

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

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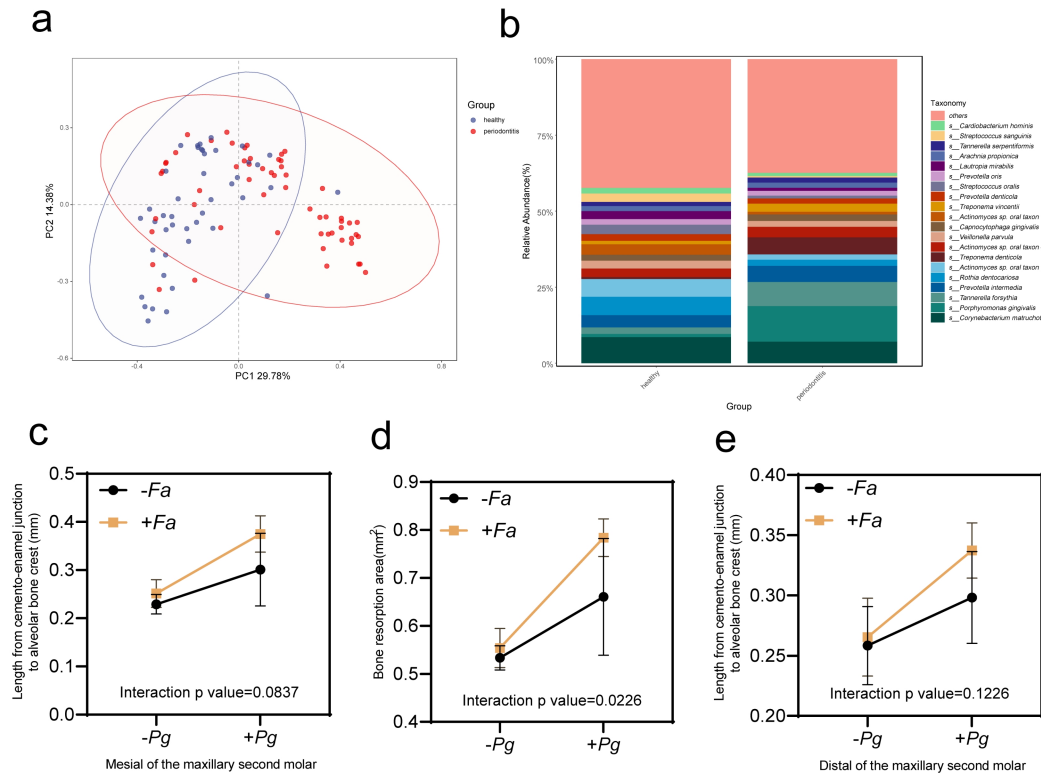
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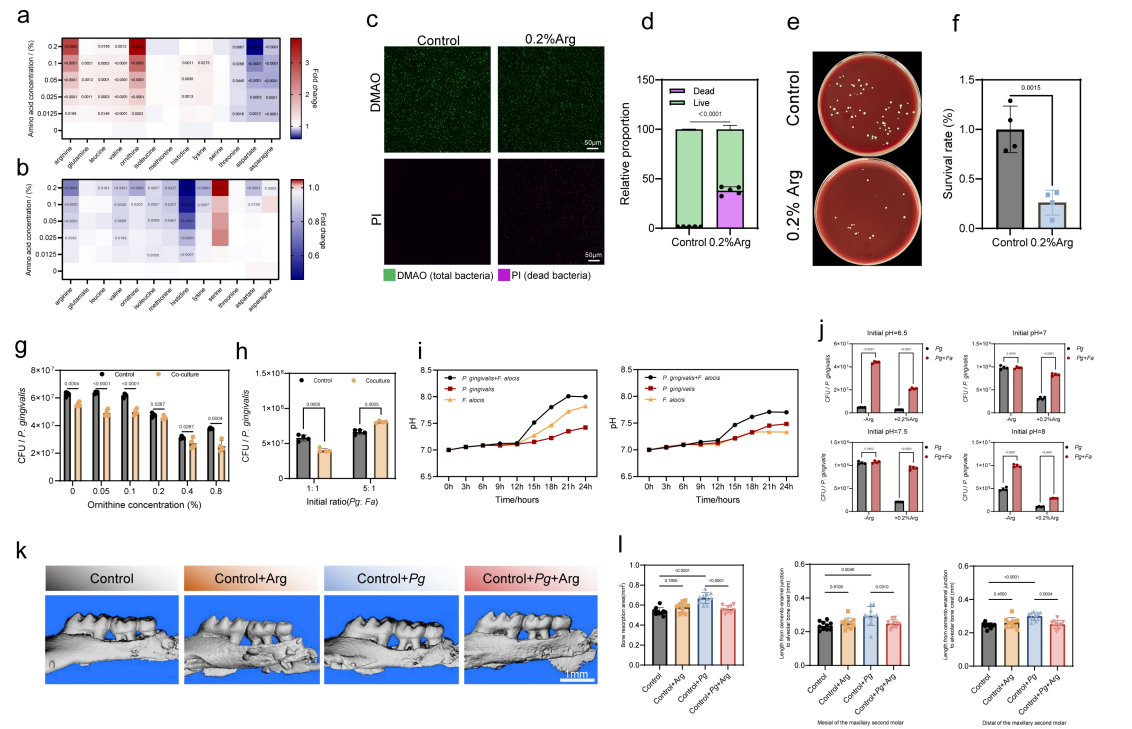
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Supplementary materials



Supplementary Fig. 1 | Subgingival microbial community differences and the combined effects of *P. gingivalis* and *F. alocis* on alveolar bone loss. **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. **c–e**, Interaction plots illustrating the combined effects of *Pg* and *Fa* on alveolar bone resorption area (**c**), mesial CEJ–ABC distance (**d**), and distal CEJ–ABC distance (**e**) in the murine ligature-induced periodontitis model (n=10 mice, ordinary two-way ANOVA). Interaction *p* values are shown at the bottom of each panel. Data in **c**, **d** and **e** are mean $\pm$ s.d. (error bars, s.d.). Source data are provided as a Source Data file. *Pg*, *P. gingivalis*; *Fa*, *F. alocis*.

