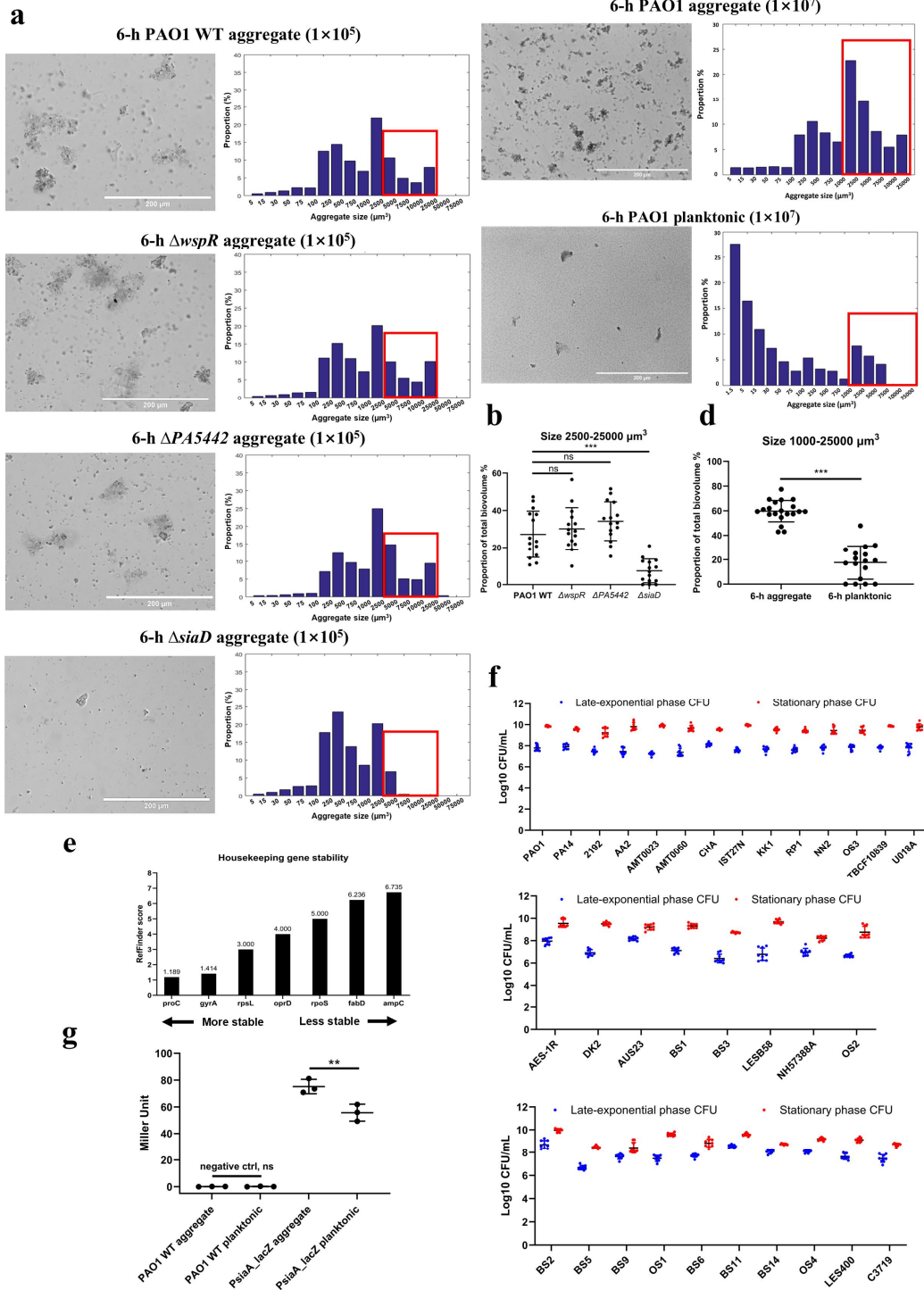


Supplementary Figure 1

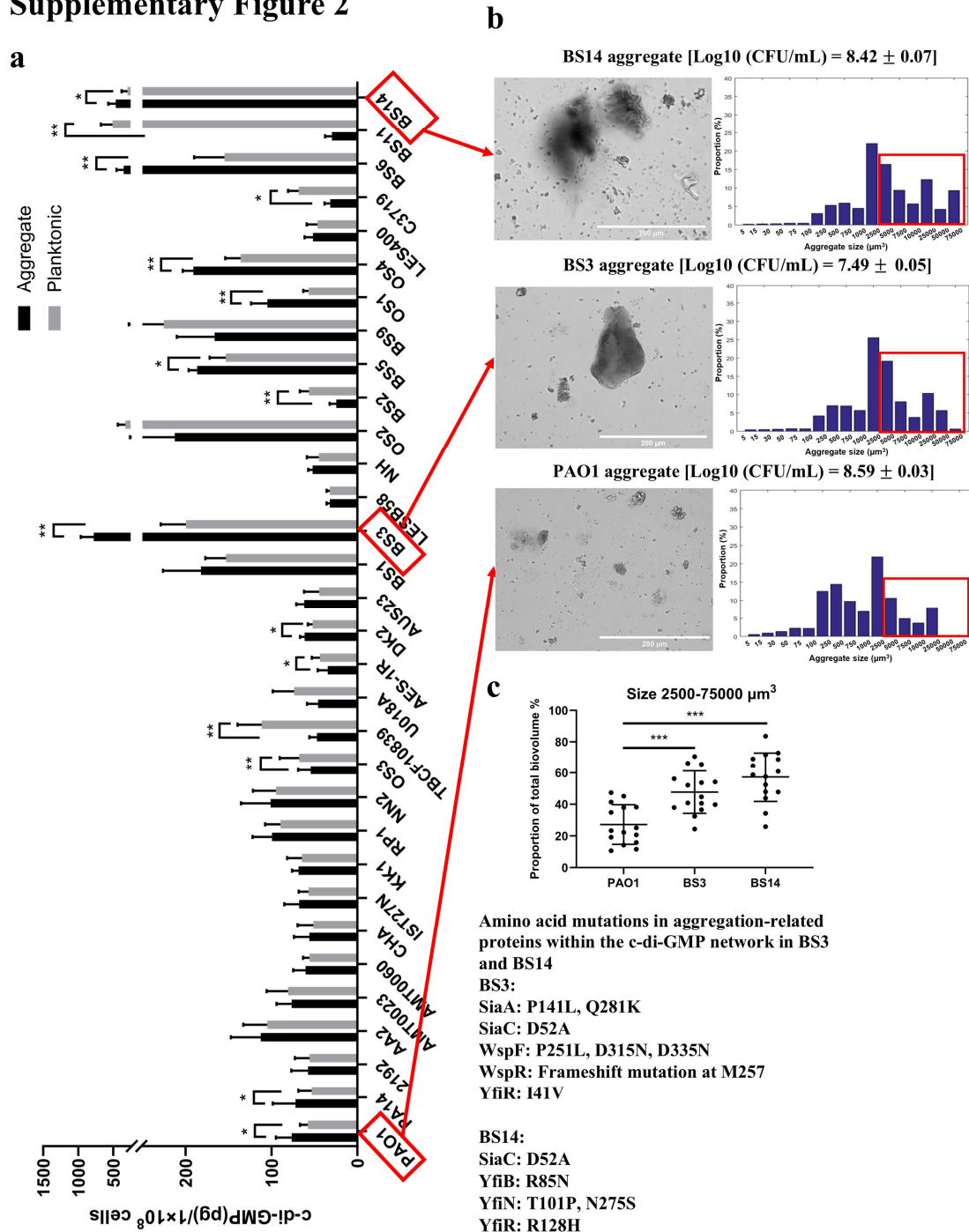


1

2 **Supplementary Figure 1. Cell morphologies, cell densities, and the stability of**
 3 **different housekeeping genes in *P. aeruginosa* aggregates and/or planktonic**
 4 **cultures. a) Representative micrographs of *P. aeruginosa* PAO1 WT, $\Delta wspR$, $\Delta PA5442$,**
 5 **and $\Delta siaD$ aggregates (initial inoculum 1×10^5 CFU/mL) grown in SCFM2 for 6 hrs**
 6 **(Scale bar = 200 μm) and the corresponding distribution of the sizes of aggregates. The**

biovolume (μm^3) of aggregates were grouped into 16 categories. Histograms represent the mean proportion value of all samples collected from 3 individual experiments (n=3), where micrographs were obtained from at least 3 random locations in each sample. Numerical data of mean proportions and standard deviation are shown in Supplementary Table 1. **b)** Proportion of aggregates in a) with sizes ranging from 2500 to 25000 μm^3 in total biovolume. ***, $p < 0.001$ (Student's t-test). **c)** Representative micrographs of *P. aeruginosa* PAO1 WT aggregates (in SCFM2) and planktonic cells (in SCFM2 without mucin and DNA) grown for 6 hrs with an initial inoculum size of 1×10^7 CFU/mL (Scale bar = 200 μm) and distribution of the size of 6-h PAO1 WT aggregates and planktonic cells. Histograms represent the mean proportion value of all samples collected from 3 individual experiments (n=3), where micrographs were obtained from at least 3 random locations in each sample. Numerical data and standard deviations are shown in Supplementary Table 1. **d)** Proportion of aggregates in c) with sizes ranging from 1000 to 25000 μm^3 in total biovolume. ***, $p < 0.001$ (Student's t-test). **e)** Stability of expression of 7 candidate reference genes for qPCR normalization as determined with RefFinder. The stability index of each gene is shown above each bar. **f)** Cell density of late-exponential phase aggregate culture of each strain after the desired incubation time in SCFM2. The starting inoculum concentrations for all strains were 1×10^5 CFU/mL, and the CFU counts were determined by agar plating. The late-exponential phase growth was defined when the CFU count was 1-2-log lower than the corresponding stationary phase cultures. **g)** The relative expression level of *siaA* in aggregates vs planktonic cells determined by β -galactosidase assay and quantified as Miller Units. PAO1 WT without the *lacZ* reporter plasmid was used as the negative control.

