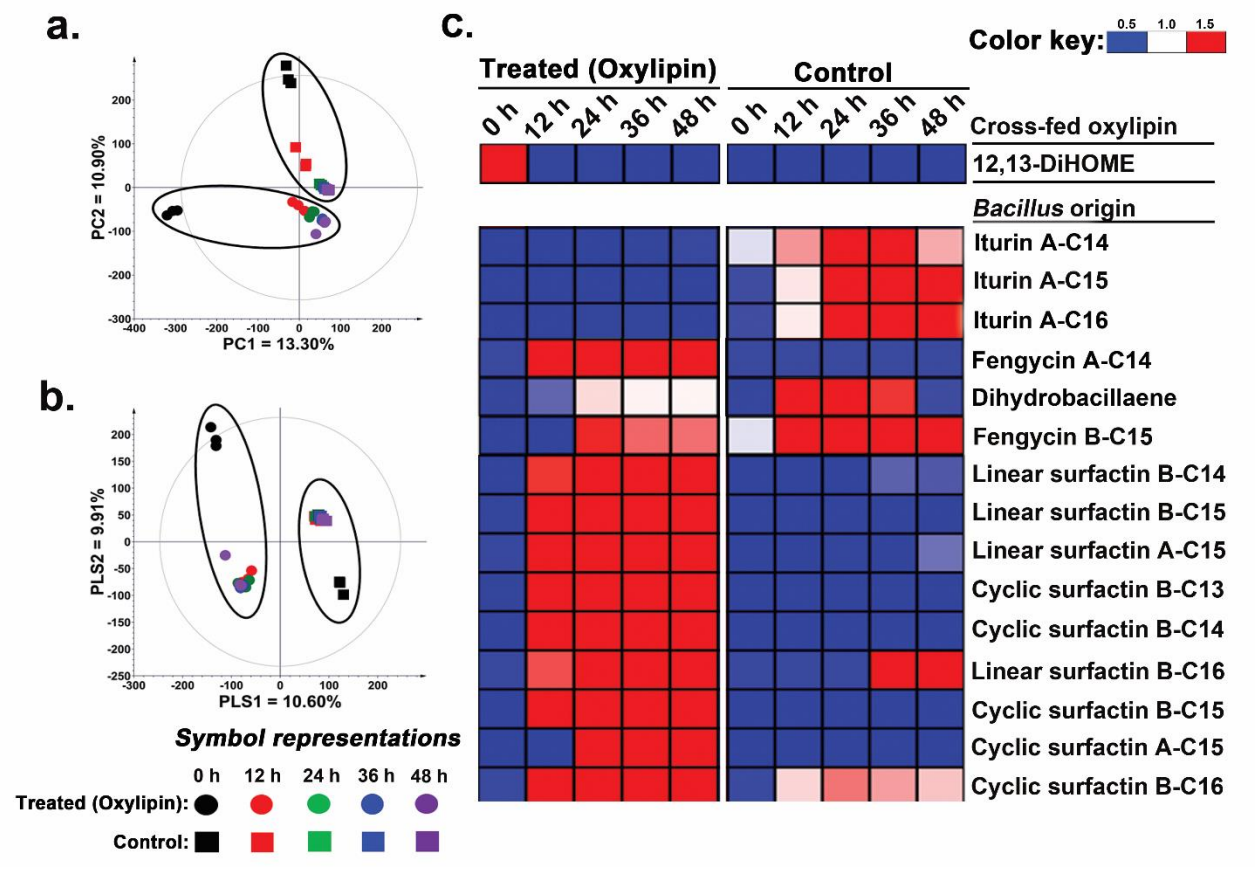
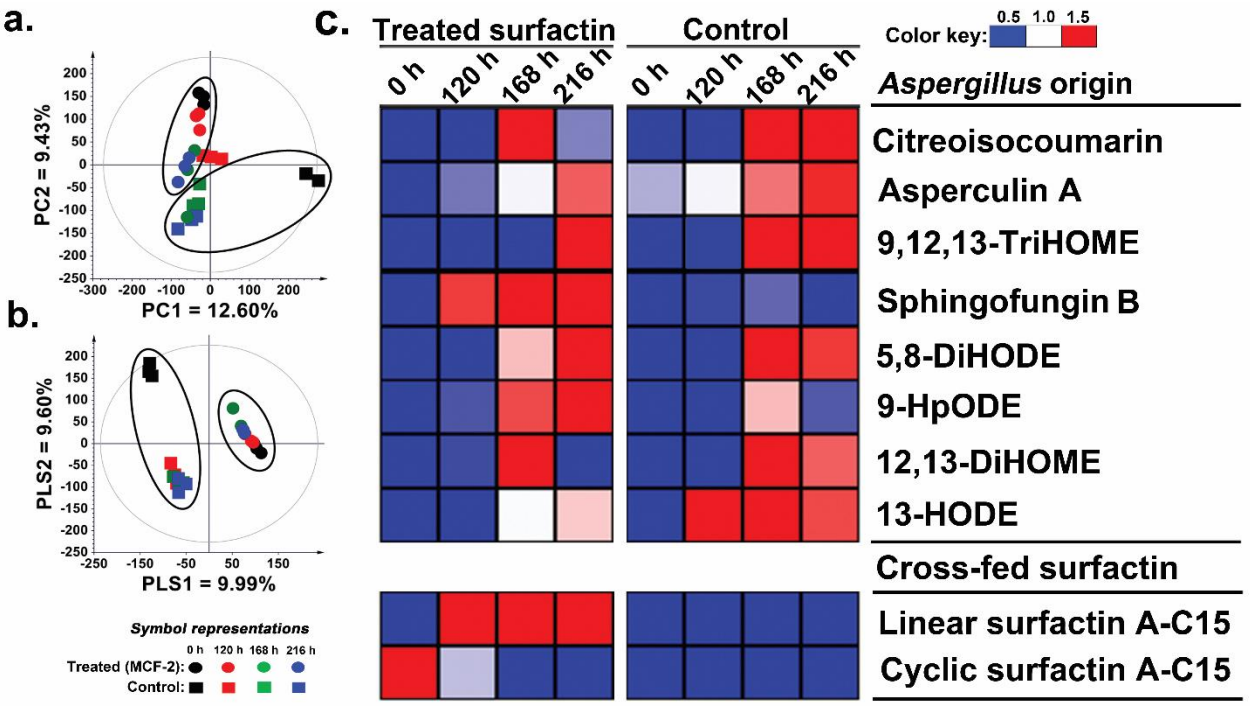
Supplementary information

Non-obligate pairwise metabolite cross-feeding