Supplementary Information

Metabolomics study of the synergistic killing of polymyxin B in combination with amikacin against polymyxin-susceptible and -resistant *Pseudomonas aeruginosa*

Maytham Hussein[1,2], Mei-Ling Han[1], Yan Zhu[1], Qi (Tony) Zhou[3], Yu-Wei Lin[1], Robert. E.W. Hancock[4], Daniel Hoyer,[2,5,6] Darren J Creek[7], Jian Li[1*], Tony Velkov[2*]

[1]Monash Biomedicine Discovery Institute, Department of Microbiology, School of Biomedical Sciences, Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, Australia; [2]Department of Pharmacology & Therapeutics, School of Biomedical Sciences, Faculty of Medicine, Dentistry and Health Sciences, The University of Melbourne, Parkville, VIC, 3010, Australia; [3]Department of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University, West Lafayette, IN 47907, USA. [4]Department of Microbiology and Immunology, Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, British Columbia, Canada; [5]The Florey Institute of Neuroscience and Mental Health, The University of Melbourne, 30 Royal Parade, Parkville, VIC, 3052, Australia; [6]Department of Molecular Medicine, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, USA; [7]Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne 3052, Australia.

[†]**Correspondence:** Tony Velkov, Phone: + 61 3 83449846. Fax: +61 3 9903 9583. E-mail: Tony.Velkov@unimelb.edu.au OR Jian.Li@monash.edu

Multivariate analysis of global metabolic changes for *P. aeruginosa* FADDI-PA111 & *LESB58* after polymyxin B, amikacin, and their combination treatment

Untargeted metabolomics was employed to profile the metabolic changes in *P. aeruginosa* FADDI-PA111 and LESB58 treated with polymyxin B and amikacin monotherapy or in combination at 15 min, 1 and 4 h. Four biological replicates were independently prepared from different cultures on separate days, and all of the samples were analyzed in a single LC-MS run. The analytical and technical variations in the experiment were monitored based on periodic analysis of pooled biological quality control (PBQC) samples in the run. The median relative standard deviation (RSD) of the PBQC, an indicator for analytical reproducibility, was 16% for FADDI-PA111 and 18% for LESB58; which are well within the acceptable limits for metabolomics data (**Supplementary Tables 1A&B**).[1] In addition, the principle component analysis (PCA) plots for both test strains showed that the PBQC samples tightly clustered together, indicating well-controlled technical variability (**Supplementary Figure S1**). The intragroup median RSD varies from 17% to 30% for FADDI-PA111, and from 20% to 30% for LESB58 (**Supplementary Tables 1A and 1B**). A total of 1142 and 541 metabolites were putatively identified in FADDI-PA111 and LESB58, respectively, including carbohydrates, energy metabolites, amino acids, nucleotides, lipids and peptides. Partial least squares discriminant analysis (PLSDA) plots (**Supplementary Figures S2A and S2B**) and heatmaps (**Supplementary Figures S3A and S3B**) revealed the extent of global metabolic changes in FADDI-PA111 and LESB58 induced by antibiotic treatment at each time point. One-way ANOVA analysis of FADDI-PA111 revealed that approximately 4-10%, 3-4.7% and 8-17% of metabolites were significantly altered following polymyxin B, amikacin and their combination, respectively (**Supplementary Figure S3A**). On the other hand, a marginal response was observed with the highly resistant strain LESB58 in which only 4.6-7% and

0.5-3.3% of its metabolome underwent a significant perturbation following polymyxin B mono- or combination therapy and amikacin alone, respectively (**Supplementary Figure S4B**).

Treatment of FADDI-PA111 with the polymyxin B-amikacin combination perturbed 46 and 118 additional unique metabolites (i.e. that were not altered by either polymyxin B or amikacin monotherapy) at 15 min and 1 h, respectively (**Supplementary Figure 5A**). Similarly, the combination perturbed 16 and 15 additional metabolites in LESB58 that were not affected by each antibiotic monotherapy (**Supplementary Figure 5B**). At early time points (15 min and 1 h) polymyxin B monotherapy and the combination treatment induced a significant suppression of the levels of the perturbed metabolites; then at the latter time point (4 h), polymyxin B monotherapy induced a marked elevation in metabolite levels (**Supplementary Figures S6A and S6B)**.

Supplementary Table 1. Data precision of individual samples represented as the median relative standard deviation (RSD) for all metabolites of **(A)** *P. aeruginosa* FADDI-PA111 & **(B)** LESB58 based on all replicates (n=4) of each group (n=19 for technical replicates of PBQCs).

A.

15 min	Median RSD %
Control	27
Polymyxin B	24
Amikacin	22
Combination	21
1 h	
Control	17
Polymyxin B	29
Amikacin	22
Combination	22
4 h	
Control	24
Polymyxin B	24
Amikacin	25
Combination	21
PBQCs	16

B.

15 min	Median RSD %
Control	30
Polymyxin B	30
Amikacin	29
Combination	30
1 h	
Control	29
Polymyxin B	26
Amikacin	26
Combination	32
4 h	
Control	20
Polymyxin B	29
Amikacin	24
Combination	27
PBQCs	18

Supplementary Table 2. The influenced purine/pyrimidine nucleotide pools after treatment with polymyxin B, amikacin, and the combination at 1 hr (≥ 1.0-log2-fold, $p \leq 0.05$; FDR ≤ 0.1). Note: The significant metabolites represented in italic and bold font.

Purine metabolism				**Pyrimidine metabolism**		
Anabolism			***Catabolism***	***Catabolism***		***Anabolism***
Ribose 5-phosphate→IMP	**IMP→AMP[3]**	**IMP→GMP**	**Hypoxanthine [3]**	Uracil	Thymine	Carbamoyl phosphate
Ribose 5-phosphate [3]	Adenylosuccinate	Xanthine monophosphate	**Xanthine [1, 3]**	**Dihydrouracil [3], 5,6-Dihydrouracil[3]**	Dihydrothymine	Carbamoyl aspartic acid
Phosphoribosyl pyrophosphate	**ADP[3], ADP-ribose[3], dADP [3]**	GDP, dGDP	**Urate [3]**	3-Ureidopropionic acid	β-Ureidoisobutyric acid	4,5-Dihydroorotic acid
Phosphoribosylamine [2]	ATP, dATP	GTP, dGTP	5-Hydroxyisourate	β-Alanine	3-Aminoisobutyric acid	**Orotate [1]**
5'-Phosphoribosyl-4-carboxy-5-aminoimidazole		**GMP [3], dGMP[3]**	**Allantoate[3]**		**5-Methylcytosine**	**Uridine monophosphate (UMP)[3], UDP[3]**
5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)		**Guanine[3]**			**2',3'-Cyclic CMP[3], CMP[3]**	**dTMP [1,3], dTDP [1,3], dCMP[3], dAMP[3],** dTTP, dCDP, dCTP

[1] PMB affected metabolites.
[2] AMK affected metabolites.
[3] COM affected metabolites.

Supplementary Table 3. Significant metabolites identified following exposure to polymyxin B and amikacin alone and in combination in polymyxin-resistant LESB58. Significant fold-changes are highlighted in bold and italic. One-way ANOVA for multiple comparisons, FDR ≤ 0.1; Fisher's LSD, $p \leq 0.05$.

Mass	RT (min)	Formula	Putative metabolite	IDEOM Confidence	Map	Pathway	Log2-fold change			FDR
							Polymyxin B	Amikacin	Combination	
							15 min			
165.0462	13.10	C5H11NO3S	L-Methionine S-oxide	7	Amino acid metabolism	Methionine metabolism	***-7.5999***	***-7.1219***	0.88466	6.401E-05
612.1521	16.98	C20H32N6O12S2	Glutathione disulfide	8	Amino acid metabolism	Glutathione metabolism	***-2.0721***	-1.44	-0.59261	1.401E-05
312.0225	15.72	C9H16N2O5Se	gamma-Glutamyl-Se-methylselenocysteine	7	Amino acid metabolism	Selenoamino acid metabolism	***-0.99182***	-0.62288	-0.48509	2.137E-02
160.0846	10.77	C6H12N2O3	D-Alanyl-D-alanine	6	Amino acid metabolism	D-Alanine metabolism; Peptidoglycan biosynthesis	***-0.64792***	-0.33231	-0.12347	1.368E-02
147.0532	14.22	C5H9NO4	O-Acetyl-L-serine	10	Amino acid metabolism	Cysteine metabolism;Selenoamino acid metabolism	***-1.1389***	-0.19854	-0.48976	5.137E-03
147.0532	14.22	C5H9NO4	L-Glutamate	10	Amino acid metabolism	Arginine and proline metabolism; Glutathione metabolism	***-1.1389***	-0.46318	0.21307	1.755E-03
426.0881	16.59	C13H22N4O8S2	S-glutathionyl-L-cysteine	8	Amino acid metabolism	Cysteine metabolism	***-1.4395***	0.54329	0.12453	5.435E-02
161.0477	8.25	C9H7NO2	4,8-Dihydroxyquinoline	7	Amino acid metabolism	Tryptophan metabolism	-1.3968	-0.88934	***-0.68394***	1.928E-02