Supplementary Figure 2



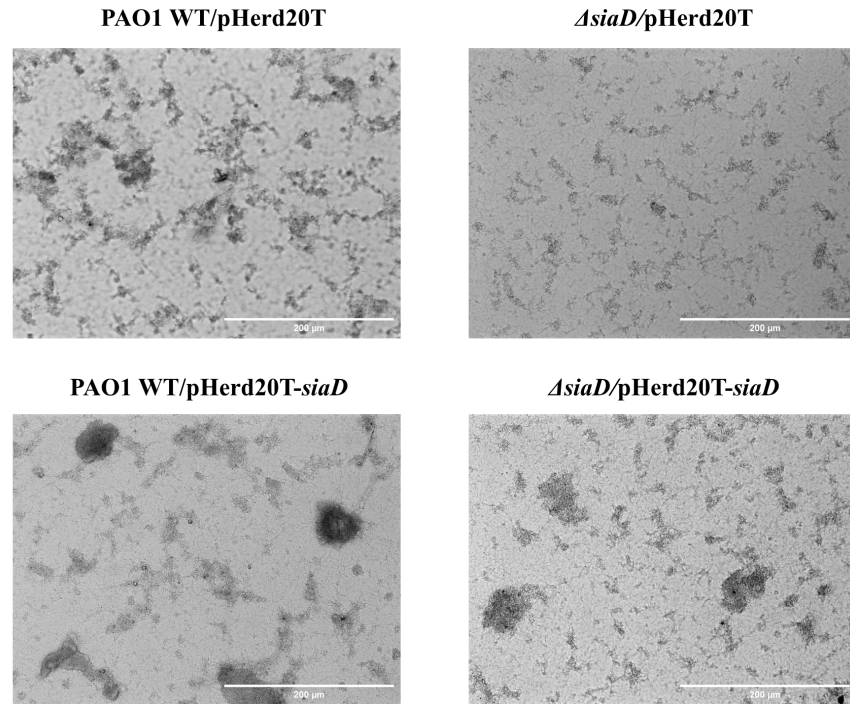
31

32 **Supplementary Figure 2. Intracellular c-di-GMP levels and aggregation patterns**
 33 **of *P. aeruginosa* clinical strains.** a) Intracellular c-di-GMP levels for 32 *P. aeruginosa*
 34 strains grown as aggregates (in SCFM2) or planktonic cells (in SCFM2 without DNA
 35 and mucin). Intracellular c-di-GMP concentrations (as determined by ELISA) were
 36 normalized to CFU counts (determined by plating). Data were acquired from 3
 37 independent experiments with 2 technical replicates and the mean values were shown.
 38 Error bars indicate standard deviation. *, $p < 0.05$; **, $p < 0.01$ (Ratio paired t-test

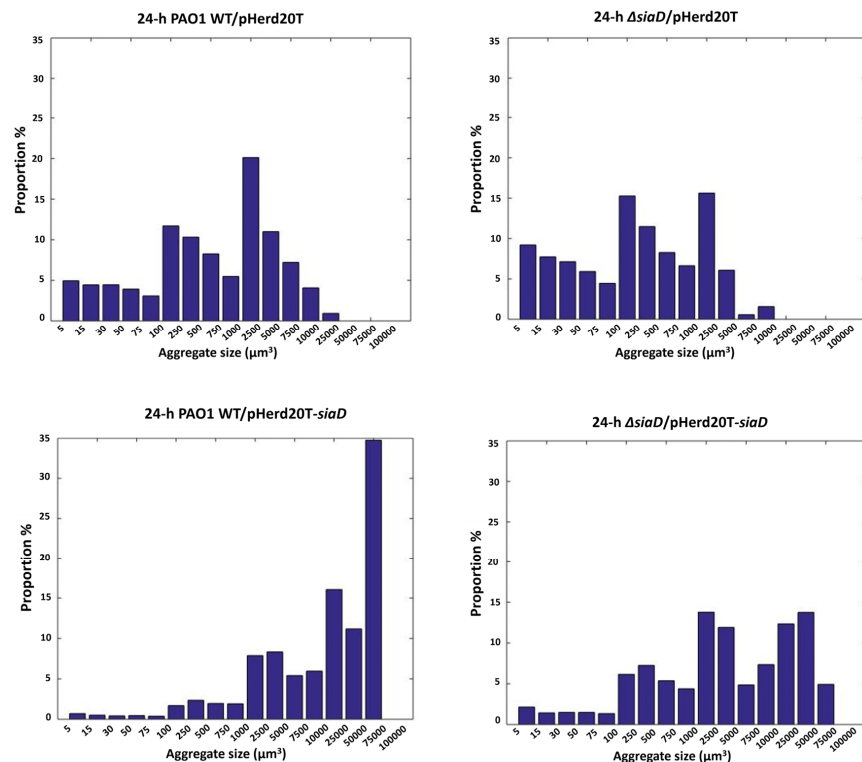
39 between two groups). **b)** Representative micrographs of *P. aeruginosa* PAO1, BS3, and
40 BS14 aggregates (initial inoculum 1×10^5 CFU/mL) grown in SCFM2 until the late-
41 exponential phase (Scale bar = 200 μm) and the corresponding distribution of the size
42 of aggregates (final cell density at the point of sampling labelled above, $n=3$). The
43 biovolume (μm^3) of aggregates were grouped into 16 categories. Histograms represent
44 the mean proportion value of all samples collected from 3 individual experiments ($n=3$),
45 where micrographs were obtained from at least 3 random locations in each sample.
46 Numerical data of mean proportions and standard deviation are shown in
47 Supplementary Table 1. **c)** Proportion of aggregates in b) with sizes ranging from 2500
48 to 75000 μm^3 in total biovolume. ***, $p < 0.001$ (Student's t-test).

Supplementary Figure 3

a



b

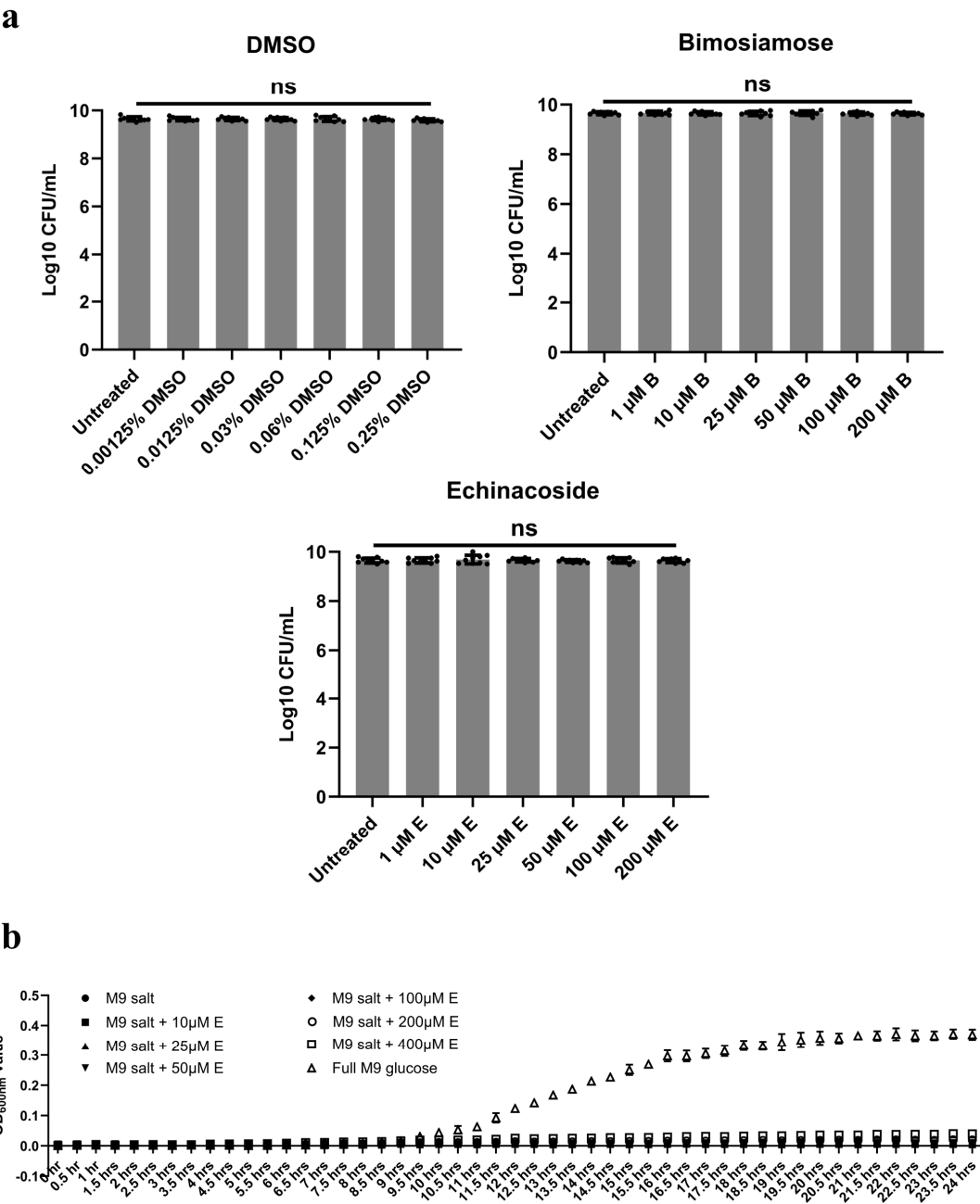


49

50 **Supplementary Figure 3. The aggregate morphologies of PAO1 WT and *ΔsiaD***
 51 **complemented with empty vector or *siaD*. a)** Representative micrographs of PAO1
 52 WT/pHerd20T, *ΔsiaD*/pHerd20T, PAO1 WT/pHerd20T-*siaD* and *ΔsiaD*/pHerd20T-
 53 *siaD* aggregates grown for 24 h in SCFM2. Scale bar = 200 μm. Plasmids were