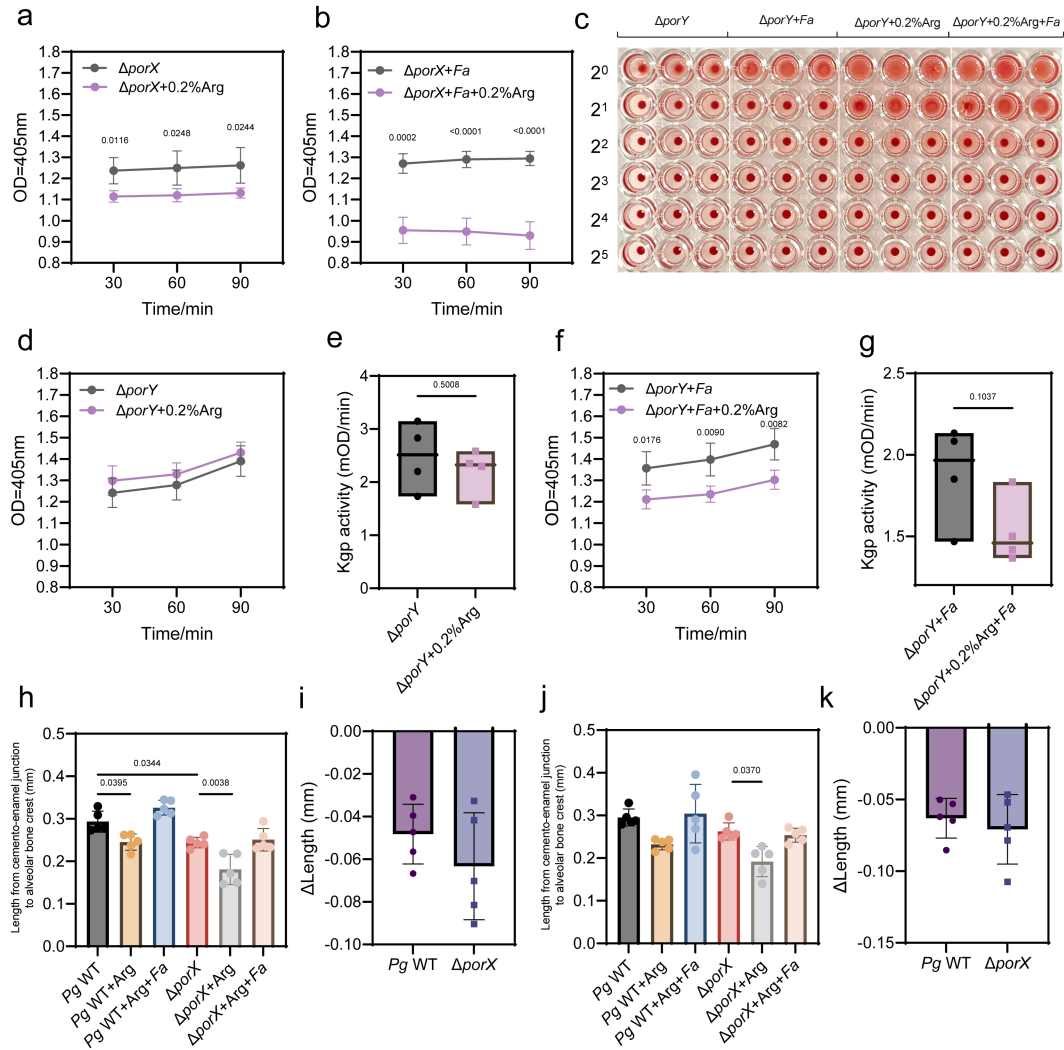


Supplementary Fig. 2 | Amino acid-dependent growth responses and the effects of arginine and *F. alocis* on *P. gingivalis* viability, growth and alveolar bone loss.

Effects of 13 common amino acids on *Pg* (a) and *Fa* (b) growth (n=4; one-way ANOVA with Dunnett's multiple-comparisons test). Each heat-map square shows the OD₆₀₀ fold change versus untreated control at each concentration. Red and blue indicate increased and decreased growth, respectively; color intensity reflects effect magnitude. Exact *p* values are shown within the corresponding squares for comparisons with *p*<0.05. c,d, Representative live/dead staining images (c) and quantification (d) of *Pg* following arginine treatment. Stacked bars show the proportions of live and dead bacteria, with dead bacteria shown in red (n=5; one field quantified per culture; two-sided unpaired *t*-test). e,f, Representative colony images (e) and relative viability (f) of *Pg* following arginine treatment (n=4; one plate quantified per culture; two-sided unpaired *t*-test). g, Effect of 0–0.8% (w/v) ornithine on *Pg* growth in *Pg*-*Fa* coculture (n=4; multiple two-sided unpaired *t*-tests). h, Effect of *Fa* on the growth of *Pg* in 0.2% arginine. *Pg* and *Fa* are cocultured for 24 h at initial *Pg*:*Fa* ratios of 1:1 and 5:1 (n=4; multiple two-sided unpaired *t*-tests). i, Dynamic changes in pH over 24 h in *Pg* monoculture, *Fa* monoculture, and coculture systems with (left) or without (right) the treatment of 0.2% arginine (n=3). j, Effects of 0.2% arginine and *Fa* on the growth of *Pg* under different pH conditions (pH=6.5, 7.0, 7.5, 8.0) (n=4; multiple two-sided unpaired *t*-tests). k, Representative 3D-reconstructed micro-CT images of maxillae (buccal view) from the Control, Control+Arg, Control+Pg and Control+Pg+Arg groups (n=5 mice). l, Bone loss area (left) and CEJ