612.1521	16.98	C20H32N6O12S2	Glutathione disulfide	8	Amino acid metabolism	Glutamate metabolism; Glutathione metabolism	-2.0721	-1.44	***-1.2132***	2.013E-02
194.0426	13.95	C6H10O7	2-Dehydro-D-gluconate	8	Carbohydrate metabolism	Pentose phosphate pathway	***-1.1452***	-0.77251	***-0.76351***	5.435E-04
104.0109	13.95	C3H4O4	2-Hydroxy-3-oxopropanoate	6	Carbohydrate metabolism	Ascorbate and aldarate metabolism;Glyoxylate and dicarboxylate metabolism	***-1.0926***	-0.70987	-0.51012	7.175E-02
185.9929	16.37	C3H7O7P	3-Phospho-D-glycerate	10	Carbohydrate metabolism	Glycolysis / Gluconeogenesis, Glycine, serine and threonine ,Glycerolipid metabolism	***-0.84335***	-0.50782	0.07307	4.798E-04
563.0555	15.29	C15H23N3O16P2	UDP-L-Ara4FN	8	Carbohydrate metabolism	Nucleotide sugars metabolism	***-0.89003***	-0.44922	0.03993	7.798E-04
179.0794	14.54	C6H13NO5	D-Glucosamine	7	Carbohydrate metabolism	Aminosugars metabolism	***-1.2901***	-1.2614	***-0.87236***	3.798E-04
621.0610	17.14	C17H25N3O18P2	UDP-N-acetyl-2-amino-2-deoxy-D-glucuronate	7	Carbohydrate metabolism	Aminosugars metabolism	***-0.91453***	-0.30889	-0.61197	9.798E-02
580.0345	18.40	C15H22N2O18P2	UDP-glucuronate	8	Carbohydrate metabolism	Pentose and glucuronate interconversions; Nucleotide sugars metabolism	***-0.90056***	-0.30897	-0.02344	4.769E-02
180.0634	14.58	C6H12O6	D-Glucose	8	Carbohydrate metabolism	Glycolysis / Gluconeogenesis; Pentose phosphate pathway; Galactose ,Starch and sucrose metabolism	-0.8497	-0.50118	***-0.86691***	7.889E-08
150.0528	14.53	C5H10O5	L-Arabinose	6	Carbohydrate metabolism	Pentose and glucuronate interconversions; Nucleotide sugars metabolism	-0.41275	-0.6668	***-0.74672***	1.078E-07
290.0405	15.73	C7H15O10P	D-Sedoheptulose 7-phosphate	8	Carbohydrate	Pentose phosphate	***-0.85096***	-0.45427	-0.50056	1.180E-

					metabolism	pathway; Carbon fixation				02
743.0757	16.27	C21H28N7O17P3	NADP	10	Energy metabolism	Photosynthesis; Glutathione metabolism; Nicotinate and nicotinamide metabolism	***-0.67683***	-0.25445	0.28846	1.459E-02
663.1095	13.84	C21H27N7O14P2	NAD	10	Energy metabolism	Oxidative phosphorylation; Glutamate metabolism; Nicotinate and nicotinamide metabolism	***-0.63447***	-0.21837	0.29254	1.749E-02
515.6802	7.51	C39H68N7O17P3S	Oleoyl-CoA	6	Lipid metabolism	Biosynthesis of unsaturated fatty acids	***-1.0863***	-0.98142	-0.91111	3.149E-03
216.0401	14.35	C5H13O7P	2-C-Methyl-D-erythritol 4-phosphate	6	Lipid metabolism	Biosynthesis of steroids	-1.4587	-.98341	***-1.0548***	2.104E-04
173.1417	6.24	C9H19NO2	[FA amino(9:0)] 3R-aminononanoic acid	7	Lipid metabolism	Amino Fatty Acids	-1.4395	0.48062	***-1.3023***	2.226E-03
188.1412	4.07	C10H20O3	[FA hydroxy(10:0)] 9-hydroxy-decanoic acid	6	Lipid metabolism	Fatty Acids and Conjugates	-0.060985	-0.00763	***-2.4996***	2.037E-02
287.2458	4.35	C16H33NO3	Lauroyl diethanolamide	7	Lipid metabolism	Fatty Acyls	-0.13359	-0.082231	***-1.1383***	2.374E-03
340.2608	4.21	C20H36O4	[FA (20:2)] hydroperoxy-eicosadienoic acid	7	Lipid metabolism	Fatty Acids and Conjugates	-0.6777	-0.76651	***1.11***	2.374E-06
258.1830	4.34	C14H26O4	Tetradecanedioic acid	7	Lipid metabolism	Fatty Acids and Conjugates	-0.22685	-0.35927	***1.0139***	1.374E-04
257.1030	14.39	C8H20NO6P	sn-glycero-3-Phosphocholine	10	Lipid metabolism	Glycerophospholipid metabolism; Ether lipid metabolism	-0.19634	0.026812	***0.94275***	7.420E-02
482.9844	17.62	C9H16N3O14P3	CTP	10	Nucleotide metabolism	Pyrimidine metabolism	***-1.0393***	-0.49808	0.13509	1.221E-02
403.0183	16.45	C9H15N3O11P2	CDP	8	Nucleotide metabolism	Pyrimidine metabolism	***-0.71695***	-0.28482	0.31577	1.254E-02

136.0386	9.99	C5H4N4O	Hypoxanthine	10	Nucleotide metabolism	Purine metabolism	-0.54538	-0.24737	***1.2739***	1.204E-03
405.1385	18.06	C15H23N3O10	Glu-Glu-Glu	7	Peptides metabolism	Acidic peptide	***-7.5786***	***-3.8461***	***-0.84025***	1.840E-02
534.1816	19.09	C20H30N4O13	Glu-Glu-Glu-Glu	7	Peptides metabolism	Acidic peptide	***-6.2528***	***-5.7748***	***-5.7988***	3.486E-02
276.0960	16.72	C10H16N2O7	Glu-Glu	7	Peptides metabolism		***-1.7927***	-0.92497	0.016992	3.582E-02
460.4640	15.68	C18H31N5O9	L-Ala-D-Glu-meso-A2pm	6	Peptides metabolism	Peptidoglycan biosynthesis	***-0.63806***	-0.37585	0.22932	4.069E-02
224.0589	5.24	C13H8N2O2	phenazine-1-carboxylate	8	Undefined	phenazine-1-carboxylate & 2-hydroxyphenazine biosynthesis	***-3.5536***	-0.89754	***-1.3836***	1.860E-07
231.1259	4.91	C14H17NO2	Leiokinine A	6	Undefined	Undefined	-1.3018	-1.6398	***-6.1463***	4.999E-03
326.1918	3.80	C18H30O3S	2-Dodecylbenzenesulfonic acid	6	Undefined	Undefined	-0.021896	0.48381	***0.93657***	2.308E-05
137.9987	7.48	C3H6O4S	2-oxopropane sulfonate	6	Undefined	Undefined	0.54672	-0.9506	***3.6557***	4.811E-03
266.1551	3.81	C12H26O4S	sodium dodecyl sulfate	7	Undefined	Undefined	-1.0399	-0.64365	***3.0042***	6.667E-02
113.9447	20.90	H2O3S2	H2S2O3	8	Undefined	Undefined	0.41202	0.014432	***2.0416***	7.681E-03
							1h			
129.0427	7.46	C5H7NO3	L-1-Pyrroline-3-hydroxy-5-carboxylate	6	Amino acid metabolism	Arginine and proline metabolism	***-1.1644***	0.1669	-0.001232	2.340E-02
138.0429	10.37	C6H6N2O2	Urocanate	10	Amino acid metabolism	Histidine metabolism	***-2.2423***	-1.5838	-0.45621	1.100E-06
165.0462	13.10	C5H11NO3S	L-Methionine S-oxide	7	Amino acid metabolism	Methionine metabolism	***0.62124***	-0.63594	0.26735	1.200E-06