maintained by adding 200 µg/mL carbenicillin and expression was induced by 0.5% (v/v) arabinose. **b)** Distribution of the size of 24-h PAO1 WT/pHerd20T, *ΔsiaD*/pHerd20T, PAO1 WT/pHerd20T-*siaD* and *ΔsiaD*/pHerd20T-*siaD* aggregates grown in SCFM2. The biovolume (µm³) of single cells and aggregates were grouped into 17 categories. Histograms represent the mean proportion value of all samples collected from 3 individual experiments (n=3), where micrographs were obtained from at least 3 random locations in each sample. Numerical data of mean proportions and standard deviation are shown in Supplementary Table 1.

Supplementary Figure 4

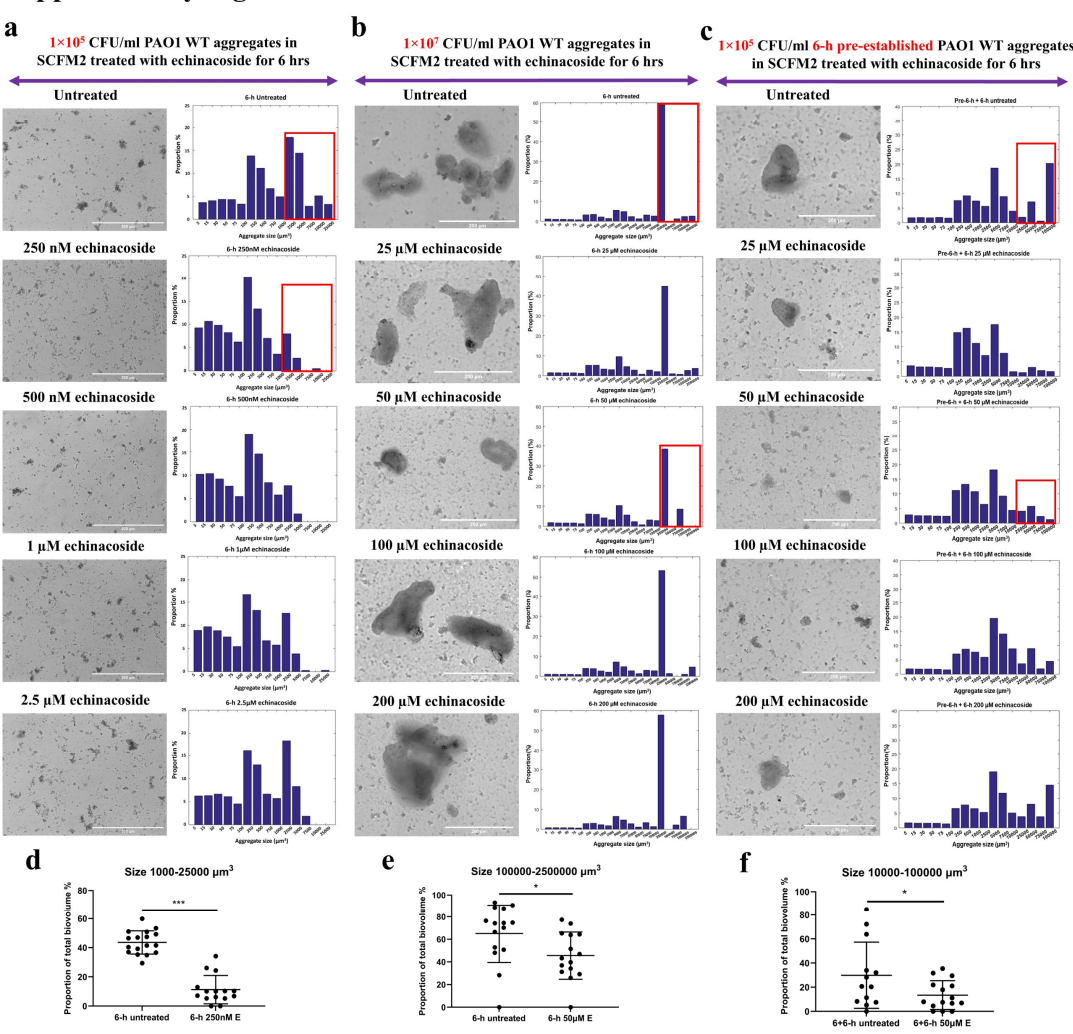


63

64 **Supplementary Figure 4. Potential anti-biofilm compounds do not interfere with**
 65 **the growth of PAO1. a) Bactericidal tests for DMSO (solvent for bimosiamose),**
 66 **bimosiamose, and echinacoside against 6-h pre-established PAO1 aggregates grown in**
 67 **SCFM2. Data are expressed as the mean number of CFU remaining after an additional**
 68 **18-h incubation (3 independent experiments with 3 technical replicates. No statistically**
 69 **significant difference was found comparing untreated and treated samples (One-way**

ANOVA). **b)** Echinacoside is not a nutrient source that promotes *P. aeruginosa* cell growth. 1×10^5 CFU/mL PAO1 WT cells were inoculated into M9 minimal medium without glucose, M9 minimal medium without glucose but with 25, 50, 100, 200, and 400 μ M echinacoside (the same concentration range used in combination treatment assay), and M9 minimal medium (with glucose). The OD_{600nm} measurement was performed every 30 mins over 24-h incubation at 37°C, and the growth curve was plotted with data from 3 independent experiments with 3 technical replicates (n=3).

Supplementary Figure 5

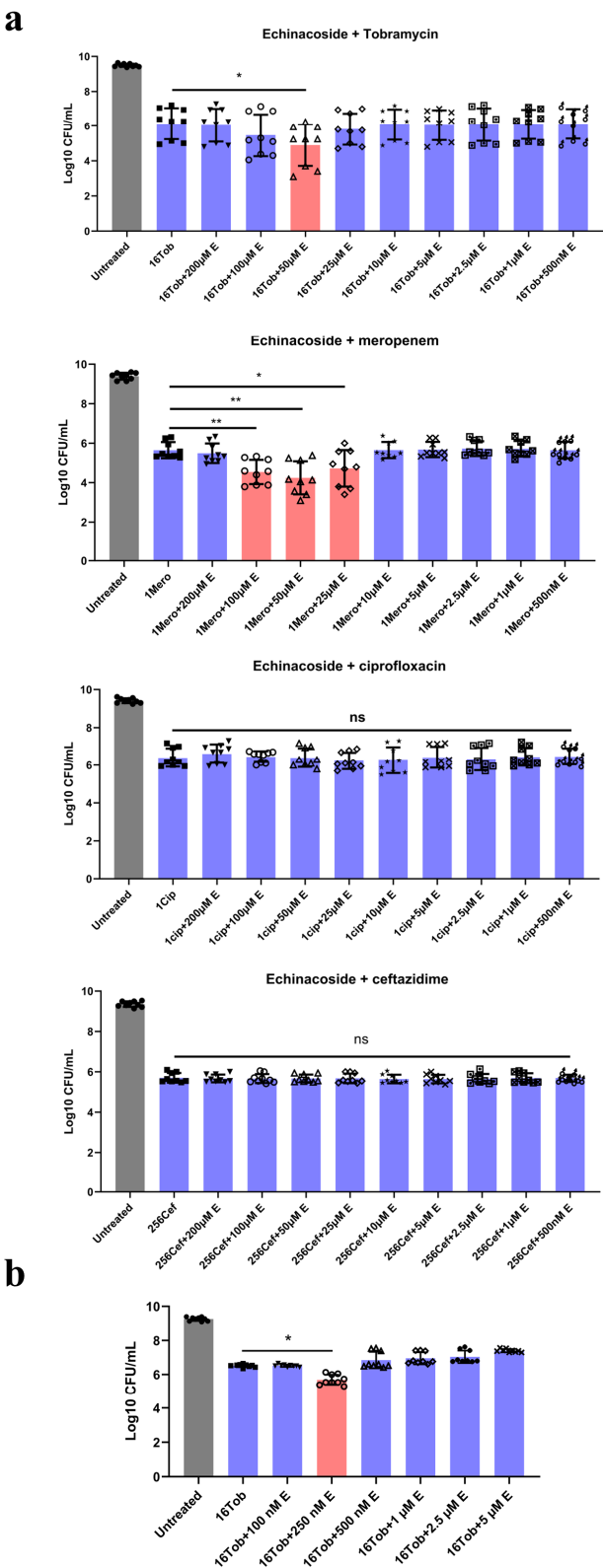


77

Supplementary Figure 5. Echinacoside inhibits aggregation in a concentration-dependent manner. **a)** Representative micrographs of 6-h *P. aeruginosa* PAO1 aggregates (initial inoculum 1×10^5 CFU/mL) grown in SCFM2 treated with different concentrations of echinacoside (Scale bar = 200 μ m) and the corresponding distribution of the size of aggregates. The biovolume (μm^3) of aggregates were grouped into 14