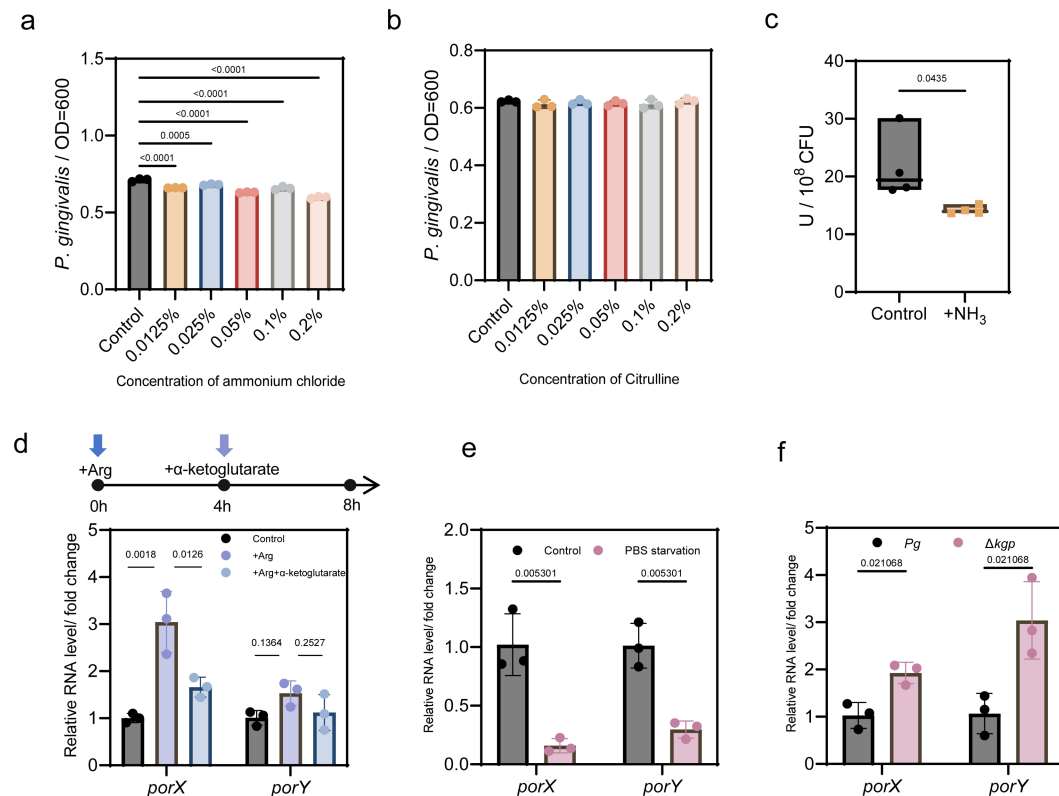
to-alveolar crest distance were mesiodistally (middle and right) measured across experimental groups (n=5 mice; one-way ANOVA with Tukey's multiple comparison test). Unless stated, n denotes biologically independent cultures. Data in **d, f, g, h, i, j** and **l** are mean $\pm$ s.d. (error bars, s.d.). Only selected pairwise comparisons are shown; exact *p* values are above comparison bars. Source data are provided as a Source Data file. *Pg*, *P. gingivalis*; *Fa*, *F. alocis*; Arg, Arginine.

correction.). **e**, Bubble plot of differentially abundant amino acids/metabolites and glycerophospholipids (*Pg*+0.2%Arg vs. *Pg*+0.2%Arg+*Fa*; $p<0.05$, VIP>1, multiple two-sided unpaired *t*-tests with FDR correction.). **f**, Effect of arginine on the RNA transcript levels of channel proteins associated with amino acid and oligopeptide transport systems in *Pg*. RA, dimeric RagA₂B₂ complex RagA transporter; RB, dimeric RagA₂B₂ complex RagB cap; ST, serine/threonine transporter SstT; OPT, oligopeptide transporter; POT, proton-dependent oligopeptide transporter (n=4; multiple two-sided unpaired *t*-tests). **g**, Effects of arginine and *Fa* on hemagglutination function. The experiment was independently repeated twice with similar results. **h,i**, Time-resolved Kgp activity in the *Pg* WT strain under four conditions: *Pg* alone, *Pg*+0.2%Arg, *Pg*+*Fa*, and *Pg*+0.2%Arg+*Fa* (n=3; multiple two-sided unpaired *t*-tests). Time-resolved Rgp activity (**j,l**) and the initial reaction velocity (V_0) of Rgp activity (**k,m**) in the *Pg* WT strain under four conditions: *Pg* alone, *Pg*+0.2% Arg, *Pg*+*Fa*, and *Pg*+0.2% Arg+*Fa*. V_0 was calculated from the linear portion of the absorbance–time curve and is expressed as mOD/min (n=3; **j, l**: multiple two-sided unpaired *t*-tests; **k, m**: two-sided unpaired *t*-test). Unless stated, n denotes biologically independent cultures. Data in **f, h, i, j** and **l** are mean $\pm$ s.d. (error bars, s.d.). Data in **k** and **m** are floating min-to-max plots (center lines, medians; limits, minima and maxima). Only selected pairwise comparisons are shown; exact *p* values are above comparison bars. Source data are provided as a Source Data file. *Pg*, *P. gingivalis*; *Fa*, *F. alocis*; Arg, Arginine.