129.0427	9.89	C5H7NO3	5-Oxoproline	9	Amino acid metabolism	Glutathione metabolism	-1.1644	***-1.4879***	-0.39902	4.860E-04
138.0429	10.37	C6H6N2O2	Urocanate	10	Amino acid metabolism	Histidine metabolism	-1.2423	***-2.5838***	-0.02316	4.529E-04
132.0898	22.10	C5H12N2O2	L-Ornithine	10	Amino acid metabolism	Arginine and proline metabolism; D-Arginine and D-ornithine metabolism; Glutathione metabolism	-0.48701	-0.05431	***-0.82265***	4.635E-04
342.1163	14.81	C12H22O11	1-alpha-D-Galactosyl-myo-inositol	7	Carbohydrate metabolism	Galactose metabolism	-0.21342	-0.38994	***-0.79281***	6.600E-05
262.0449	14.36	C6H15O9P	D-Mannitol 1-phosphate	7	Carbohydrate metabolism	Fructose and mannose metabolism	0.46098	-0.5467	***-0.98358***	5.421E-03
164.0686	7.47	C6H12O5	L-Rhamnulose	6	Carbohydrate metabolism	Fructose and mannose metabolism	0.00213	0.03216	***2.5703***	7.683E-03
182.0791	13.70	C6H14O6	Mannitol	10	Carbohydrate metabolism	Fructose and mannose metabolism	-0.77644	-0.38216	***2.8228***	5.600E-05
188.1412	4.07	C10H20O3	[FA hydroxy(10:0)] 9-hydroxy-decanoic acid	6	Lipid metabolism	Fatty Acids and Conjugates	***-2.9594***	-0.13905	-0.20017	4.502E-02
351.3137	4.22	C22H41NO2	N-(11Z,14Z-eicosadienoyl)-ethanolamine	7	Lipid metabolism	Fatty amides	***-7.0566***	-0.37887	-0.43527	3.000E-06
281.2720	4.32	C18H35NO	[FA (18:1)] 9Z-octadecenamide	7	Lipid metabolism	Fatty amides	***-0.91243***	-0.26163	***-1.2172***	5.600E-05
118.0631	7.45	C5H10O3	Methyl 3-hydroxybutyrate	7	Lipid metabolism	Undefined	***0.63759***	0.24605	-0.11484	5.670E-04
258.1830	4.34	C14H26O4	[FA (14:0/2:0)] Tetradecanedioic acid	7	Lipid metabolism	Fatty Acids and Conjugates	***1.1663***	0.11072	-0.68301	2.200E-03
173.1417	7.09	C9H19NO2	[FA amino(9:0)] 3R-aminononanoic acid	7	Lipid metabolism	Amino Fatty Acids	***1.5666***	**1.0313**	***-0.83526***	3.334E-03
501.6649	7.61	C37H64N7O17P3S	PalmitoleylCoA	6	Lipid metabolism	Fatty acids biosynthesis	***-0.81337***	0.45368	0.28209	3.410E-04

510.2956	3.99	C24H47O9P	[PG (18:1)] 1-(9E-octadecenoyl)-sn-glycero-3-phospho-(1'-sn-glycerol)	6	Lipid metabolism	Glycerophosphoglycerols	***1.1122***	0.16879	***0.8169***	3.660E-04
576.3790	3.64	C30H57O8P	PA(27:1)	6	Lipid metabolism	Glycerophosphates	***0.76572***	0.073461	0.23124	7.690E-02
493.3168	7.44	C24H48NO7P	LysoPC(16:1(9Z))	6	Lipid metabolism	Glycerophosphocholines	***0.79872***	0.48033	-0.088884	5.255E-03
229.2405	5.07	C14H31NO	[SP (14:0)] 1-deoxy-tetradecasphinganine	7	Lipid metabolism	Sphingoid bases	***-0.79962***	-1.0244	***-1.4779***	8.764E-03
257.1030	14.39	C8H20NO6P	sn-glycero-3-Phosphocholine	10	Lipid metabolism	Glycerophospholipid metabolism	***0.77398***	**0.62314**	-0.15812	4.566E-03
515.6802	7.51	C39H68N7O17P3S	Oleoyl-CoA	6	Lipid metabolism	Biosynthesis of unsaturated fatty acids	***-0.82446***	-0.66111	-0.28644	8.902E-03
225.9871	12.08	C5H7O8P	[PA] 1,2-diacyl-sn-glycero-3-phosphate	6	Lipid metabolism	GlycerophosphoLipid	***0.62447***	0.56901	0.02437	4.557E-04
674.4888	3.84	C37H71O8P	PA(34:1)	7	Lipid metabolism	Glycerophosphates	0.83948	***0.76673***	-0.52988	2.100E-05
150.1044	4.72	C10H14O	[PR] (-)-Carvone	7	Lipid metabolism	Monoterpenoid biosynthesis; Limonene and pinene degradation	-0.69269	-0.28857	***-0.8422***	8.401E-02
222.1620	4.34	C14H22O2	7E,9E,11-Dodecatrienyl acetate	6	Lipid metabolism	Fatty esters	-0.24987	-0.37112	***-0.92367***	6.758E-03
142.0994	4.57	C8H14O2	FA (8:1)	7	Lipid metabolism	Fatty Acids and Conjugates	-0.34502	-0.22322	***-0.98658***	5.350E-04
298.2873	3.80	C19H38O2	FA methyl(18:0)	7	Lipid metabolism	Fatty Acids and Conjugates	-0.56431	0.043401	***-1.0455***	7.861E-03
168.0283	11.96	C5H4N4O3	Urate	10	Nucleotide metabolism	Purine metabolism	***1.0698***	-0.34379	-0.25133	7.200E-04
306.0856	9.21	C14H14N2O6	2-oxindole-3-acetyl-asp	7	Undefined	Undefined	***1.3531***	***1.503***	***0.84977***	4.400E-05

149.1052	9.35	C6H15NO3	Triethanolamine	7	Undefined	Undefined	***-1.6516***	***-1.3446***	***-1.4302***	4.400E-05
243.1831	4.83	C13H25NO3	N-Undecanoylglycine	7	Undefined	Undefined	***-1.5017***	-0.97803	***-0.92982***	5.044E-02
215.1521	5.24	C11H21NO3	N-Nonanoylglycine	7	Undefined	Undefined	***-1.4957***	-1.1484	***-1.411***	2.311E-03
309.3035	4.27	C20H39NO	N-Hexadecanoylpyrrolidine	6	Undefined	Undefined	***-1.3586***	0.47073	***-1.6755***	5.941E-03
229.1678	5.04	C12H23NO3	N-Decanoylglycine	6	Undefined	Undefined	***-1.2343***	***-1.5254***	***-0.83041***	3.459E-02
266.1551	3.81	C12H26O4S	sodium dodecyl sulfate	7	Undefined	Undefined	***-1.1206***	-0.49145	***1.6589***	1.600E-06
86.0730	4.72	C5H10O	3-Methylbutanal	8	Undefined	leucine degradation III	0.97557	***-1.8471***	0.35249	5.111E-03
231.1259	4.91	C14H17NO2	Leiokinine A	6	Undefined	Undefined	-0.45431	***-1.2529***	***-1.0201***	9.210E-02
302.2246	4.22	C20H30O2	Retinyl ester	7	Undefined	Undefined	-0.76867	***-1.2349***	-0.71977	4.561E-04
138.0317	5.04	C7H6O3	Sesamol	7	Undefined	Undefined	0.91138	***-1.5701***	2.5703	4.500E-04
137.9987	7.48	C3H6O4S	2-oxopropane sulfonate	6	Undefined	Undefined	0.43217	0.01543	***5.4643***	5.012E-02
299.2250	4.54	C20H29NO	2-Undecyl-4(1H)-quinolinone	6	Undefined	Undefined	0.45691	0.50698	***0.7021***	4.516E-03
138.0430	7.47	C6H6N2O2	4-Nitroaniline	6	Undefined	Undefined	-1.5077	-1.7301	***-2.6327***	2.130E-04
134.0731	5.26	C9H10O	Indan-1-ol	6	Undefined	Undefined	-0.13608	-0.1011	***1.6025***	5.003E-02
439.4027	4.16	C27H53NO3	Pentacosanoylglycine	6	Undefined	Undefined	-0.501452	-0.1475	***-0.8168***	9.679E-02

298.1418	7.45	C15H22O6	Toxin T2 tetrol	7	Undefined	Undefined	0.00541	0.76546	***7.1457***	7.858E-04
137.0477	7.51	C7H7NO2	Anthranilate	10	Xenobiotic biodegradation	Benzoate degradation via hydroxylation; Phenylalanine &Tryptophan metabolism;	-0.48712	-0.49344	***0.73156***	2.430E-04
							4h			
129.0427	9.89	C5H7NO3	5-Oxoproline	9	Amino Acid Metabolism	Glutathione metabolism	***1.0977***	1.0309	0.391	1.646E-03
165.0462	13.10	C5H11NO3S	L-Methionine S-oxide	7	Amino Acid Metabolism	Methionine metabolism	***1.3273***	0.5536	1.1297	2.054E-03
221.0901	11.73	C8H15NO6	N-Acetyl-D-glucosamine	8	Amino Acid Metabolism	Glutamate metabolism; Aminosugars metabolism	0.67253	0.49892	0.070738	5.410E-04
612.1521	16.98	C20H32N6O12S2	Glutathione disulfide	8	Amino Acid Metabolism	Glutamate metabolism; Glutathione metabolism	0.22323	***1.2833***	0.26186	5.361E-02
149.0511	11.36	C5H11NO2S	L-Methionine	10	Amino Acid Metabolism	Methionine metabolism	0.76187	***1.0339***	0.45203	1.543E-02
165.0462	13.10	C5H11NO3S	L-Methionine S-oxide	7	Amino Acid Metabolism	Methionine metabolism	1.3273	***1.5536***	1.1297	6.545E-02
161.0688	11.11	C6H11NO4	O-Acetyl-L-homoserine	8	Amino Acid Metabolism	Methionine metabolism; Sulfur metabolism	0.84504	***1.3323***	0.16532	5.555E-04
138.0429	10.37	C6H6N2O2	Urocanate	10	Amino Acid Metabolism	Histidine metabolism	0.05647	***1.9377***	0.10879	3.623E-02
189.0425	7.52	C10H7NO3	Kynurenate	10	Amino Acid Metabolism	Tryptophan metabolism	0.45771	0.12079	***0.66313***	7.372E-04
179.0794	14.54	C6H13NO5	D-Glucosamine	7	Carbohydrate Metabolism	Aminosugars metabolism	***1.0616***	***1.6558***	***0.96637***	1.375E-03
180.0634	14.58	C6H12O6	D-Glucose	8	Carbohydrate Metabolism	Glycolysis / Gluconeogenesis; Pentose phosphate pathway;	***1.1627***	1.7737	***1.2773***	7.110E-04