83 categories. **b)** Representative micrographs of 6-h *P. aeruginosa* PAO1 aggregates
 84 (initial inoculum 1×10^7 CFU/mL) grown in SCFM2 treated with different
 85 concentrations of echinacoside (Scale bar = 200 μm) and the corresponding distribution
 86 of the size of aggregates. The biovolume (μm^3) of aggregates were grouped into 20
 87 categories. **c)** Representative micrographs of 6-h pre-established *P. aeruginosa* PAO1
 88 aggregates (initial inoculum 1×10^5 CFU/mL) grown in SCFM2 treated with different
 89 concentrations of echinacoside for another 6 hrs (Scale bar = 200 μm) and the
 90 corresponding distribution of the size of aggregates. The biovolume (μm^3) of
 91 aggregates were grouped into 16 categories. Histograms represent the mean proportion
 92 value of all samples collected from 3 individual experiments ($n=3$), where micrographs
 93 were obtained from at least 3 random locations in each sample. Numerical data of mean
 94 proportions and standard deviation are shown in Supplementary Table 1. **d)** Proportion
 95 of aggregates in (a) with sizes ranging from 1000 to 25000 μm^3 in total biovolume. ***,
 96 $p < 0.001$ (Student's t-test). **e)** Proportion of aggregates in b) with sizes ranging from
 97 100000 to 2500000 μm^3 in total biovolume. *, $p < 0.05$. **f)** Proportion of aggregates in c)
 98 with sizes ranging from 10000 to 100000 μm^3 in total biovolume. *, $p < 0.05$

Supplementary Figure 6

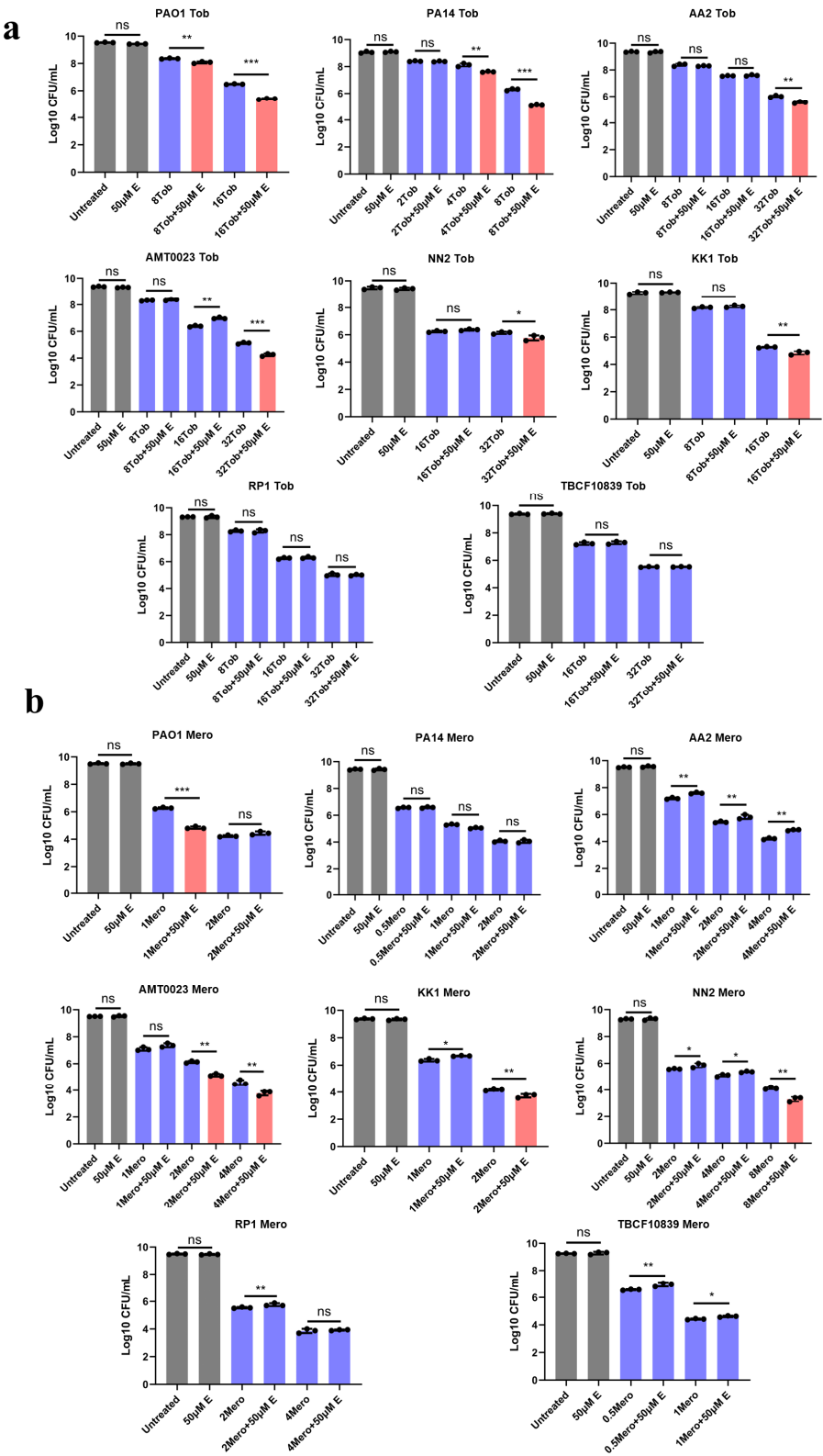


99

100 **Supplementary Figure 6. Combination treatments of echinacoside and different**
101 **antibiotics against *P. aeruginosa* PAO1 aggregates. a) Antimicrobial activity of the**

combination of echinacoside with tobramycin, meropenem, ciprofloxacin, and ceftazidime, respectively, against 6-h pre-established *P. aeruginosa* PAO1 aggregates grown in SCFM2. Data are expressed as the mean number of CFU remaining after an additional 18-h incubation (3 independent experiments with 3 technical replicates; error bars indicate standard deviation). Red bars highlighted the successful combination treatment that potentiated the efficacy of corresponding antibiotics. *, $p < 0.05$; **, $p < 0.01$ (Student's t-test). **b)** Antimicrobial activity of tobramycin against aggregates pre-treated with echinacoside for 6 hours. Data are expressed as the mean number of CFU remaining after an additional 18-h incubation with tobramycin treatment (3 independent experiments with 3 technical replicates; error bars indicate standard deviation). Red bars highlighted the successful combination treatment that potentiated the efficacy of corresponding antibiotics. *, $p < 0.05$ (Student's t-test).