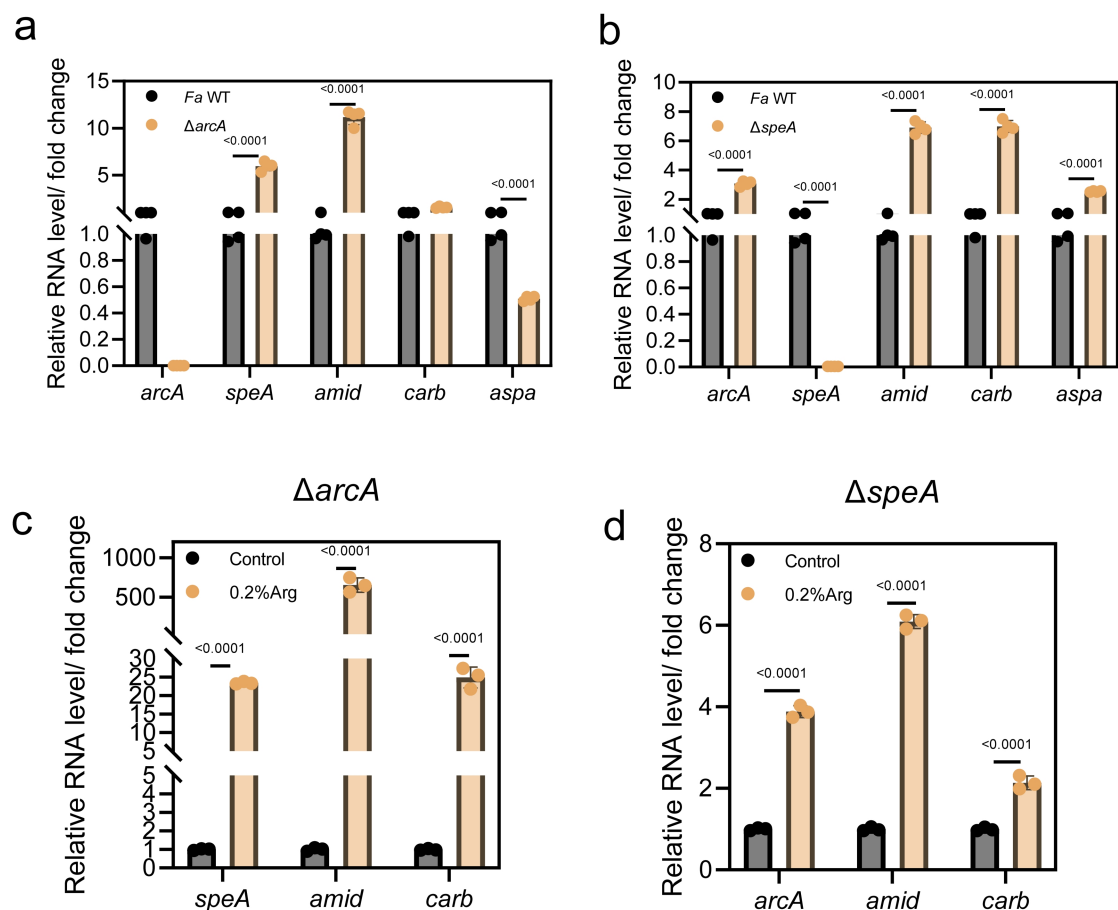


Supplementary Fig. 4 | PorX and PorY modulate arginine- and *F. alocis*-dependent T9SS functions, and PorX contributes to alveolar bone loss. **a,b**, Time-resolved Kgp activity in the $\Delta porX$ strain under four conditions: $\Delta porX$ alone, $\Delta porX+0.2\%$ Arg, $\Delta porX+Fa$, and $\Delta porX+0.2\%$ Arg+Fa (n=4; multiple two-sided unpaired *t*-tests). **c**, Effects of arginine and Fa on hemagglutination function in the $\Delta porY$ strain. The experiment was independently repeated twice with similar results. Time-resolved Kgp activity (**d,f**) and the initial reaction velocity (V_0) of Kgp activity (**e,g**) in the $\Delta porY$ strain under four conditions: $\Delta porY$ alone, $\Delta porY+0.2\%$ Arg, $\Delta porY+Fa$, and $\Delta porY+0.2\%$ Arg+Fa. V_0 was calculated from the linear portion of the absorbance–time curve and is expressed as mOD/min (n=4; **d, f**: multiple two-sided unpaired *t*-tests; **e, g**: two-sided unpaired *t*-test). **h,i,j,k**, Effects of arginine and Fa on alveolar bone destruction induced by Pg WT and $\Delta porX$ infection. CEJ-to-alveolar crest distance measurements at mesial (**h**) and distal (**j**) sites across experimental groups (n=5 mice; one-way ANOVA with Tukey's multiple comparison test). Measurement of arginine-attenuated CEJ-to-alveolar crest distance at mesial (**i**) and

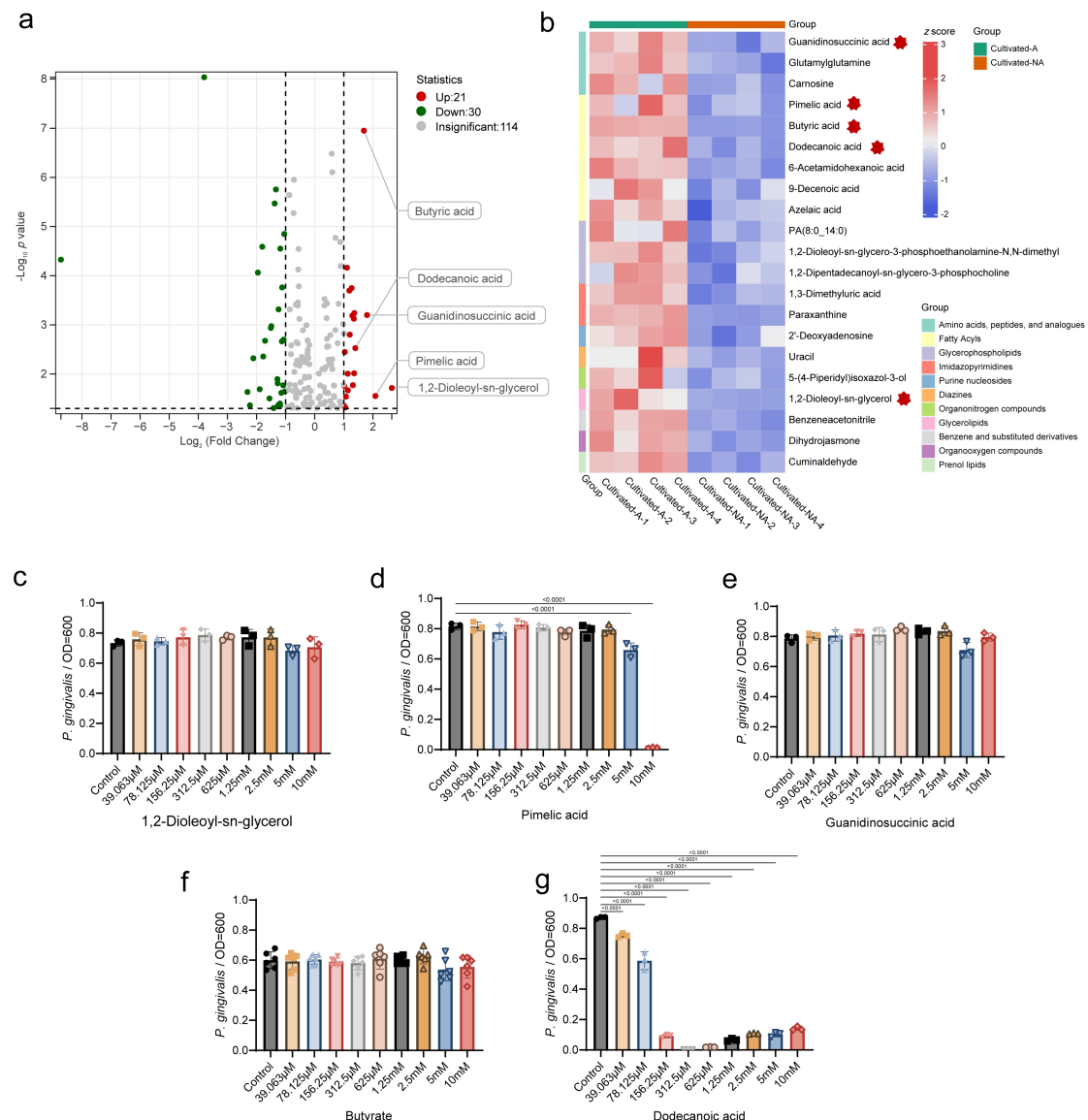
distal (**k**) sites in *Pg* WT and $\Delta porX$ infections (n=5 mice, two-sided unpaired *t*-test). Unless stated, n denotes biologically independent cultures. Data in **a**, **b**, **d**, **f**, **h**, **i**, **j** and **k** are mean $\pm$ s.d. (error bars, s.d.). Data in **e** and **g** are floating min-to-max plots (center lines, medians; limits, minima and maxima). Only selected pairwise comparisons are shown; exact *p* values are above comparison bars. Source data are provided as a Source Data file. *Pg*, *P. gingivalis*; *Fa*, *F. alocis*; Arg, Arginine; WT, wild type; $\Delta porX$, *porX* deletion mutant; $\Delta porY$, *porY* deletion mutant.