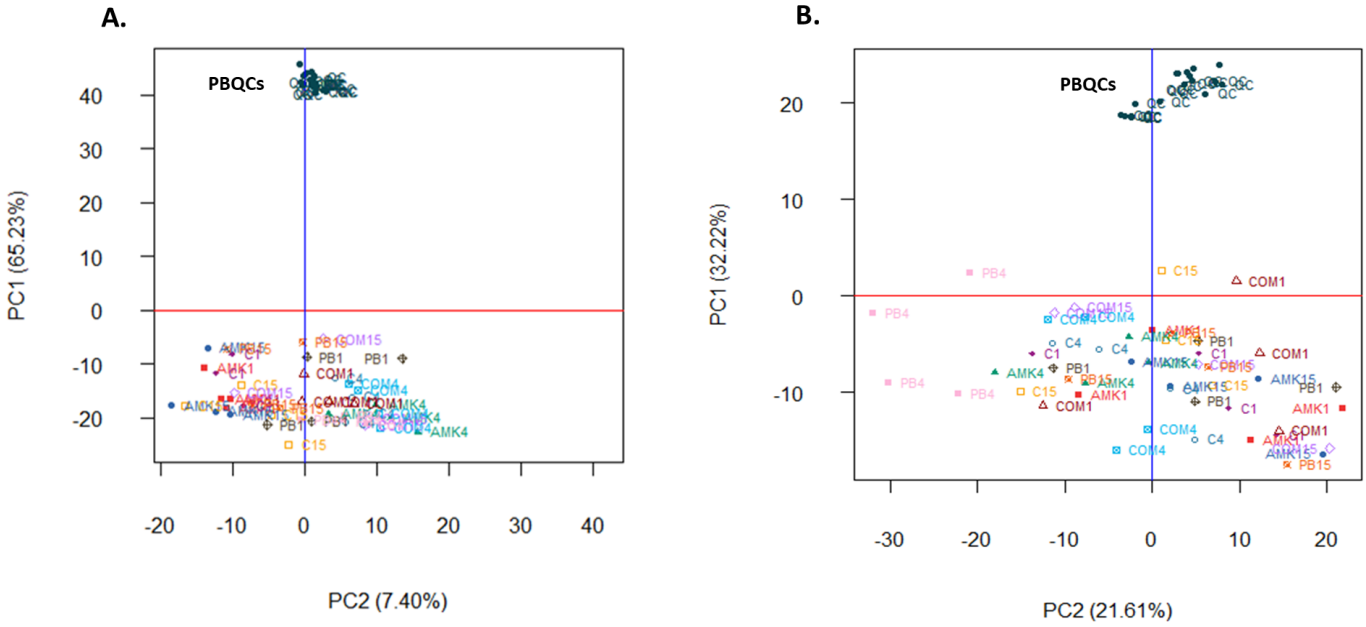
275.1007	7.82	C11H17NO7	1,6-anhydro-N-acetylmuramate	8	Carbohydrate metabolism	Aminosugars metabolism	***0.75264***	0.48842	0.012913	6.640E-04
342.1163	14.81	C12H22O11	1-alpha-D-Galactosyl-myo-inositol	7	Carbohydrate metabolism	Galactose metabolism	***0.59541***	0.50981	0.45865	4.646E-02
88.0523	7.46	C4H8O2	Butanoic acid	6	Carbohydrate Metabolism	Butanoate metabolism	***0.65677***	0.37058	0.072155	4.605E-02
150.0528	14.53	C5H10O5	L-Arabinose	6	Carbohydrate Metabolism	Pentose and glucuronate interconversions; Nucleotide sugars metabolism	***0.671***	0.07632	0.04512	1.054E-02
293.1107	9.89	C11H19NO8	N-Acetylmuramate	8	Carbohydrate metabolism	Aminosugars metabolism	***1.4331***	0.90324	0.56312	1.054E-04
192.0271	17.50	C6H8O7	Citrate	10	Carbohydrate Metabolism	Citrate cycle (TCA cycle);Glutamate metabolism; Alanine and aspartate metabolism;Glyoxylate and dicarboxylate metabolism; Reductive carboxylate cycle (CO2 fixation)	-0.23258	-0.04710	***-0.61015***	2.687E-02
281.2720	4.32	C18H35NO	[FA (18:1)] 9Z-octadecenamide	7	Lipid metabolism	Fatty amides	***1.8107***	***2.358***	***2.6155***	5.757E-04
216.0401	14.35	C5H13O7P	2-C-Methyl-D-erythritol 4-phosphate	6	Lipid Metabolism	Biosynthesis of steroids	***1.1641***	0.6837	***0.99432***	1.230E-04
312.3031	3.90	C20H40O2	FA (20:0)	7	Lipid Metabolism	Fatty Acids and Conjugates	***1.0331***	0.1782	***1.2207***	6.540E-04
300.2666	4.04	C18H36O3	FA hydroxy(18:0)	6	Lipid Metabolism	Fatty Acids and Conjugates	***1.4768***	1.1219	***1.3136***	2.500E-05
142.0994	4.57	C8H14O2	FA (8:1)	7	Lipid Metabolism	Fatty Acids and Conjugates	-0.91316	***-0.73133***	***-0.88279***	5.000E-05
256.2401	3.97	C16H32O2	FA(16:0)	8	Lipid Metabolism	Fatty acid biosynthesis; Fatty acid elongation in	0.71791	***0.82066***	***0.9288***	1.235E-02

						mitochondria; Biosynthesis of unsaturated fatty acids				
287.2458	4.35	C16H33NO3	Lauroyl diethanolamide	7	Lipid Metabolism	Undefined	-0.63925	***-1.2017***	***-1.3047***	2.438E-04
284.2715	3.93	C18H36O2	Octadecanoic acid	8	Lipid Metabolism	Fatty acid biosynthesis; Biosynthesis of unsaturated fatty acids	***1.1257***	***1.2103***	***1.3226***	1.460E-03
576.3790	3.64	C30H57O8P	PA(27:1)	6	Lipid Metabolism	Glycerophosphates	***1.2251***	-0.00356	***1.1006***	7.746E-04
258.1830	4.34	C14H26O4	FA methyl(15:0) tridecanedioic acid	7	Lipid Metabolism	Fatty Acids and Conjugates	***1.2117***	1.5271	1.8673	2.980E-02
327.3137	5.12	C20H41NO2	N,N-Dimethylsphing-4-enine	6	Lipid metabolism	Sphingoid bases	***0.60779***	0.29783	0.70324	1.054E-02
515.6802	7.51	C39H68N7O17P3S	Oleoyl-CoA	6	Lipid Metabolism	Biosynthesis of unsaturated fatty acids	***1.3827***	0.14697	0.23106	6.057E-04
173.1417	6.24	C9H19NO2	[FA amino(9:0)] 3R-aminononanoic acid	7	Lipid Metabolism	Amino Fatty Acids	0.25533	***-0.88406***	0.1592	5.285E-02
340.3341	3.88	C22H44O2	Docosanoic acid	6	Lipid Metabolism	Biosynthesis of unsaturated fatty acids	0.7739	0.82107	***1.0437***	5.687E-02
270.2560	3.95	C17H34O2	FA (17:0)	6	Lipid Metabolism	Fatty Acids and Conjugates	0.78007	0.89118	***1.0869***	5.908E-02
214.1932	4.08	C13H26O2	FA methyl(12:0) dodecanoic acid	5	Lipid Metabolism	Fatty Acids and Conjugates	0.62896	0.10098	***1.0196***	6.097E-03
242.2247	4.00	C15H30O2	FA methyl(14:0)	5	Lipid Metabolism	Fatty Acids and Conjugates	0.66988	0.82051	***1.012***	1.097E-02
338.3185	3.88	C22H42O2	FA (22:1)	5	Lipid Metabolism	Fatty Acids and Conjugates	-0.36074	0.43925	***1.2306***	2.147E-03
246.0506	12.29	C6H15O8P	Glycerophosphoglycerol	7	Lipid Metabolism	Glycerophospholipid metabolism	-0.055426	0.43727	***-0.70544***	9.557E-02