Supplementary Figure 7

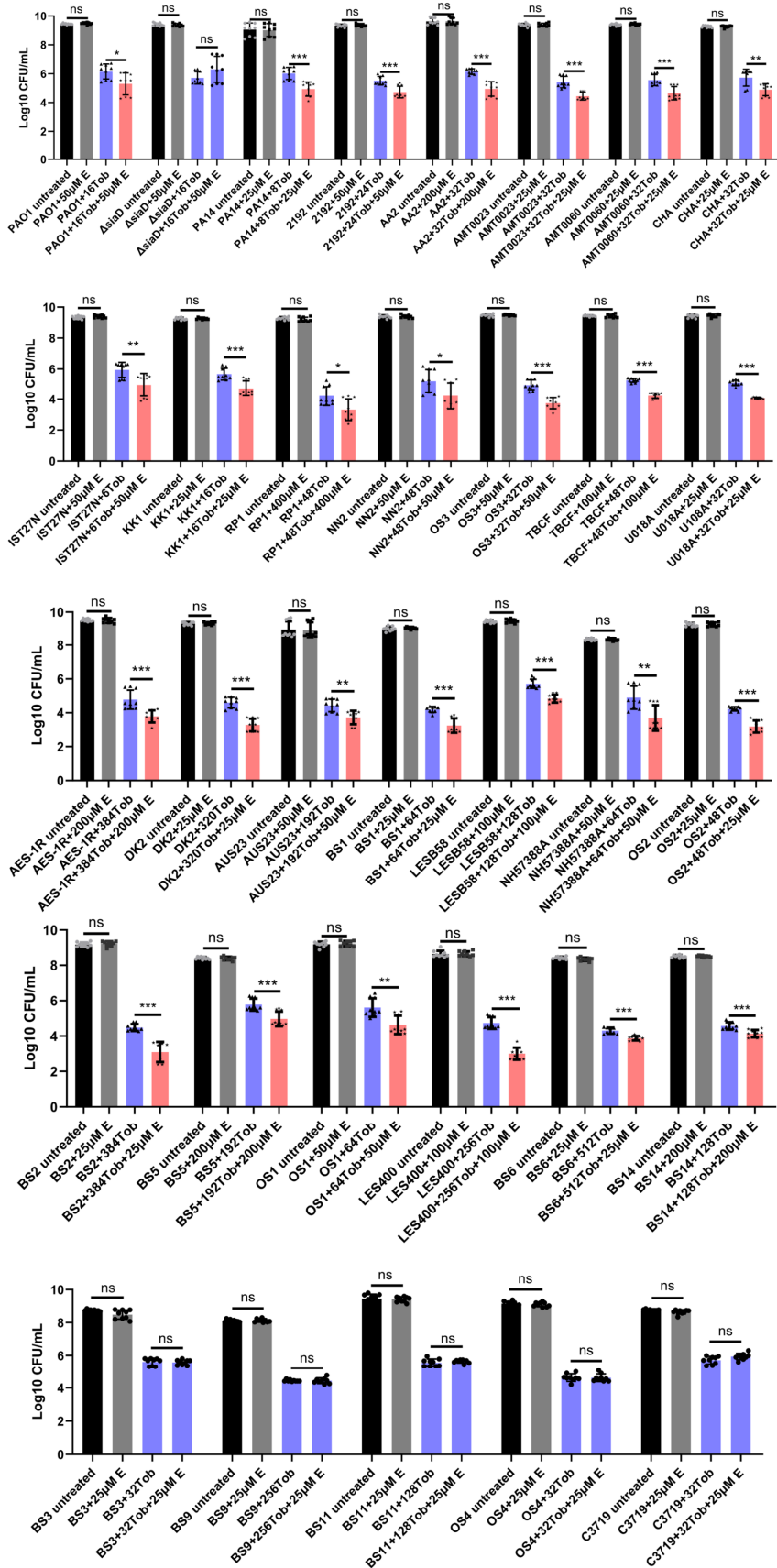


114

115 Supplementary Figure 7. Combination treatments of echinacoside and
 116 tobramycin/meropenem against different *P. aeruginosa* strains. Efficacy test for the

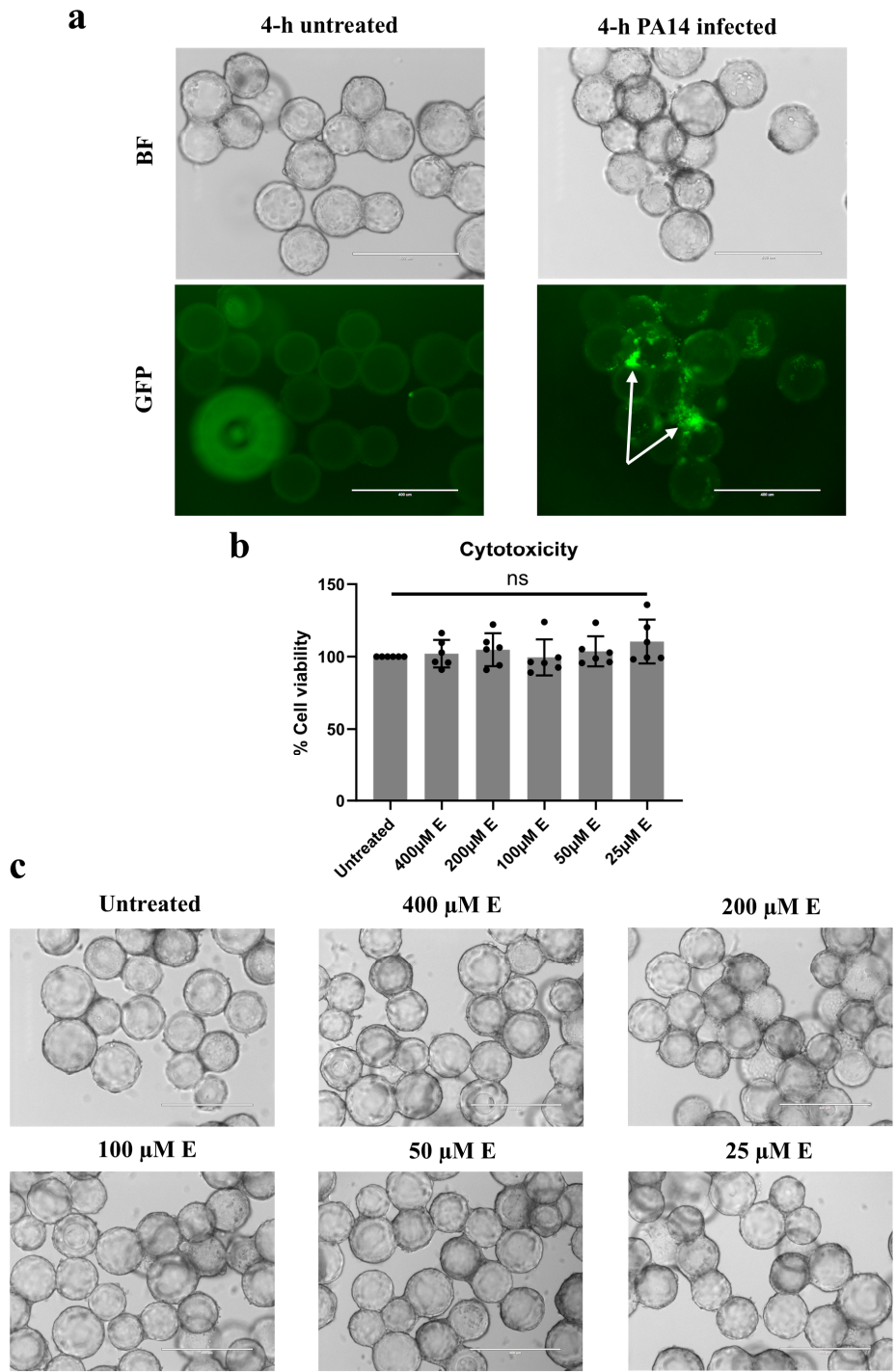
combination treatment of 50 μ M echinacoside with different concentrations of **a)** tobramycin or **b)** meropenem against pre-established aggregates formed by 8 different *P. aeruginosa* strains in SCFM2. Data are expressed as the mean number of CFU remaining after an additional 18-h incubation (3 independent experiments with 3 technical replicates; error bars indicate standard deviation). Red bars highlighted the successful combination treatment that potentiated the efficacy of corresponding antibiotics. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Student's t-test).

Supplementary Figure 8



Supplementary Figure 8. The synergistic killing effect of echinacoside and tobramycin against aggregates formed by different *P. aeruginosa* strains in SCFM2. The optimal dosages for successful combination treatments where echinacoside potentiated the efficacy of tobramycin against pre-established aggregates grown in SCFM2 for 27 *P. aeruginosa* strains and the representative data for unsuccessful combination treatments where echinacoside cannot potentiate the efficacy of tobramycin at any tested concentrations. Data are expressed as the mean number of CFU remaining after an additional 18-h incubation (3 independent experiments with 3 technical replicates; error bars indicate standard deviation). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant (Student's t-test).

Supplementary Figure 9



Supplementary Figure 9. Echinacoside is not cytotoxic to human A549 cells. a) Representative transmission light micrographs (upper panel) of 3-D A549 cells attached to/detached from collagen-treated carrier beads and fluorescent micrographs (lower panel) of GFP-tagged PA14 attached to A549 cells. After 4-h infection, PA14 aggregates (highlighted with white arrows) were found attached to A549 cells with

strong fluorescent signals. Healthy cells in the untreated group showed a smooth morphology and remained firmly attached to beads, while cells infected with PA14 were stressed (rough and patchy morphology at the surface of beads). Scale bar = 400 μm . **b)** Cytotoxicity test for different concentrations of echinacoside. The viability of 3-D A549 cells challenged with different concentrations of echinacoside for 18 hrs was measured by LDH assay and normalized to untreated groups. Data were acquired from 6 independent experiments with 4 technical replicates. No statistically significant difference was found comparing untreated and treated cells (One-way ANOVA). **c)** Representative transmission light micrographs of 3-D A549 cells attached to collagen-treated carrier beads challenged with different concentrations of echinacoside. Healthy cells in all groups showed a smooth morphology and remained firmly attached to beads without difference in attachment patterns. Scale bar = 400 μm .