Supplementary Fig. 5 | Nitrogen metabolic perturbations and restricted nutrient acquisition affect *P. gingivalis* growth and PorX/PorY transcription. **a**, Effects of ammonium chloride on *Pg* growth (n=3, one-way ANOVA with Dunnett's multiple-comparisons test). **b**, Effects of citrulline on *Pg* growth (n=3, one-way ANOVA with Dunnett's multiple-comparisons test). **c**, Effects of ammonium chloride on the activity of glutamate dehydrogenase in *Pg* (n=4, two-sided unpaired *t*-test). **d**, Effect of α-ketoglutarate supplementation on *porX* and *porY* transcription in *Pg* (n=3, one-way ANOVA with Tukey's multiple comparison test). **e**, Effect of PBS starvation on *porX* and *porY* transcription in *Pg* (n=3, two-sided unpaired *t*-test). **f**, Changes in *porX* and *porY* transcription in the protein acquisition-restricted mutant Δkgp compared with the *Pg* WT (n=3, two-sided unpaired *t*-test). Unless stated, n denotes biologically independent cultures. Data in **a**, **b**, **d**, **e** and **f** are mean ± s.d. (error bars, s.d.). Data in **c** is shown as floating min-to-max plots, with the center line indicating the median and the lower and upper limits representing the minimum and maximum values, respectively. Only selected pairwise comparisons are shown; exact *p* values are above comparison bars. Source data are provided as a Source Data file. *Pg*, *P. gingivalis*; Arg, Arginine; $\Delta porX$, *porX* deletion mutant; $\Delta porY$, *porY* deletion mutant; Δkgp , *kgp* deletion mutant.

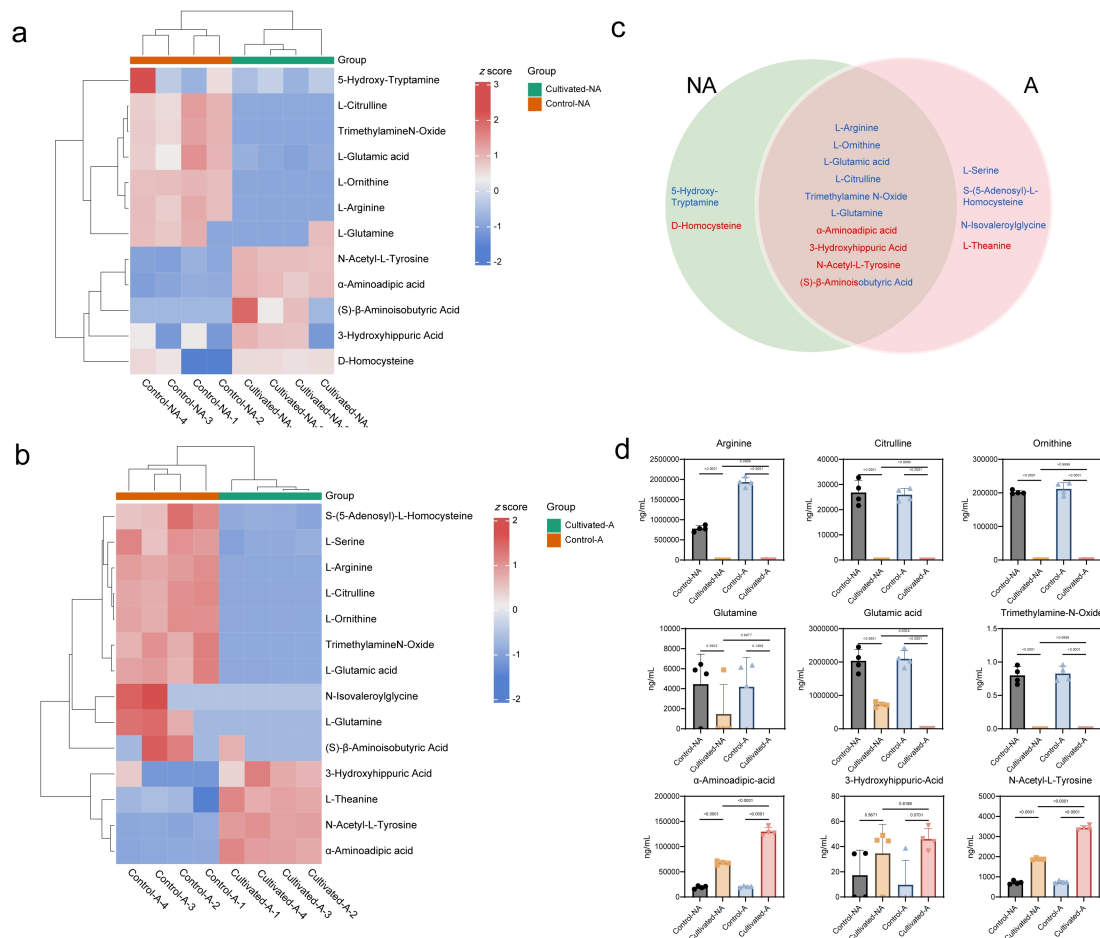


Supplementary Fig. 6 | Deletion of *arcA* or *speA* alters basal and arginine-responsive transcription of arginine metabolism genes in *F. alocis*. Changes in the transcript levels of arginine metabolism-related genes in the *ΔarcA* (a) and *ΔspeA* (b) mutants compared with those in the *Fa* wild type (n=4; multiple two-sided unpaired *t*-tests). Effect of 0.2% arginine on the mRNA expression of arginine metabolism-related genes in the *ΔarcA* (c) and *ΔspeA* (d) mutants (n=3; multiple two-sided unpaired *t*-tests). Unless stated, n denotes biologically independent cultures. Data in a, b, c and d are mean ± s.d. (error bars, s.d.). Only selected pairwise comparisons are shown; exact *p* values are above comparison bars. Source data are provided as a Source Data file. Arg, Arginine; WT, wild type; *ΔarcA*, *arcA* deletion mutant; *ΔspeA*, *speA* deletion mutant. *Fa*, *F. alocis*.