327.3137	5.12	C20H41NO2	Stearoyl-EA	6	Lipid metabolism	Fatty amides	0.60779	0.27481	***0.70324***	7.838E-02
102.0429	7.46	C3H6N2O2	N-Formiminoglycine	6	Nucleotide metabolism	Purine metabolism	***0.59541***	0.04371	0.05692	5.057E-02
390.1756	16.21	C15H26N4O8	L-Ala-D-Glu-meso-A2pm		Peptides metabolism	Peptidoglycan biosynthesis	***0.6232***	0.23399	0.031243	5.054E-02
86.0730	4.72	C5H10O	3-Methylbutanal	8	Undefined	leucine degradation III	-0.9642	***-5.8974***	***-1.2316***	8.752E-03
243.1831	4.83	C13H25NO3	N-Undecanoylglycine	7	Undefined	Undefined	***1.2175***	***1.7638***	***2.9454***	3.425E-02
145.0525	4.76	C9H7NO	Quinolin-4-ol	6	Undefined	Undefined	***0.79119***	0.04566	***0.75885***	7.746E-02
243.1624	5.25	C16H21NO	Chalciporone	7	Undefined	Undefined	***0.63178***	-0.33865	0.17718	4.867E-02
376.1632	8.43	C19H24N2O6	Diacetylfusarochromanone	7	Undefined	Undefined	***0.59973***	0.06512	0.53263	4.867E-02
289.1682	4.52	C17H23NO3	Dihydroferuperine	6	Undefined	Undefined	***1.0645***	-0.01693	0.92506	4.920E-02
478.1803	10.23	C19H30N2O12	glcNAc-1,6-anhMurNAc	8	Undefined	Undefined	***0.74994***	0.32095	0.25752	2.054E-02
287.1885	5.21	C18H25NO2	Isolobinine	7	Undefined	Undefined	***1.1992***	0.96339	1.1972	4.054E-02
263.1523	3.85	C15H21NO3	N-(Heptan-4-yl)benzo[d][1,3]dioxole-5-carboxamide	6	Undefined	Undefined	***0.58836***	-0.15104	0.39269	5.054E-02
306.0856	9.21	C14H14N2O6	2-oxindole-3-acetyl-asp	7	Undefined	Undefined	0.02346	***-0.74065***	0.03415	5.357E-02
269.3083	7.47	C18H39N	Octadecylamine	7	Undefined	Undefined	0.41772	***-0.70035***	0.11838	5.573E-03
138.0317	5.04	C7H6O3	Sesamol	7	Undefined	Undefined	-0.69245	***-2.2051***	-0.71272	6.232E-

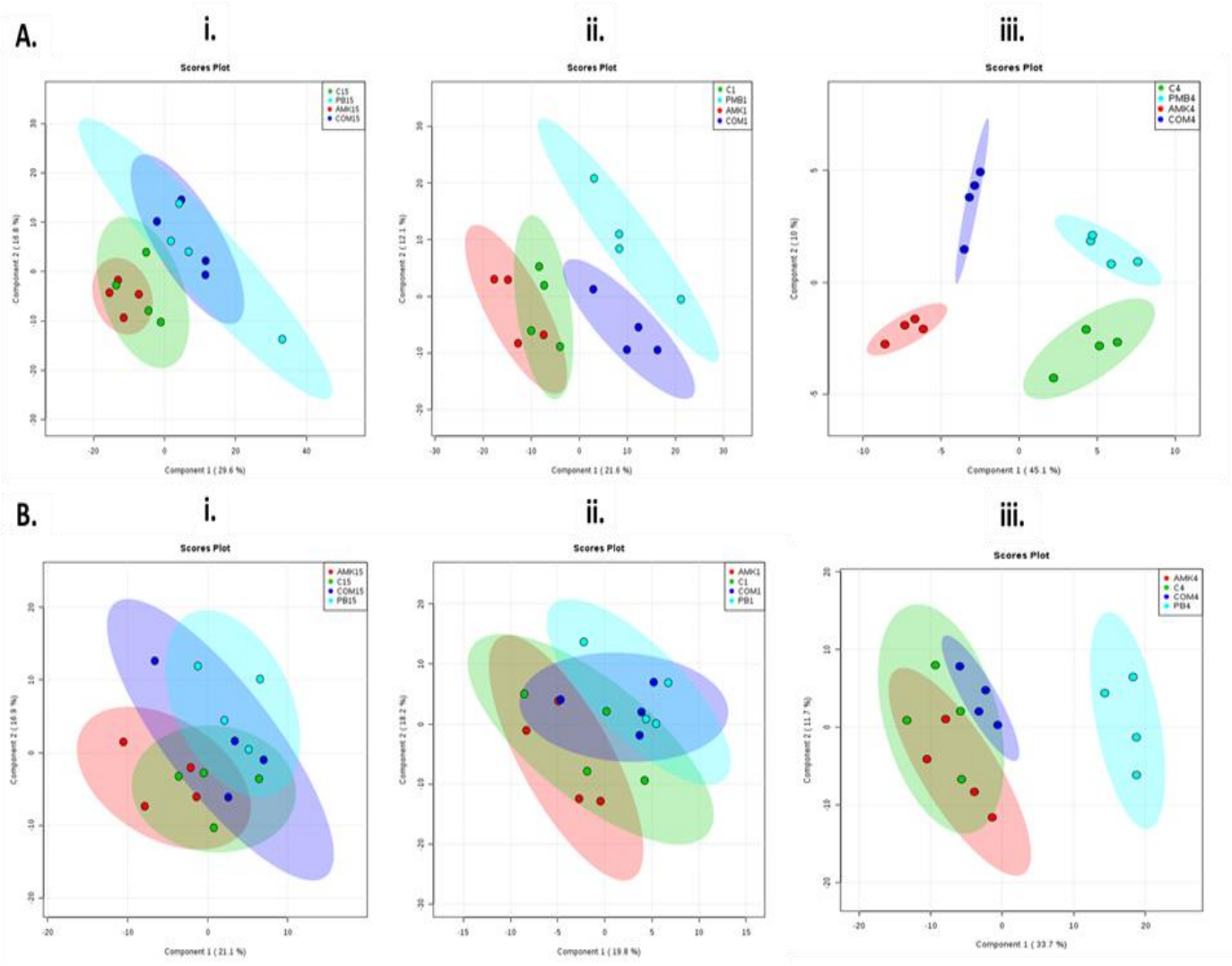
										03
716.5143	3.65	C43H73O6P	2,3-Bis-O-(geranylgeranyl)glycerol 1-phosphate	6	Undefined	Undefined	0.62384	0.046334	***0.80838***	5.623E-02
102.0681	6.73	C5H10O2	Ethylmethylaceticacid	7	Undefined	isoleucine biosynthesis V;epoxypseudoisoeugenol-2-methylbutyrate biosynthesis	0.61402	0.37385	***0.67823***	5.882E-02
215.1886	6.69	C12H25NO2	Heptanoylcholine	7	Undefined	Undefined	1.0943	1.0439	***0.95255***	6.557E-02
241.2770	11.85	C16H35N	Hexadeceylamin	7	Undefined	Undefined	0.75812	0.15706	***0.76861***	9.858E-03
285.1730	6.07	C18H23NO2	Isococculidine	7	Undefined	Undefined	0.4595	0.31762	***0.63449***	8.858E-02
229.1678	5.04	C12H23NO3	N-Decanoylglycine	6	Undefined	Undefined	-0.55201	0.41797	***0.78051***	7.932E-02
309.3035	4.27	C20H39NO	N-Hexadecanoylpyrrolidine	7	Undefined	Undefined	0.1572	0.03189	***1.9085***	8.140E-02
215.1521	5.24	C11H21NO3	N-Nonanoylglycine	7	Undefined	Undefined	-0.67396	-0.52231	***0.77007***	9.358E-03

Supplementary Figure S1. PCA score plots of all metabolites of **(A)** *P. aeruginosa* FADDI-PA111 & **(B)** LESB58 treated with polymyxin B and amikacin alone and in combination. Nineteen PBQCs were analysed throughout the LC-MS batch. Each dataset represents four biological replicates of antibiotic-treated and untreated control samples of all the time points. Pooled biological quality controls = PBQCs.