Supplementary Table 1. Mean fraction (%) of aggregates within each size category in total biovolume. SD, standard deviation. N=3

6-h aggregates vs planktonic (initial inoculum 1×10^7 CFU/mL)				
Size (μm^3)	aggregate		planktonic	
	mean	SD	mean	SD
1.5-5	N/A	N/A	27.64	10.31
5-15	1.44	0.48	16.44	4
15-30	1.39	0.43	10.92	3.94
30-50	1.52	0.48	7.26	2.73
50-75	1.64	0.49	4.65	2.25
75-100	1.48	0.5	2.82	1.56
100-250	7.89	3.04	5.38	3.21
250-500	10.56	2.59	3.23	1.55
500-750	8.31	2.36	2.81	2.14
750-1000	6.5	2.1	1.27	1.66
1000-2500	22.77	4.95	7.72	7.73
2500-5000	14.56	3.99	5.74	7.18
5000-7500	8.58	5.3	4.14	6.99
7500-10000	5.48	4.29	0	0
10000-25000	7.87	5.59	0	0
25000-50000	0	0	0	0

6-h aggregates (initial inoculum 1×10^5 CFU/mL)								
Size (μm^3)	WT		<i>AwspR</i>		<i>APA5442</i>		<i>AsiaD</i>	
	mean	SD	mean	SD	mean	SD	mean	SD
5-15	0.55	0.42	0.43	0.21	0.36	0.17	0.49	0.33
15-30	0.92	0.84	0.67	0.37	0.42	0.17	0.98	0.87
30-50	1.34	1.23	0.93	0.58	0.62	0.35	1.72	1.28
50-75	2.25	2.19	1.43	0.78	0.91	0.54	2.63	1.81
75-100	2.20	2.11	1.59	0.83	1.02	0.59	2.81	1.83
100-250	12.53	9.41	11.11	5.64	7.14	4.10	17.76	8.44
250-500	14.44	7.17	15.20	4.75	12.41	5.36	23.54	4.96
500-750	9.77	4.06	10.98	2.50	9.69	3.05	13.81	2.71
750-1000	6.88	2.09	7.32	2.69	7.84	1.94	8.59	2.98
1000-2500	21.89	11.44	20.17	5.44	24.97	5.64	20.25	8.09
2500-5000	10.65	7.30	10.06	5.42	14.82	5.09	6.78	5.71
5000-7500	4.91	4.75	5.51	4.21	5.09	5.08	0.44	0.94
7500-10000	3.67	3.92	4.45	4.39	4.87	3.75	0.20	0.77
10000-25000	7.99	5.61	10.15	5.47	9.47	5.81	0	0
25000-50000	0.00	0	0	0	0.37	1.44	0	0
50000-75000	0.00	0	0	0	0	0	0	0

6-h aggregates (initial inoculum 1×10 ⁵ CFU/mL)										
Size (µm ³)	Untreated		250 nM E		500 nM E		1 µM E		2.5 µM E	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
5-15	3.67	1.2	9.29	2.93	10.21	4.5	9.21	4.31	6.24	2.28
15-30	4.07	1.78	10.69	2.6	10.34	2.68	9.87	3.9	6.31	1.83
30-50	4.37	1.79	9.85	1.82	9.18	2.61	8.81	3.16	6.64	2.15
50-75	4.32	1.34	8.26	1.27	7.65	1.88	7.59	2.38	6.11	1.88
75-100	3.32	0.76	6.25	0.84	5.44	1.83	5.5	1.74	4.53	2.22
100-250	13.84	3.29	20.37	3.11	18.99	3.58	16.69	3.95	16.26	3.6
250-500	11.15	3.51	13.36	2.82	14.56	4.06	12.83	2.6	12.99	3.37
500-750	6.72	2.45	7.03	1.76	8.41	4.41	6.82	2.97	6.65	1.73
750-1000	4.93	1.85	3.63	2.11	5.77	4.12	5.62	2.73	5.71	3.28
1000-2500	17.89	5.23	8	6.1	7.76	5.11	12.12	9	18.38	9.19
2500-5000	14.44	9.06	2.77	3.84	1.71	3.03	4.2	5.29	8.29	7.39
5000-7500	2.87	3.86	0	0	0	0	0.44	1.09	1.88	3.85
7500-10000	5.13	5.09	0.51	1.97	0	0	0	0	0	0
10000-25000	3.28	4.37	0	0	0	0	0.3	1.33	0	0

[illegible]

Pre-6-h aggregate + 6-h treatments (initial inoculum 1×10 ⁵ CFU/mL)										
Size (μm ³)	Untreated		25 μM E		50 μM E		100 μM E		200 μM E	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
5-15	1.72	0.75	3.58	0.71	2.88	0.75	1.90	0.51	1.69	0.72
15-30	1.79	0.79	3.21	0.66	2.59	0.64	1.83	0.53	1.61	0.68
30-50	1.72	0.76	3.19	0.68	2.57	0.71	1.83	0.54	1.60	0.71
50-75	1.83	0.81	3.02	0.64	2.45	0.92	1.72	0.43	1.58	0.64
75-100	1.60	0.71	2.74	0.74	2.43	1.03	1.55	0.51	1.38	0.57
100-250	7.59	3.34	14.84	4.02	11.18	4.35	7.10	2.21	6.58	2.44
250-500	9.22	4.12	16.29	3.57	13.26	5.02	8.77	2.63	7.80	3.42
500-750	7.43	3.77	11.20	2.20	10.78	3.79	7.74	2.28	6.53	2.53
750-1000	5.63	2.99	7.06	1.53	6.46	1.78	5.96	1.72	5.26	2.26
1000-2500	18.68	7.67	17.52	3.98	18.20	5.28	19.54	4.31	19.01	5.87
2500-5000	8.93	3.97	7.82	4.30	9.27	4.37	14.07	2.78	11.78	4.27
5000-7500	3.96	2.64	1.58	2.23	4.41	5.60	8.90	2.91	5.06	2.71
7500-10000	1.89	1.72	1.27	2.11	4.12	3.76	3.68	3.04	3.81	3.02
10000-25000	7.14	4.99	3.05	5.21	5.77	5.92	8.97	5.66	8.03	4.04
25000-50000	0.61	2.18	1.98	5.23	2.34	4.92	1.92	3.47	3.79	5.08
50000-75000	20.26	29.32	1.64	6.35	1.26	4.90	4.51	8.65	14.50	20.94
75000-100000	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

late-exponential phase aggregates (initial inoculum 1×10 ⁵ CFU/mL)						
Size (μm ³)	WT		BS3		BS14	
	mean	SD	mean	SD	mean	SD
5-15	0.55	0.42	0.42	0.12	0.21	0.11
15-30	0.92	0.84	0.49	0.20	0.29	0.23
30-50	1.34	1.23	0.57	0.27	0.36	0.31
50-75	2.25	2.19	0.72	0.50	0.48	0.55
75-100	2.20	2.11	0.69	0.46	0.48	0.68
100-250	12.53	9.41	4.21	2.48	3.09	4.42
250-500	14.44	7.17	7.00	3.22	5.32	4.50
500-750	9.77	4.06	6.92	2.51	5.92	4.59
750-1000	6.88	2.09	5.71	1.81	4.50	2.29
1000-2500	21.89	11.44	25.55	5.86	22.01	7.07
2500-5000	10.65	7.30	19.16	5.58	16.39	6.28
5000-7500	4.91	4.75	8.09	3.73	9.39	4.20
7500-10000	3.67	3.92	3.81	3.36	5.72	3.05
10000-25000	7.99	5.61	10.37	4.27	12.27	7.73
25000-50000	0.00	0	5.68	5.15	4.24	4.35
50000-75000	0.00	0	0.64	1.72	9.32	6.25

[illegible]

Supplementary Table 2. Bacterial strains and plasmids used. Clinical strains were either collected from Ghent University Hospital (BS and OS) or selected from the international *P. aeruginosa* reference panel collection^{1,2}. All strains were obtained from different patients.

Clinical strains		
<i>P. aeruginosa</i> isolates (accession number of genome sequence)	Description	Source or reference
PAO1 (NC_002516)	Wound isolate (Australia)	3
PA14 (NC_008463)	Burn wound isolate (US)	4
2192 (NZ_CH482384)	CF isolate (US)	5
AA2 (MCMJ000000000)	CF isolate (Germany)	6,7
AES-1R (MCML000000000)	CF isolate (Australia)	7,8
AMT0023-30 (MCM100000000)	CF isolate (US)	7,9
AMT0060-3 (MCMZ000000000)	CF isolate (US)	7,9
AUS23 (MCMN000000000)	CF isolate (Australia)	7,10
BS1 (PRJNA1072279)	CF isolate (Belgium)	This study
BS2 (PRJNA1072279)	CF isolate (Belgium)	This study
BS3 (PRJNA1072279)	CF isolate (Belgium)	This study
BS5 (PRJNA1072279)	CF isolate (Belgium)	This study
BS6 (PRJNA1072279)	CF isolate (Belgium)	This study
BS9 (PRJNA1072279)	CF isolate (Belgium)	This study
BS11 (PRJNA1072279)	CF isolate (Belgium)	This study
BS14 (PRJNA1072279)	CF isolate (Belgium)	This study
C3719 (MCM000000000)	CF isolate (UK)	5,7
CHA (MCMG000000000)	CF isolate (France)	7,11
DK2 (NC_018080)	CF isolate (Denmark)	12
IST27N (MCMW000000000)	CF isolate (Portugal)	7,13
KK1 (MCMB000000000)	CF isolate (Germany)	6,7
LESB58 (NC_011770)	CF isolate (UK)	14
LES400 (NZ_CP006982)	CF isolate (UK)	15
NH57388A (MCMT000000000)	CF isolate (Denmark)	7,16
NN2 (NZ_LT883143.1)	CF isolate (Germany)	17,18
OS1 (PRJNA1072279)	CF isolate (Belgium)	This study
OS2 (PRJNA1072279)	CF isolate (Belgium)	This study
OS3 (PRJNA1072279)	CF isolate (Belgium)	This study
OS4 (PRJNA1072279)	CF isolate (Belgium)	This study
RP1 (LNB000000000)	CF isolate (Germany)	19
TBCF10389 (MCLZ000000000)	CF isolate (Germany)	7,20
U018A (MCM000000000)	CF isolate (Australia)	7,21
<i>P. aeruginosa</i> PAO1 isogenic mutants		
<i>ΔwspR</i>	<i>ΔwspR</i> deletion mutant	This study
<i>ΔsiaD</i>	<i>ΔsiaD</i> deletion mutant	This study
<i>Δpa5442</i>	<i>Δpa5442</i> deletion mutant	This study
PAO1 WT/pHerd20T	PAO1 carrying empty pHerd20T, Cb ^R	This study
<i>ΔsiaD</i> /pHerd20T	<i>ΔsiaD</i> mutant carrying empty pHerd20T, Cb ^R	This study
PAO1 WT/pHerd20T- <i>siaD</i>	PAO1 carrying pHerd20T with <i>siaD</i> ORF inserted, Cb ^R	This study
<i>ΔsiaD</i> /pHerd20T- <i>siaD</i>	<i>ΔsiaD</i> mutant carrying pHerd20T with <i>siaD</i> ORF inserted, Cb ^R	This study
PAO1 <i>PsiaA::lacZ</i>	PAO1 with <i>PsiaA-lacZ</i> and a Gm ^R cassette inserted in the <i>att::Tn7</i> site downstream of <i>glmS</i>	This study
GFP-labelled PA14		
PA14/pBK-miniTn7-gfp2	Chromosomal labelled PA14 with pBK-miniTn7-gfp2. Gm ^R ,	This study
<i>E. coli</i> strains		
HB101	K-12/B hybrid; Sm ^r <i>recA thi pro leu hsdRM</i> ⁺	22
DH5α	F ⁻ ϕ 80dlacZΔM15 Δ(lacZYA-argF)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44 λ⁻ thi-1 gyrA96 relA1</i>	23
XL1Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^q ZAM15 Tn10</i> (Tet ^R)]	24
Plasmids		
pBK-miniTn7-gfp2	pUC19-based delivery plasmid for miniTn7- <i>gfp2</i> . Gm ^R , Cm ^R , Ap ^R , mob ⁺	25
pRK600	ColE1 RK2-Mob ⁺ RK2-Tra ⁺ ; helper plasmid; Cm ^R	24
pUX-BF13	R6K replicon-based helper plasmid, providing the Tn7 transposition function in trans, Ap ^R mob ⁺	24
pK18-Gm-mobsacB	Suicide knockout plasmid, Gm ^R	26
pHerd20T	pUCP20T Plac replaced with 1.3-kb AflIII-EcoRI fragment of <i>araC</i> -PBAD cassette, Amp ^R	27