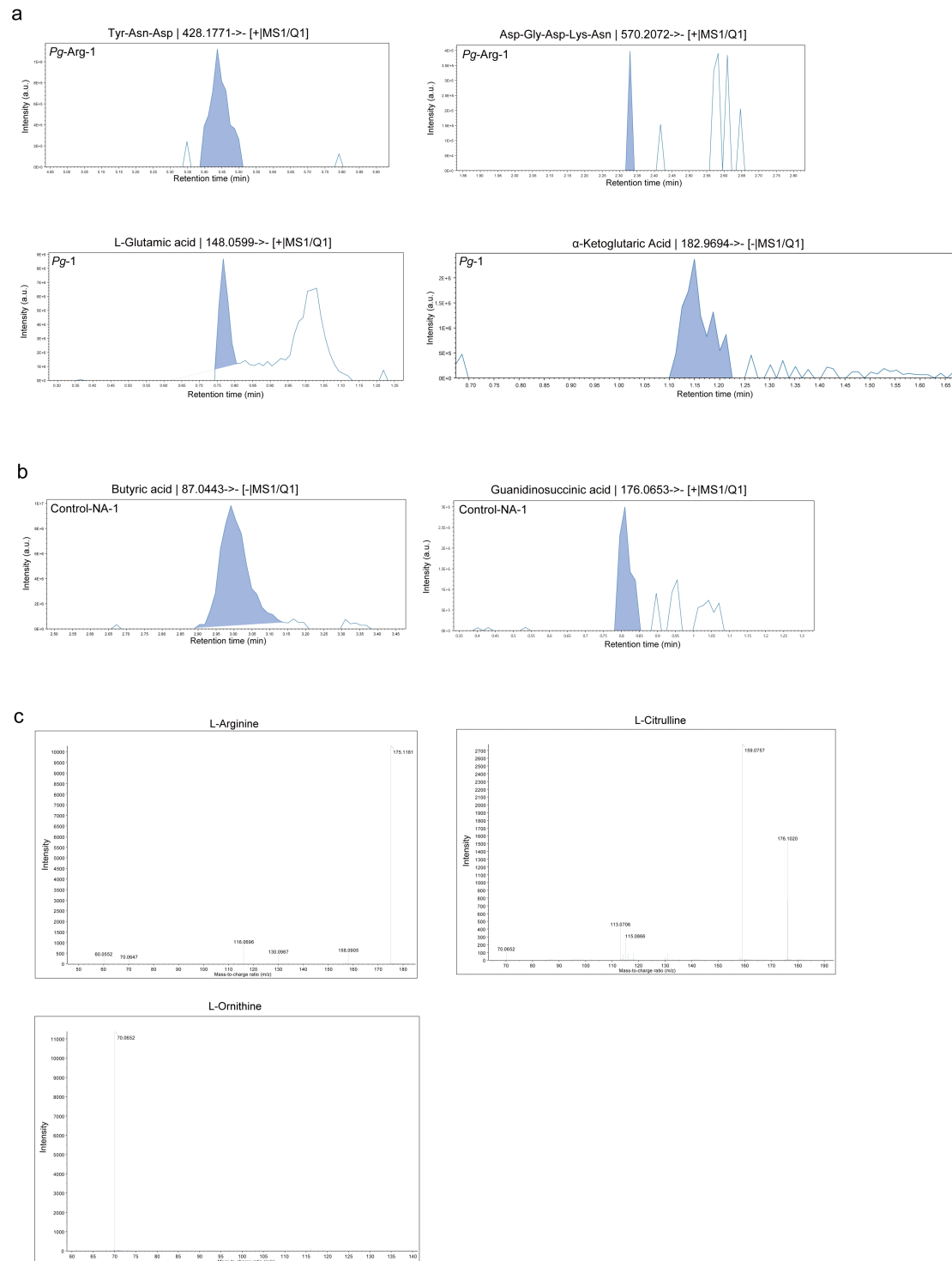


Supplementary Fig. 7 | Arginine alters the extracellular metabolite profile of *F. alocis*, and selected metabolites modulate *P. gingivalis* growth. **a**, Volcano plot of differentially abundant metabolites in the culture supernatants of *F. alocis* grown in medium with or without 0.2% arginine (n=4). **b**, Top 20 differentially abundant metabolites in the culture supernatants, ranked by fold change, between the *Fa* and *Fa*+0.2% arginine groups (n=4). 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 *Pg* (n=3/6, one-way ANOVA with Dunnett's multiple-comparisons test). Unless stated, n denotes biologically independent cultures. Data in **c**, **d**, **e**, **f** and **g** are mean $\pm$ s.d. (error bars, s.d.). Only selected pairwise comparisons are shown; exact *p* values are above comparison bars. Source data are provided as a Source Data file. A, Arginine; NA, No Arginine. *Pg*, *P.*

gingivalis.



Supplementary Fig. 8 | *F. alocis* remodels amino acid-related metabolites in the culture medium in an arginine-dependent manner. 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 (n=4, one-way ANOVA with Tukey's multiple comparison test). Unless stated, n denotes biologically independent cultures. Data in **d** is presented as the mean $\pm$ s.d, error bars represent s.d. Only selected pairwise comparisons are shown; exact *p* values are above comparison bars. Source data are provided as a Source Data file. A, Arginine; NA, No Arginine.



Supplementary Fig. 9 | Representative chromatographic and mass spectrometric profiles of key metabolites from untargeted and targeted metabolomics analyses.

a, Representative extracted ion chromatograms (XICs) of Tyr–Asn–Asp, Asp–Gly–Asp–Lys–Asn, L-glutamic acid and α -ketoglutarate from the intracellular untargeted metabolomics analysis of *P. gingivalis*. The representative sample identifier is shown in the upper-left corner of each plot. **b**, Representative XICs of butyric acid and guanidinosuccinic acid from the untargeted metabolomics analysis of

F. alocis culture supernatants. The representative sample identifier is shown in the upper-left corner of each plot. **c**, Representative tandem mass spectrometry (MS/MS) spectra of L-arginine, L-citrulline and L-ornithine from the targeted amino acid metabolomics analysis of *F. alocis* culture supernatants. Positive- or negative-ion acquisition modes are indicated in the individual plots. *Pg*, *P. gingivalis*; *Fa*, *F. alocis*; Arg, arginine; NA, no arginine.

Supplementary Table 1-Strains used in this study.

Strain	Description	Reference
<i>P. gingivalis</i> W83	Strain obtained from the ATCC	-
<i>F. alocis</i> ATCC35896	Strain obtained from the ATCC	-
<i>F. alocis</i> Δ <i>arcA</i>	Isogenic <i>arcA</i> deletion in <i>F. alocis</i>	This study
<i>F. alocis</i> Δ <i>speA</i>	Isogenic <i>speA</i> deletion in <i>F. alocis</i>	This study
<i>P. gingivalis</i> Δ <i>porX</i>	Isogenic <i>porX</i> deletion in <i>P. gingivalis</i>	This study
<i>P. gingivalis</i> Δ <i>porY</i>	Isogenic <i>porY</i> deletion in <i>P. gingivalis</i>	This study
<i>P. gingivalis</i> Δ <i>kgp</i>	Isogenic <i>kgp</i> deletion in <i>P. gingivalis</i>	This study

Supplementary Table 2-Culture mediums used in this study.