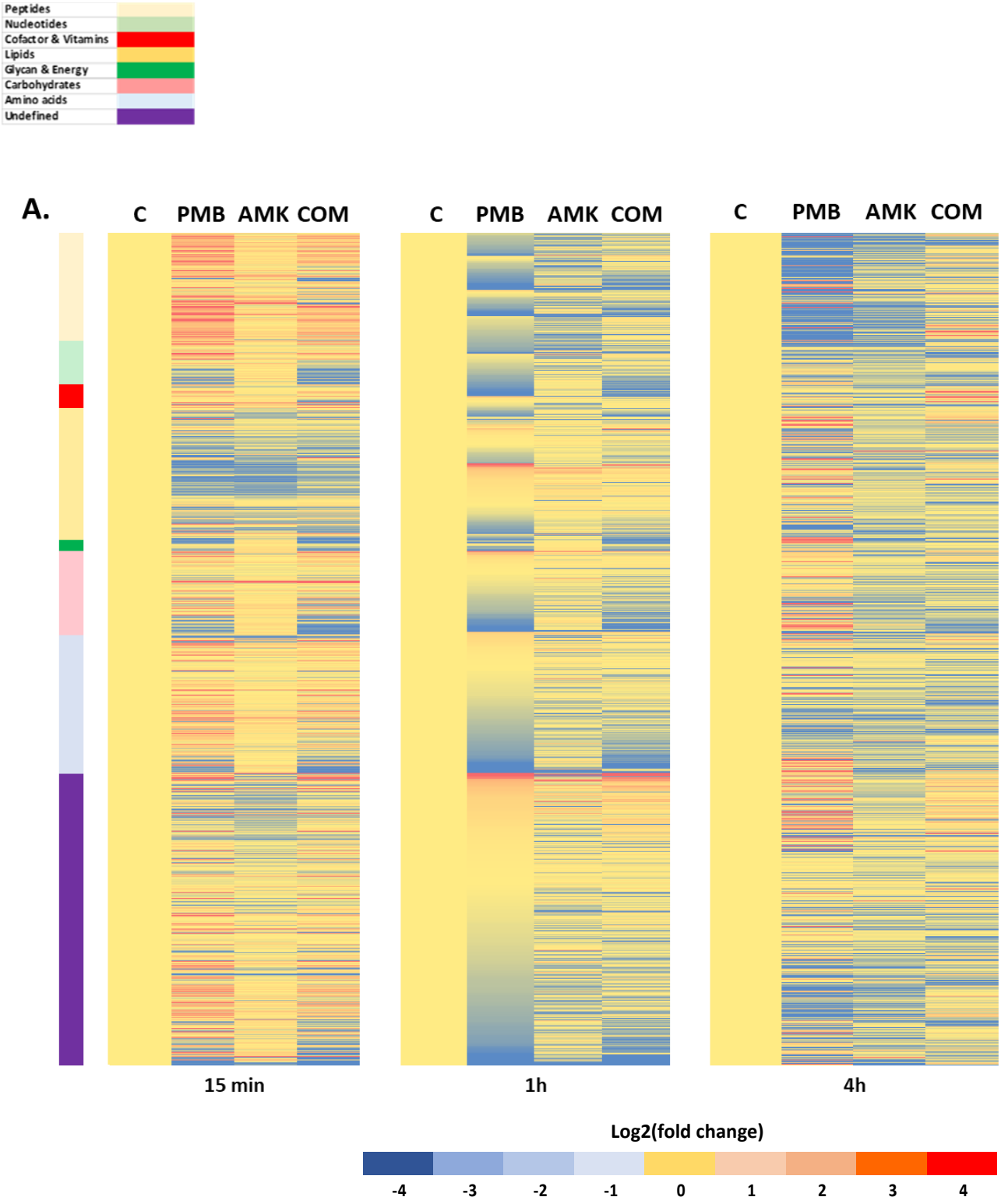


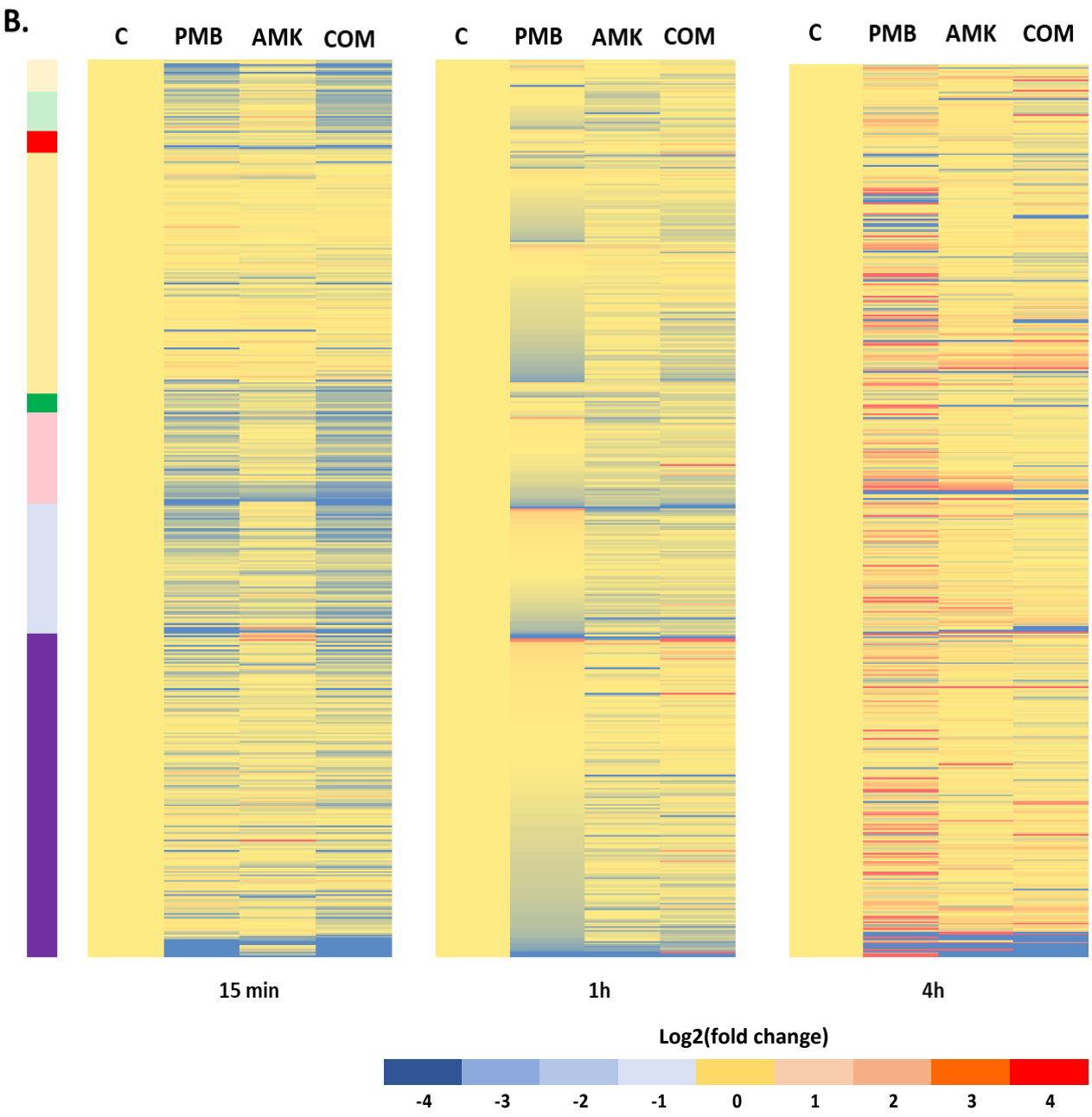
C	**Control**
PB	**Polymyxin B**
AMK	**Amikacin**
COM	**Combination**

Supplementary Figure S2. Multivariate analyses of global metabolic changes. PLSDA score plots for metabolite levels of (A) FADDI-PA111 and (B) LESB58 samples treated with polymyxin B, amikacin and the combination at 15 min, 1 h, and 4 h. Each data set represents a total of 16 samples of 4 biological replicates of each condition. Green = untreated control (C); Cyan = polymyxin B alone (PMB); Red = amikacin (AMK); Purple = polymyxin B and amikacin combination (COM).