pHerd20T- <i>siaD</i> pUC18-mini-Tn7T-Gm- <i>lacZ</i>	pHerd20T plasmid carrying <i>siaD</i> ORF Plasmid containing a mini-Tn7 system with single attTn7 site for integration in <i>P. aeruginosa</i> chromosome and a promoterless <i>lacZ</i> Helper plasmid for integration of pUC18-mini-Tn7T-Gm pUC18-mini-Tn7T-Gm- <i>PsiaA-lacZ</i>	This study ²⁸
pTNS2 pUC18-mini-Tn7T-Gm- <i>PsiaA-lacZ</i>		²⁸ This study

Supplementary Table 3. The presence of c-di-GMP related genes in different *P. aeruginosa* strains. +, gene is present; -, gene is absent.

	PAO1	PA14	2192	AA2	AES-1R	AMT0023-30	AMT0060-3	AUS23	C3719	CHA
<i>siaD</i>	+	+	+	+	+	+	+	+	+	+
<i>pa0290</i>	+	+	+	+	+	+	+	+	+	+
<i>pa0338</i>	+	+	+	+	+	+	+	+	+	+
<i>pa0847</i>	+	+	+	+	+	+	+	+	+	+
<i>roeA</i>	+	+	+	+	+	+	+	+	+	+
<i>pa1851</i>	+	+	+	+	+	+	+	+	+	+
<i>pa2771</i>	+	-	+	+	+	+	+	+	+	+
<i>pa2870</i>	+	+	+	+	+	+	+	+	+	+
<i>pa3177</i>	+	+	+	+	+	+	+	+	+	+
<i>hsbD</i>	+	+	+	+	+	+	+	+	+	+
<i>sadC</i>	+	+	+	+	+	+	+	+	+	+
<i>pa4396</i>	+	+	+	+	+	+	+	+	+	+
<i>gcbA</i>	+	+	+	+	+	+	+	+	+	+
<i>pa4929</i>	+	+	+	+	+	+	+	+	+	+
<i>dgcH</i>	+	+	+	+	+	+	+	+	+	+
<i>tpbB</i>	+	+	+	+	+	+	+	+	+	+
<i>wspR</i>	+	+	+	+	+	+	+	+	+	+
<i>pa2133</i>	+	+	+	+	+	+	+	+	+	+
<i>pa2200</i>	+	+	+	+	+	+	+	+	+	+
<i>pa2572</i>	+	+	+	+	+	+	+	+	+	+
<i>arr</i>	+	-	-	-	-	-	+	-	-	-
<i>pa3825</i>	+	+	+	+	+	+	+	+	+	+
<i>rocR</i>	+	+	+	+	+	+	+	+	+	+
<i>pa4108</i>	+	+	+	+	+	+	+	+	+	+
<i>pa4781</i>	+	+	+	+	+	+	+	+	+	+
<i>pvrR</i>	-	+	-	-	-	-	-	-	-	-

[illegible]

[illegible]

[illegible]

Supplementary Table 4. Strain cultivation time

Strain	Aggregate culture	Planktonic culture
PAO1	6 h	6 h
PA14	6 h	6 h
2192	6 h	6 h
AA2	6 h	6 h
AMT0023-30	6 h	6 h
AMT0060-3	6 h	6 h
CHA	6 h	6 h
IST27N	6 h	6 h
KK1	6 h	6 h
NN2	6 h	6 h
OS3	6 h	6 h
RP1	6 h	6 h
TBCF10839	6 h	6 h
U018A	6 h	6 h
AES-1R	9 h	9 h
AUS23	9 h	9 h
BS1	9 h	9 h
BS3	9 h	9 h
DK2	9 h	9 h
LESB58	9 h	9 h
NH57388A	9 h	9 h
OS2	9 h	9 h
BS2	12 h	12 h
BS5	12 h	12 h
BS9	12 h	12 h
OS1	12 h	12 h
C3719	18 h	9 h
LES400	18 h	18 h
OS4	18 h	18 h
BS6	24 h	24 h
BS11	24 h	9 h
BS14	24 h	24 h

Supplementary Table 5. MIC ($\mu\text{g/mL}$) of tobramycin and meropenem against different strains and mucoidy on PIA plates.

	Tobramycin	Meropenem	Mucoidy on PIA
PAO1	2	1	no
PA14	1	0.5	no
2192	1	0.5	no
AA2	2	1	no
AES-1R	8	8	no
AMT0023-30	1	0.5	no
AMT0060-3	1	0.25	no
AUS23	2	16	no
BS1	1	1	yes
BS2	1	0.125	no
BS3	16	4	yes
BS5	32	4	yes
BS6	2	1	yes
BS9	16	1	yes
BS11	2	4	yes
BS14	0.5	0.125	yes
CHA	2	0.5	no
C3719	4	64	no
DK2	32	32	no
IST27N	1	0.0625	no
KK1	1	0.5	no
LES400	8	8	no
LESB58	4	2	no
NH57388A	2	0.5	no
NN2	1	1	no
RP1	1	1	no
OS1	2	1	yes
OS2	2	0.25	yes
OS3	1	1	no
OS4	0.5	0.125	yes
TBCF10839	1	0.5	no
U018A	1	2	no