Culture medium	Description
BHI broth	Brain heart infusion
BHIV broth	Brain heart infusion, 5 µg/ml hemin and 1 µg/ml menadione
BB broth	Brain heart infusion, 5 µg/ml hemin, 1 µg/ml menadione and 0.1% cysteine
BF broth	Brain heart infusion, 5 µg/ml hemin, 1 µg/ml menadione 0.1% cysteine and 0.1% arginine
Blood agar plate	Brain heart infusion, 1.5% agar, 5% sheep blood, 5 µg/ml hemin and 1 µg/ml menadione
BF plate	Brain heart infusion, 1.5% agar, 5% sheep blood, 5 µg/ml hemin, 1 µg/ml menadione, 0.1% cysteine and 0.1% arginine

Supplementary Table 3-Primers used in this study.

Primer	Sequence (5' – 3')
Mutagenesis (<i>P. gingivalis</i>)	
<i>kgp</i> -Up-F	CCGGTAAGACTGTAAGTCAG
<i>kgp</i> -Up-R	GCAATAGCGGAAGCTATCGGACTTTAAAACAATTTATGGT
<i>kgp</i> -Down-F	GCAAATTGGCGATGGAGCGGAAACGGCAAGTCTTACGTAGAGAA
<i>kgp</i> -Down-R	AGAATGTTTTGAGTATGGGT
<i>porX</i> -Up-F	AATCGTCGTCCTCCTCAACT
<i>porX</i> -Up-R	GCAATAGCGGAAGCTATCGGAATTATGTTCTTCTCTATTT
<i>porX</i> -Down-F	GCAAATTGGCGATGGAGCGGAAACATACTTCATACATGAATACG
<i>porX</i> -Down-R	TCTTGCCACTGAACATGGAG
<i>porY</i> -Up-F	CAGACGACAGAGAAGACAAT
<i>porY</i> -Up-R	GCAATAGCGGAAGCTATCGGACCCTGTAGTTTTTCGCTTTT
<i>porY</i> -Down-F	GCAAATTGGCGATGGAGCGGAAACGTATACGAACGAAATATGGA
<i>porY</i> -Down-R	GATACCATAACCCCTCATCGT
<i>Pg</i> -ermF-F	CCGATAGCTTCCGCTATTGC
<i>Pg</i> -ermF-R	GTTTCCGCTCCATCGCCAATTTGC
Mutagenesis (<i>F. alocis</i>)	
<i>arcA</i> -Up-F	ACAGGTGTGCGTATCAAGAT

<i>arcA</i> -Up-R	ATTTATTCTCCTAGTTAGTCACACAACCACAATCCTTTCTC
<i>arcA</i> -Down-F	GTAGTACCTGGAGGGAATAATCCAGTCAAACAAGATGTTATG
<i>arcA</i> -Down-R	TATCATCTCATTGATAGCTC
<i>speA</i> -Up-F	TTGGAAGTGCCTTGTGCTCC
<i>speA</i> -Up-R	ATTTATTCTCCTAGTTAGTCACTCTTCGTCTGTTTCATATT
<i>speA</i> -Down-F	GTAGTACCTGGAGGGAATAATCGGTAGAATGAATACAATATG
<i>speA</i> -Down-R	CATCAATCCATCTTTGCCGT
<i>Fa-ermF</i> -F	TGACTAACTAGGAGGAATAAATGACAAAAAAGAAATTGCCCCG
<i>Fa-ermF</i> -R	GATTATTCCCTCCAGGTACTACGAAGGATGAAATTTTCA
qRT-PCR	
<i>Pg16S</i> -F	TGTAGATGACTGATGGTGAAAACC
<i>Pg16S</i> -R	ACGTCATCCACACCTTCCTC
<i>Fa16S</i> -F	ACCCTCAAGTTGCCAAAATTATTAT
<i>Fa16S</i> -R	TACTCCCTTTCTTCTGGTTAAATCT
<i>arcA</i> -F	ACACTTCCACGCCATTGTCT
<i>arcA</i> -R	ACCGGGAAGGGAGATTGAGA
<i>speA</i> -F	CGTCAAGCTGGGAAGTGTCT
<i>speA</i> -R	GCATGGCTCTCACTTGGGAT
<i>amid</i> -F	TTGGTCCTCCCGTTCCTCTA
<i>amid</i> -R	GAAGGGGTGCCGAGAATCAA
<i>carb</i> -F	TGGGCGTGACGGATCTTTTT
<i>carb</i> -R	CCGCTTCCAAGGGTAAACGA
<i>aspa</i> -F	AAGATGCCGGACGTGGATAC
<i>aspa</i> -R	GGATTCCCCCTCCACCTACT
<i>kgp</i> -F	TGGGAGTTGGTCTTTACGCC
<i>kgp</i> -R	TGGTCTCCACCTTTGTCAGC
<i>porX</i> -F	ACGCTACCGCAAGCACTACAG
<i>porX</i> -R	CCACGAAGTTCAGGACTATCACAT
<i>porY</i> -F	TCCAATCCAACAATAGCATCCC
<i>porY</i> -R	GCAATACCAACAGTCGCCGTA
<i>sigP</i> -F	GCGGACAATACATCCCCACC
<i>sigP</i> -R	TCTGGCTCCTTCCCCTCTGA
<i>porL</i> -F	CGCCTTGCCTCTGCCTCTTT
<i>porL</i> -R	CGCCTGCTTCAGCCAGTTTG
<i>porU</i> -F	TGCCGACCTGACCTACAACC
<i>porU</i> -R	GGAATAGAATCACATCAGCCACAT
<i>porK</i> -F	ACGACTTCCGTCTGCCCCACT
<i>porK</i> -R	CCGTCAGCCGTATAATCTCCC
<i>porP</i> -F	ATCTGTCCGTCGCCCTCCAA
<i>porP</i> -R	TCCCATCGTCACGTCCAAAC
<i>porN</i> -F	CCTACGCCCCGATTCCATGA
<i>porN</i> -R	TTTTACCACGAGCCTTTTCCA
<i>porM</i> -F	GCACAAGTGATTCCACAGAGCC

<i>porM</i> -R	CACCTGCCGTAGCCGTAAATAG
<i>ragA</i> -F	AGAGGTCGCCATCGCTAATG
<i>ragA</i> -R	AGTGCTGAGTTTCTGTCCCG
<i>ragB</i> -F	CTGGAGATACCCCAACAGCC
<i>ragB</i> -R	GCGGACCATATCACGCAAAC
<i>sstT</i> -F	ATGGCTACGGTGTGTCAGG
<i>sstT</i> -R	AAGGCTGTACTCCTTGCGAC
<i>opt</i> -F	ACTGGCCGAGAAGTACAAGC
<i>opt</i> -R	AGAAACGAACCGGCACAGAT
<i>pot</i> -F	ACCCCTGCATTACGCTCTTC
<i>pot</i> -R	ATATGGAGCCTTCCACCCCT

Supplementary Table 4-Oligonucleotide probes used in FISH.