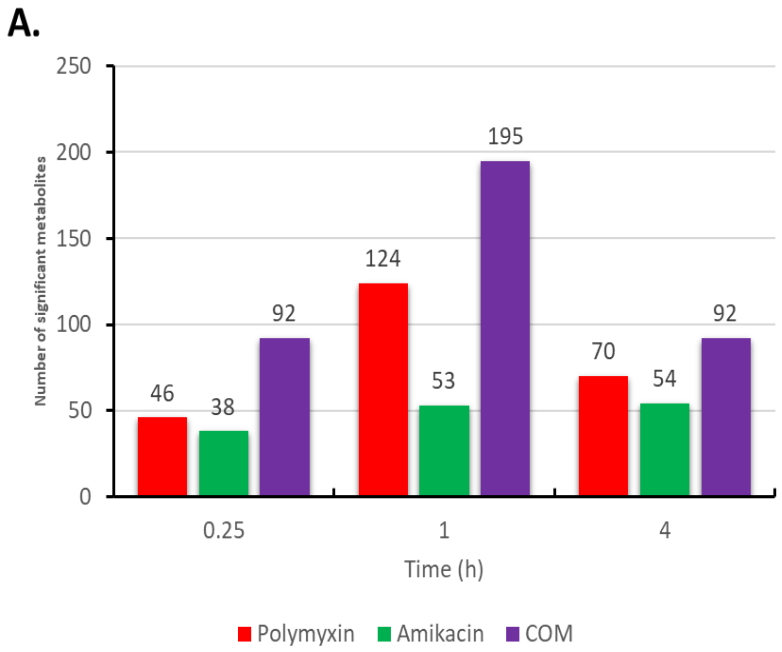


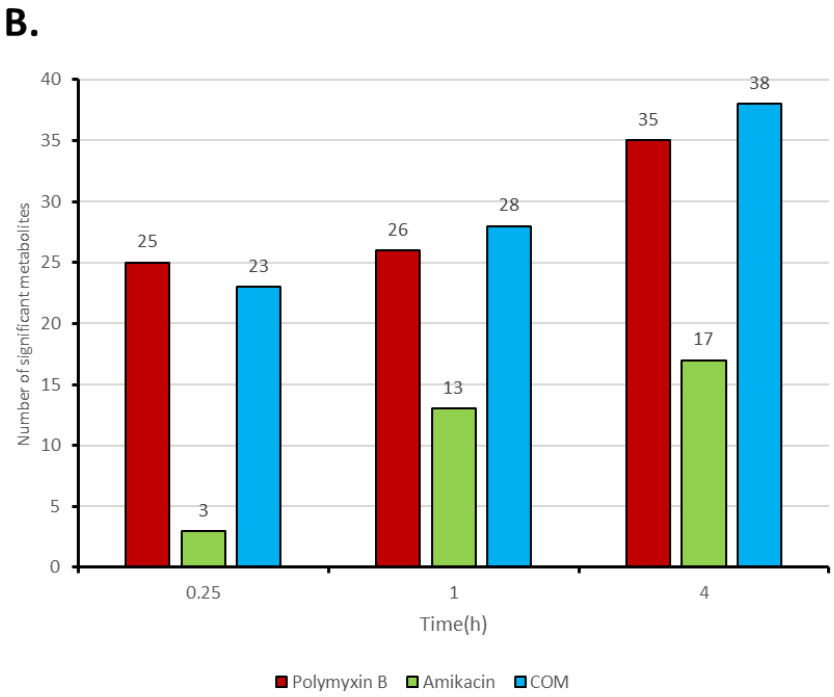
Supplementary Figure S3. Monotherapy and combination (COM) of polymyxin B (PMB) and amikacin (AMK) induce global metabolic changes. Heatmap profiles of all identified metabolites after treatment of **(A)** *P. aeruginosa* FADDI-PA111 and **(B)** LESB58 with single and combination of polymyxin B and amikacin at 15 min, 1 h, and 4 h.



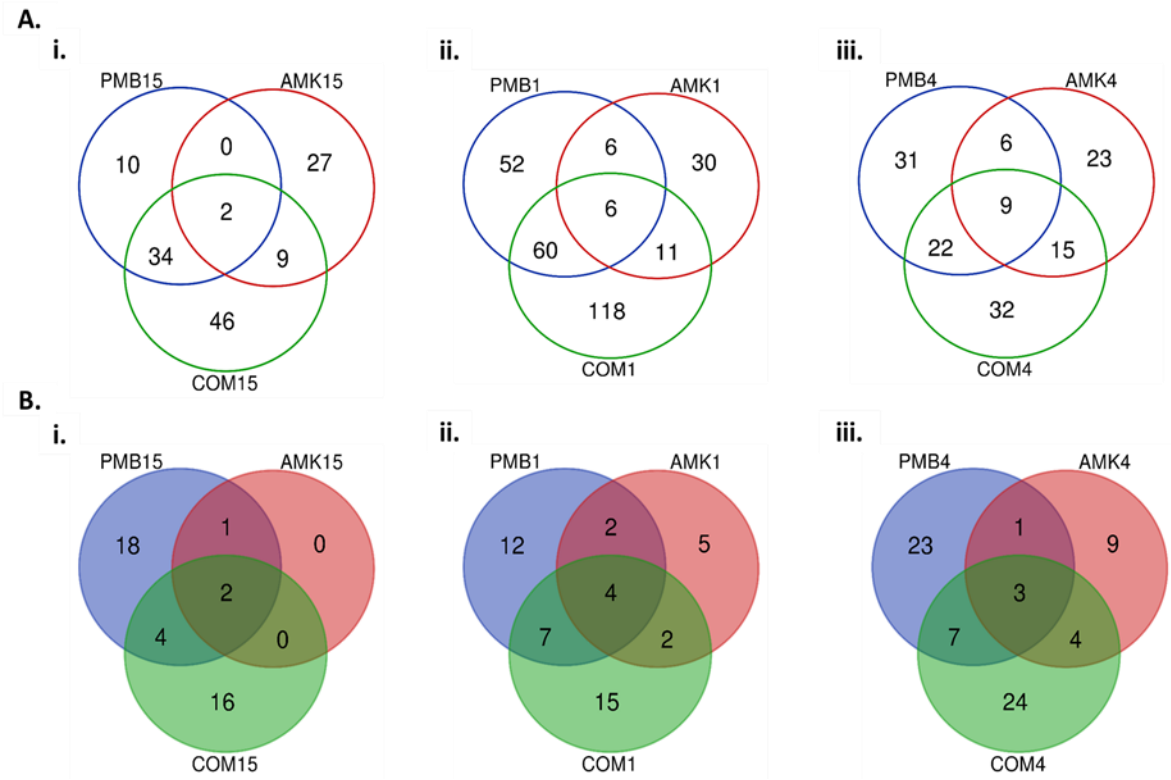

B.
C
PMB
AMK
COM
C
PMB
AMK
COM
C
PMB
AMK
COM
15 min
1h
4h
Log2(fold change)
-4
-3
-2
-1
0
1
2
3
4

Supplementary Figure S4. Summary number of significant metabolites changes after antibiotic monotherapy and combination (COM) treatment of **(A)** FADDI-PA111 **(B)** LESB58 at 15 min, 1 h and 4 h. Changes (≥ 0.59-log2-fold, $p \leq 0.05$; FDR ≤ 0.1).