Supplementary Table 6. Primers used

Primers	Sequence 5'→3'
Gene deletion	
PAO1-wspR-up-F	AGCTCGGTACCCGGGATTGTTACCGTCGATATCGAGC
PAO1-wspR-up-R	CACCGGCTGTTCCATCAGTCGTATCAAATTGAATGCTGTTT
PAO1-wspR-dn-F	GAACAGCATTCAATTTGATACGACTGATGGAACAGCCGGTG
PAO1-wspR-dn-R	CGACGGCCAGTGCCAATCTCGATCTCGATGTTGTCGTC
PAO1-siaD-up-F	AGCTCGGTACCCGGGTCTCGGCCTGCTGGACATC
PAO1-siaD-up-R	CTGGACGCCTGAGGAGGCCAGTTGCTCCAGCGATTGCTG
PAO1-siaD-dn-F	CAGCAATCGCTGGAGCAACTGGCCTCCTCAGGCGTCCAG
PAO1-siaD-dn-R	CGACGGCCAGTGCCATTCGCCATGGAGATCATCAGC
PAO1-PA5442-up-F	AGCTCGGTACCCGGGATTGATCCCGAGCAACTGCTC
PAO1-PA5442-up-R	GAGTCGAAAGTGCTACAGCATACGAACACCAGCACCCTGCTGGAG
PAO1-PA5442-dn-F	CTCCAGCAGGTGCTGGTGTTCGTATGCTGTAGCACTTTCGACTC
PAO1-PA5442-dn-R	CGACGGCCAGTGCCAAGCCTTGTCGACATTCTGC
Sequencing	
pk18-F	TGCTTCCGGCTCGTATGTTG
pk18-R	GCGAAAGGGGGATGTGCTG
pHerd20T-F	ATCGCAACTCTCTACTGTTTCT
pHerd20T-R	TGCAAGGCGATTAAGTTGGGT
PAO1-wspR-F	ATCGATGCAGCGGTGCAG
PAO1-wspR-R	AGCTGCGAAGTATACTGCACTTGC
PAO1-siaD-F	ACGACGAGTAGCCGACGATG
PAO1-siaD-R	TCGTCCTGACCCTGGAGTTG
PAO1-PA5442-F	ATCTGGAAGTTCTCGCTCAG
PAO1-PA5442-R	TTCGAGCTTCTCTTCCTCC
Expression	
SiaD-exp-F	ATGGGATCTGATAAGAATTCGTGCGGCTGGAGCGCATC
SiaD-exp-R	CGACGG CCA GTG CCA AGC TTT CAG CGC GCT GGA GCC GG
lacZ reporter fusion	
PsiaA_lacZ frag_F	GAATCCGTAATCATGGTCATGGCTATCCCTATCAGTTTCTC
PsiaA_lacZ frag_R	ATCATGCATGAGCTCACTAGGCGCAACCTGCTCGCCGG
PsiaA_lacZ vec_F	AGGCCGGCGAGCAGGTTGCGCCTAGTGAGCTCATGCATGATC
PsiaA_lacZ vec_R	GAAACTGATAGGGATAGCCATGACCATGATTACGGATTAC
Tn7-GlmS	AATCTGGCCAAGTCGGTGAC
Tn7-R109	CAGCATAACTGGACTGATTTTCAG
RT-qPCR	
qRT-siaD-F	CGCTACCAGCAGATGATG
qRT-siaD-R	GAGCGTTTCGTTCTCCTC
qRT-pipA-F	ATCTCGCAATCGCCATCG
qRT-pipA-R	TTGCGGCGAAGAAGAACAG
qRT-pa0290-F	CAACGCGAATACCGCATC
qRT-pa0290-R	GCTTCTTGTCGGTGATGTC
qRT-pa0338-F	CATCCATCCCGAGGACTATC
qRT-pa0338-R	CGCTGATCCACAGGTAATCC
qRT-rmcA-F	AACCGCTTCACCTACGTC
qRT-rmcA-R	GTAGTGGATGGCGTACTCG
qRT-pa0847-F	CATGCTGTCGCCCCATTAC
qRT-pa0847-R	TCCGACAAGAGCCAGATAC
qRT-rbdA-F	GGTGCTTCACGACATGAC
qRT-rbdA-R	TGCAGGCGATACTCGAAC
qRT-roeA-F	TGACCGGCCTGTTCAATC
qRT-roeA-R	GCGGTCGTTGATGTACTTG

qRT-yegE-F	GCCTGAACACCTGTTTCATC
qRT-yegE-R	GATCACCGAGACCAGGAAGG
qRT-pa1433-F	AGGCCTACCAGGACAGTCTC
qRT-pa1433-R	AGCCCGTTCAGGTCGTTTCAG
qRT-pa1851-F	ATGATGGCCGAACAGGAC
qRT-pa1851-R	CGACATCCACCAGGATCAG
qRT-pa2072-F	CATCGAGCAGAGCCATTTC
qRT-pa2072-R	CCGGTTTCCCAGATAGACG
qRT-pa2133-F	GTATACCCGCGAAGCACGTC
qRT-pa2133-R	TCGTCTCTCTCGAGACCTTC
qRT-pa2200-F	TGTTGTTCGGCGTCATGCTG
qRT-pa2200-R	ATGGGCTGGTAGTGCACCTC
qRT-pa2567-F	CAATGGAACGCACGGAAC
qRT-pa2567-R	GGAAAGGTCGATGTCTGATG
qRT-pa2572-F	CCTGCAACTGCTGGAAAG
qRT-pa2572-R	GTGAGCAGGATGCGAATG
qRT-pa2771-F	CCCTTCATCCGCTTCTACG
qRT-pa2771-R	ATTGAGCTGGCGTCCTTC
qRT-pa2870-F	CGTTCTACGCGTTGTTCTG
qRT-pa2870-R	TGGCGTTAAGCTCGATCAG
qRT-pa3177-F	GCCTGTTCTTCGAGGAAGTC
qRT-pa3177-R	TGTCATGGCTCAGGCGATAG
qRT-pa3258-F	ATTGCGACGAGAGCGTTC
qRT-pa3258-R	TTCGGAAAGCGAGAGGATG
qRT-nbdA-F	CAGATGGCTCGCTACGACAG
qRT-nbdA-R	TCGAGGTCGAGGAACATCAC
qRT-hsbD-F	ACGACTCCCGTTCCAATC
qRT-hsbD-R	GCTGTCTCTCCAGCATGTAG
qRT-pa3825-F	ATCGAGTCGAGCGAAGTC
qRT-pa3825-R	TGGAACCTTGCGCAGGTAG
qRT-pa4108-F	ACGTCTACGACGCGATCACC
qRT-pa4108-R	TTGACGAAGGCGCGGAACAC
qRT-sadC-F	CGGCATCTACCTGGTAGAG
qRT-sadC-R	TATGTAGCTGGCGAACAGG
qRT-pa4396-F	ACGGGCTCAACCTGAAG
qRT-pa4396-R	GCAGCAGTTTCGTCGATG
qRT-pa4781-F	ATCTTCCGCGTATCGAGC
qRT-pa4781-R	GCCGATGTCATGCAACAG
qRT-gcbA-F	TCGATCAGCGAACTCAACC
qRT-gcbA-R	GTCCTTCAGTGCCAGGTAG
qRT-pa4929-F	CGACGAACTGCTGGAATAC
qRT-pa4929-R	GCACGTACCAGAAATAGGC
qRT-dipA-F	CAGTTGTCGCTGTCGAAG
qRT-dipA-R	GGCGAGTTTCTCGATGTG
qRT-proE-F	GACATGCAGTGGCTCAAC
qRT-proE-R	CGTGCTCTTCGATCAACC
qRT-pa5442-F	TCGCGGTGATCATGCTG
qRT-pa5442-R	AGGCGAAGGTGAGGAAG
qRT-dgcH-F	ATCGATGGCAGCCTGAAC
qRT-dgcH-R	CCGCAGGTATTCTCGAAG
qRT-bifA-F	GGCGAGAACTTCGTCACCAC
qRT-bifA-R	GATCTTCGACAGCGGCTTGG
qRT-fimX-F	TCCTACATGGCGCTGTTC
qRT-fimX-R	TGGTAGCCCTTGAGGAAATC
qRT-morA-F	TCGTCCAGGTCAACGATTC
qRT-morA-R	TTGAGCTGGTTGGCTTCC
qRT-mucR-F	CAGCCGTACCAGATATCCC
qRT-mucR-R	TGGTCCTTGGCGTGATAC
qRT-RocR-F	CTGGTCGACAAGCTGTTC
qRT-RocR-R	ACCCAGTTGCGAAGGATG

qRT-tpbB-2-F	CTGATCGCCCGTTCCATCAG
qRT-tpbB-2-R	TAGACGATGGCGCTGGAGAC
qRT-wspR-F	GTGGCCAACCAGATCAAG
qRT-wspR-R	AGGACGATGATCGGGATG
qRT-proC-F	CAGGCCGGGCAGTTGCTGTC
qRT-proC-R	GGTCAGGCGCGAGGCTGTCT
qRT-ampC-F	AGATTCCCCTGCCTGTG
qRT-ampC-R	GCGGTGAAGGTCTTGCT
qRT-OprD-F	TCCGCAGGTAGCACTCA
qRT-OprD-R	AAGCCGGATTCATAGGTGG
qRT-recA-F	TCCGCAGGTAGCACTCAG
qRT-recA-R	AAGCCGGATTCATAGGTGG
qRT-gyrA-F	TGTGCTTTATGCCATGAGCGA
qRT-gyrA-R	TCCACCGAACCGAAGTTGC
qRT-fabD-F	GCATCCCTCGCATTCTGTCT
qRT-fabD-R	GGCGCTCTTCAGGACCATT

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☒ Approved the study protocol -OR- ☒ Provided guidelines for study procedures (if protocol approval is not required)

Informed consent

☒ We have obtained informed consent from all participants and this is noted in the manuscript.

Identifiable images

For publication of identifiable images of research participants, confirm that consent to publish was obtained and is noted in the Methods.

Authors must ensure that consent meets the conditions set out in the [Nature Portfolio participant release form](#).

☐ Yes ☒ No identifiable images of human research participants

Clinical studies

Policy information about [clinical studies](#)

Clinical trial registration

☒ We have provided the trial registration number from [ClinicalTrials.gov](#) or an equivalent agency in the manuscript.

Phase 2 and 3 randomized controlled trials

We have provided the [CONSORT checklist](#) with your submission.

☐ Yes ☐ No ☒ Not a phase 2/3 randomized controlled trial

Tumor marker prognostic studies

We have followed the [REMARK reporting guidelines](#).

☐ Yes ☐ No ☒ Not a tumor marker prognostic study

Archaeological, geological, and palaeontological materials

Policy information about studies involving [archaeological, geological, and palaeontological materials](#)

n/a We affirm that archaeological, geological, and palaeontological materials samples were collected (and, where applicable, exported) in a responsible manner and in accordance with relevant permits and local laws, and that this information is detailed within the manuscript.

I certify that all the above information is complete and correct.

Typed signature Yuming Cai Date 07/08/2024

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