\Delta porY$ mutant retained partial basal Kgp activity, arginine failed to enhance Kgp activity in this mutant, and the arginine-induced upregulation of T9SS-related genes was attenuated. These findings are consistent with a contribution of *PorY* to the arginine-associated regulatory

response, but do not establish the underlying biochemical mechanism. Accordingly, we now limit our conclusion to the involvement of the PorX/PorY regulatory system in the arginine-associated response of *P. gingivalis*, while direct signaling between PorY and PorX remains unresolved. We have substantially revised the corresponding description in the **Results (Lines 233–236, 246–249, 281–293)** section and have also added a **Discussion (Lines 381–392)** paragraph to clarify this point in the revised manuscript.

***Comment:** 7) Lines 253–257: For the reasons outlined above, this constitutes a significant overinterpretation of the data.*

Response: We thank the reviewer for this important comment. We agree that the original wording in Lines 253–257 was overstated. In response, we revised this part of the Results section to adopt a more cautious interpretation. The original sentence has now been replaced with: “These results suggested that arginine perturbed glutamate metabolism in *P. gingivalis*, leading to metabolic imbalance associated with depletion of key intermediates such as α -ketoglutarate. This metabolic disturbance was accompanied by changes in the PorX/PorY-regulated T9SS and increased Kgp activity” (**Results Lines 281–284**). This change was made to avoid overinterpretation and to better reflect the evidence presented.

Response to Reviewers

Manuscript title: Arginine Metabolism as a Mutualistic Strategy in the Interaction between *Porphyromonas gingivalis* and *Filifactor alocis*

Manuscript ID: NCOMMS-25-85197B

Reviewer #1

We sincerely thank Reviewer #1 for their time and consideration of our manuscript. No additional comments were provided in this round of review.

Reviewer #2 (Remarks to the Author):

Comment: Thank you for revising the paper.

*You have successfully addressed the previous concerns by conducting the necessary additional experiments and making appropriate revisions throughout the manuscript. Although the revised study does not fully elucidate the entire mutualistic relationship between *P. gingivalis* and *F. alocis* (given that their interaction may also be influenced by other microbial species and the surrounding microenvironment) the authors have convincingly demonstrated the high probability that these two bacteria maintain a unique, cooperative relationship. The presented data offer significant insights that are highly valuable for understanding the pathogenicity associated with periodontitis.*

Response: We sincerely thank Reviewer #2 for the positive assessment of our revised manuscript and for acknowledging the significance of our findings. We also appreciate the reviewer's constructive comments throughout the review process, which have helped us substantially improve the rigor and clarity of the study. We agree that the interaction between *P. gingivalis* and *F. alocis* may be further influenced by other microbial species and the surrounding microenvironment, and we consider this an important direction for future investigation.

Reviewer #3 (Remarks to the Author):

Comment: I would like to thank the authors for responding constructively to all my comments and critiques, which required conducting several experiments. The revised

data fully addressed my critique and made the manuscript acceptable for publication.

Response: We sincerely thank Reviewer #3 for the positive assessment of our revised manuscript. We greatly appreciate the reviewer's thoughtful comments and constructive critiques throughout the review process, which guided us in performing the additional experiments and substantially improved the rigor and completeness of the study. We are grateful that the revised data have fully addressed the reviewer's concerns.