Probes	Sequence(5'—3')	Target Gene
<i>P. gingivalis</i>	(Alexa Fluor 568)-CAATACTCGTATCGCCCGTTATTC	16S rRNA
<i>F. alocis</i>	(Alexa Fluor 488)-TCTTTGTCCACTATCGTTTGA	16S rRNA

Supplementary Table 5. Liquid chromatography–tandem mass spectrometry acquisition and data-processing parameters for untargeted metabolomics.

Item	Details (intracellular metabolomics)	Details (culture-supernatant metabolomics)
Ultra-high-performance liquid chromatography (UHPLC) system	Vanquish UHPLC, Thermo Fisher Scientific	Vanquish UHPLC, Thermo Fisher Scientific
Mass spectrometer	Q Exactive HF-X, Thermo Fisher Scientific	Q Exactive HF-X, Thermo Fisher Scientific
Analytical column	Waters ACQUITY Premier HSS T3, 1.8 μ m, 2.1 $\times$ 100 mm	Waters ACQUITY Premier HSS T3, 1.8 μ m, 2.1 $\times$ 100 mm
Mobile phase A	0.1% formic acid in water	0.1% formic acid in water
Mobile phase B	0.1% formic acid in acetonitrile	0.1% formic acid in acetonitrile
Flow rate	0.4 mL/min	0.4 mL/min
Column temperature	40 °C	40 °C
Injection volume	4 μ L	3 μ L
Ionization modes	Separate electrospray ionization (ESI)+ and ESI- runs	Separate ESI+ and ESI- runs

Acquisition mode	Full-scan MS with data-dependent MS/MS acquisition and dynamic exclusion	Full-scan MS with data-dependent MS/MS acquisition and dynamic exclusion
Spray voltage, ESI+/ESI–	3500/3200 V	3800/3400 V
Sheath gas, ESI+/ESI–	30/30 arbitrary units	60/60 arbitrary units
Auxiliary gas, ESI+/ESI–	5/5 arbitrary units	20/20 arbitrary units
Ion-transfer tube temperature	320 °C	320 °C
Vaporizer temperature, ESI+/ESI–	300/300 °C	350/650 °C
MS1 scan range	<i>m/z</i> 75–1000	<i>m/z</i> 70–1000
MS1 resolution	35000	60000
MS1 automatic gain control (AGC) target	1 × 10 ⁶	3 × 10 ⁶
RF-lens setting	50%	50%
MS2 scan range	<i>m/z</i> 75–1000	<i>m/z</i> 70–1000
MS2 resolution	17500	15000
MS2 AGC target	2 × 10 ⁵	1 × 10 ⁵
Stepped collision energy	30, 40 and 50	30, 40 and 50
Precursor intensity threshold	1 × 10 ⁶ cps	1 × 10 ⁶ cps
Number of selected precursors	Top 10	Top 10
Dynamic-exclusion duration	3 s	3 s
Quality-control samples	Pooled quality control (QC) samples prepared by combining sample extracts; one QC sample inserted after every ten study samples	Pooled QC samples prepared by combining sample extracts; one QC sample inserted after every ten study samples
Raw-data conversion	ProteoWizard v3.0.2	ProteoWizard v3.0.2
Peak processing	XCMS v4.8.0 for peak detection, alignment and retention-time correction	XCMS v4.8.0 for peak detection, alignment and retention-time correction
Missing-value filtering	Features with >50% missing values in an experimental group were removed	Features with >50% missing values in an experimental group were removed

Missing-value imputation	K-nearest-neighbour imputation or one-fifth of the minimum detected value, depending on missing-value frequency; R package impute v1.56.0	K-nearest-neighbour imputation or one-fifth of the minimum detected value, depending on missing-value frequency; R package impute v1.56.0
Signal correction	Support vector regression	Support vector regression
Metabolite annotation	In-house database, integrated public databases and predicted databases, supplemented by metDNA v1.23	In-house database, integrated public databases and predicted databases, supplemented by metDNA v1.23
Feature-retention criteria	Integrated annotation score >0.5 and pooled-QC coefficient of variation (CV) <0.30	Integrated annotation score >0.5 and pooled-QC CV <0.30
Orthogonal Partial Least Squares–Discriminant Analysis (OPLS-DA)	MetaboAnalystR v1.0.1; log ₂ transformation and mean centering	MetaboAnalystR v1.0.1; log ₂ transformation and unit-variance scaling
Model validation	Permutation test with 200 iterations	Permutation test with 200 iterations
Differential-metabolite criteria	Variable importance in projection (VIP) >1 and two-sided unpaired <i>t</i> -test <i>p</i> <0.05	VIP >1 and two-sided unpaired <i>t</i> -test <i>p</i> <0.05

Supplementary Table 6. Ultra-performance liquid chromatography–tandem mass spectrometry parameters for targeted amino acid metabolomics.

Item	Details
Ultra performance liquid chromatography (UPLC) system	ExionLC AD UPLC, SCIEX
Mass spectrometer	QTRAP 6500+ triple-quadrupole/linear ion-trap mass spectrometer, SCIEX
Ion source	Turbo IonSpray electrospray-ionization source
Analytical column	ACQUITY BEH Amide, 1.7 µm, 2.1 × 100 mm
Mobile phase A	Water containing 2 mM ammonium acetate and 0.04% formic acid
Mobile phase B	Acetonitrile containing 2 mM ammonium acetate and 0.04% formic acid

Liquid chromatography gradient	0–1.2 min, A/B = 10:90 (v/v); 9 min, A/B = 40:60 (v/v); 10–11 min, A/B = 60:40 (v/v); and 11.01–15 min, A/B = 10:90 (v/v)
Flow rate	0.4 mL/min
Column temperature	40 °C
Injection volume	2 µL
Ionization modes	Positive and negative electrospray ionization (ESI) modes
Source temperature	550 °C
Ion-spray voltage	5500 V in positive mode and –4500 V in negative mode
Curtain gas	35 psi
Acquisition mode	Multiple-reaction monitoring
Multiple-reaction monitoring (MRM) optimization	Declustering potential and collision energy were optimized individually for each MRM transition
MRM scheduling	Specific MRM-transition sets were monitored according to the retention-time periods of the corresponding analytes
Metabolite identification	Comparison with the MetWare Database constructed using authentic standards
Standard preparation	Individual standard stock solutions were prepared at 1 mg/mL in methanol, stored at –20 °C and diluted with methanol before analysis
Quantification	MRM peak integration and calibration curves generated using authentic standards
Acquisition software	Analyst v1.6.3, SCIEX
Quality control	Pooled quality control (QC) samples were used to monitor retention-time and signal-intensity stability