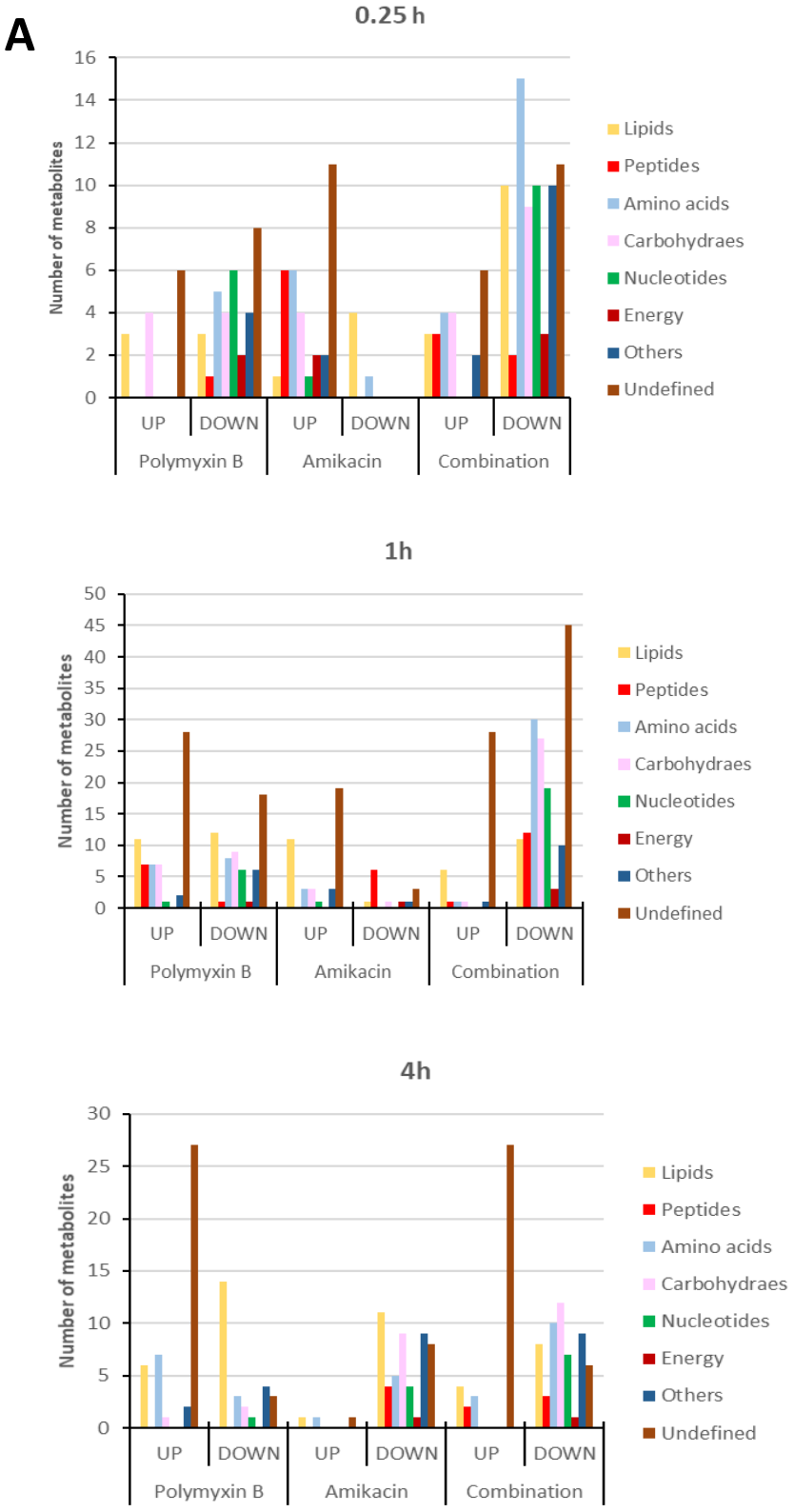




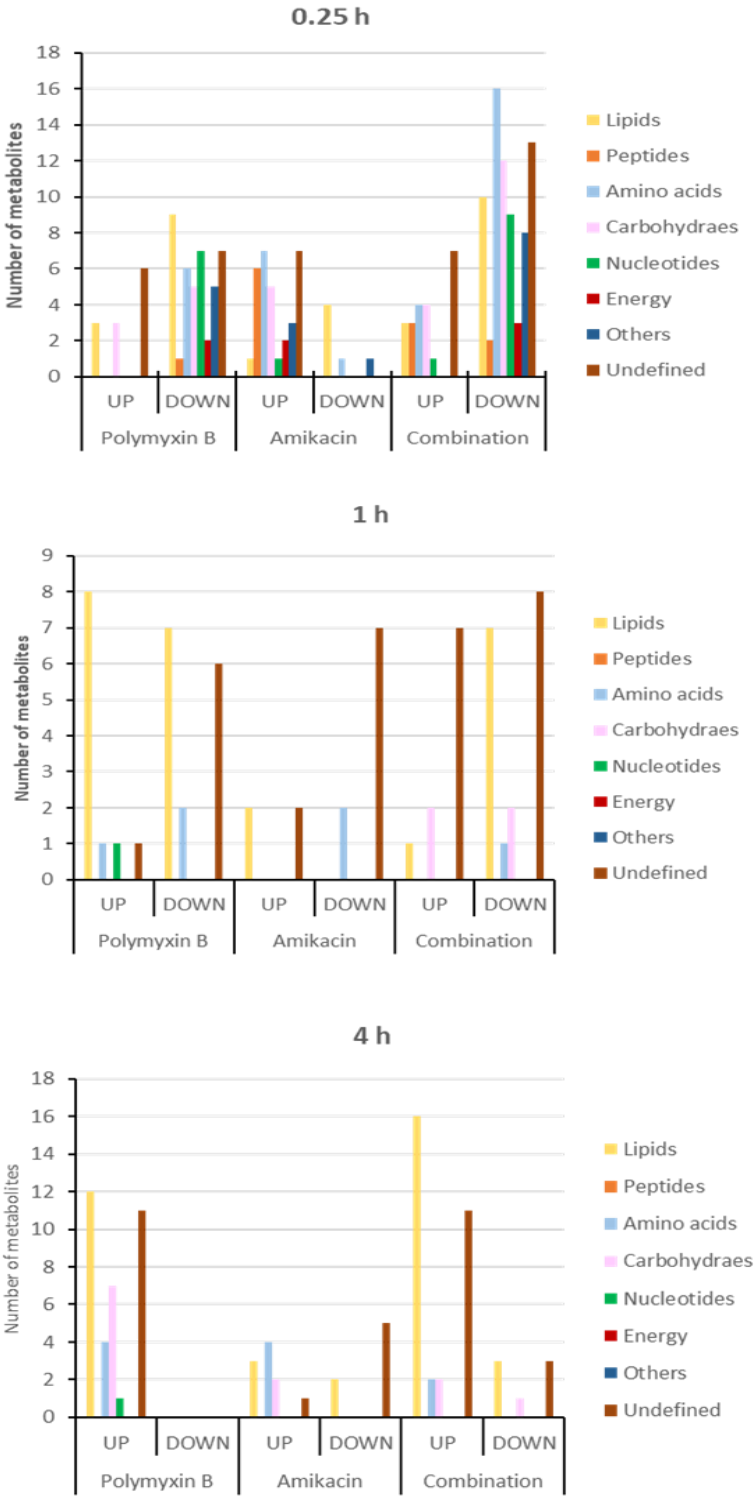
Supplementary Figure S5. Venn diagrams represent the number of metabolites significantly affected by each treatment for **(A)** FADDI-PA111 and **(B)** LESB58 at (i) 15 min, (ii) 1 h, and (iii) 4 h. Significant metabolites were selected with ($\geqslant$ 0.58553-log2-fold, p $\leqslant$ 0.05; FDR $\leqslant$ 0.1).



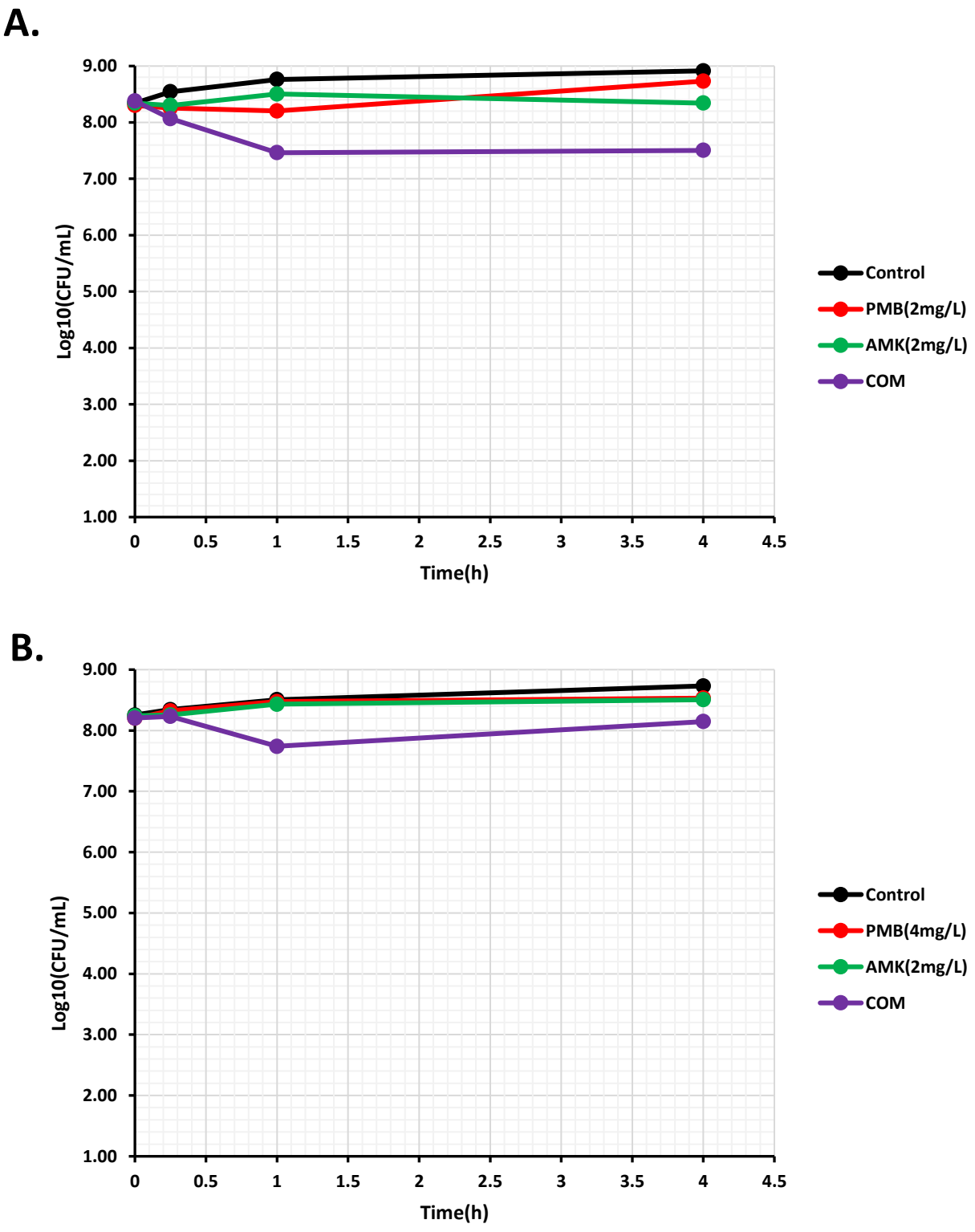
Supplementary Figure S6. Summary number of significantly changed metabolites classified according to different metabolite classes after antibiotic treatment of **(A)** FADDI-PA111 & **(B)** LESB58 at 15 min, 1 h, and 4 h. (Changes > 1.0-log2-fold, $p \leq 0.05$;FDR ≤ 0.1 for FADDI-PA111; (≥ 0.59-log2-fold, $p \leq 0.05$; FDR ≤ 0.1) for LESB58.



B.


0.25 h
Number of metabolites
18
16
14
12
10
8
6
4
2
0
UP
DOWN
UP
DOWN
UP
DOWN
Polymyxin B
Amikacin
Combination
Lipids
Peptides
Amino acids
Carbohydraes
Nucleotides
Energy
Others
Undefined
1 h
Number of metabolites
9
8
7
6
5
4
3
2
1
0
UP
DOWN
UP
DOWN
UP
DOWN
Polymyxin B
Amikacin
Combination
Lipids
Peptides
Amino acids
Carbohydraes
Nucleotides
Energy
Others
Undefined
4 h
Number of metabolites
18
16
14
12
10
8
6
4
2
0
UP
DOWN
UP
DOWN
UP
DOWN
Polymyxin B
Amikacin
Combination
Lipids
Peptides
Amino acids
Carbohydraes
Nucleotides
Energy
Others
Undefined

Supplementary Figure S7. Time-kill kinetics of polymyxin B and amikacin alone and in combination against *P. aeruginosa* FADDI-P111 **(A)** and LESB58 **(B)**.



References

1. Kirwan JA, Weber RJ, Broadhurst DI et al. Direct infusion mass spectrometry metabolomics dataset: a benchmark for data processing and quality control. *Sci Data* 2014; **1**: 140012.