suggests ammensalic interactions between *Bacillus* and *Aspergillus* species

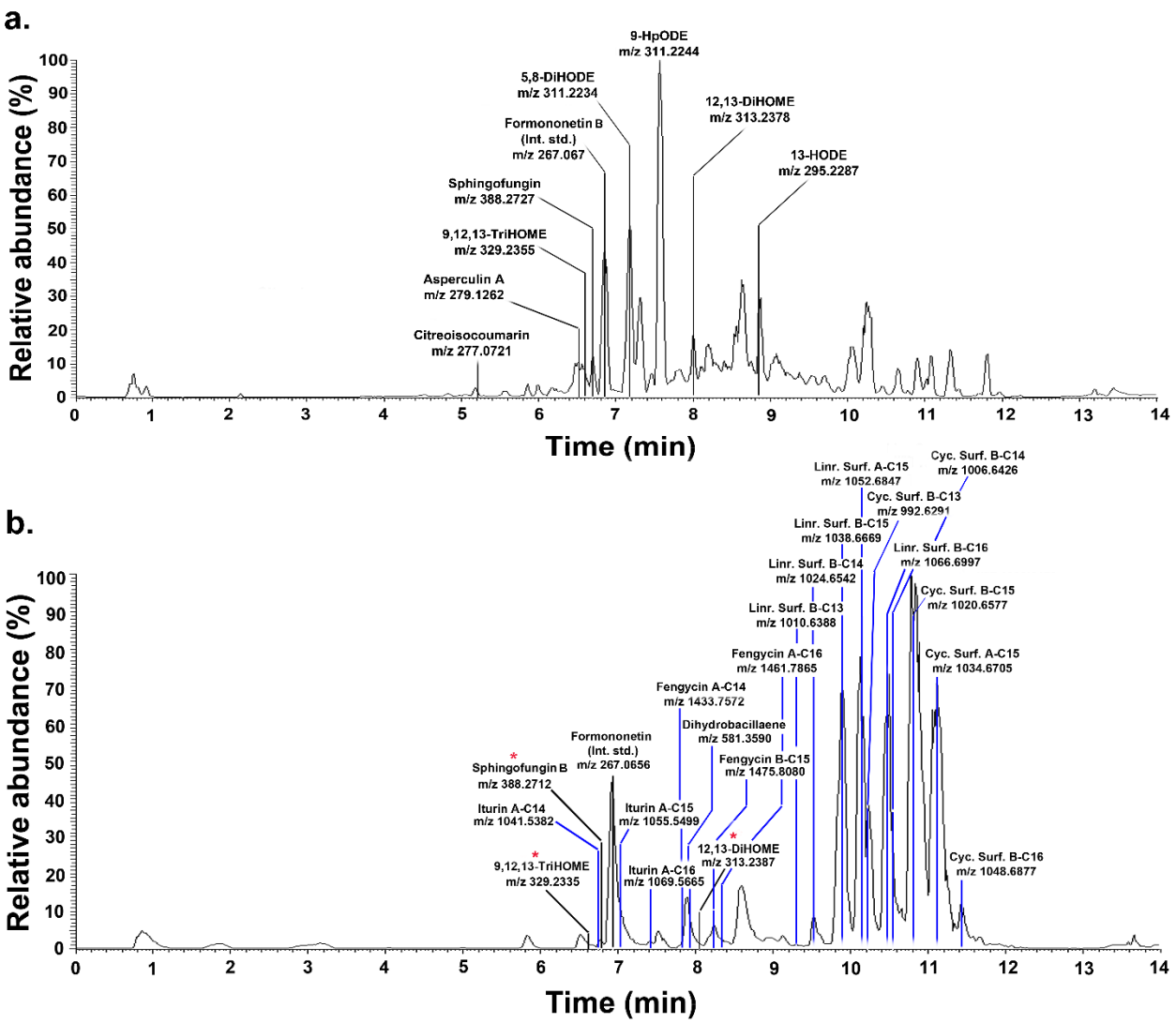
Supplementary figures



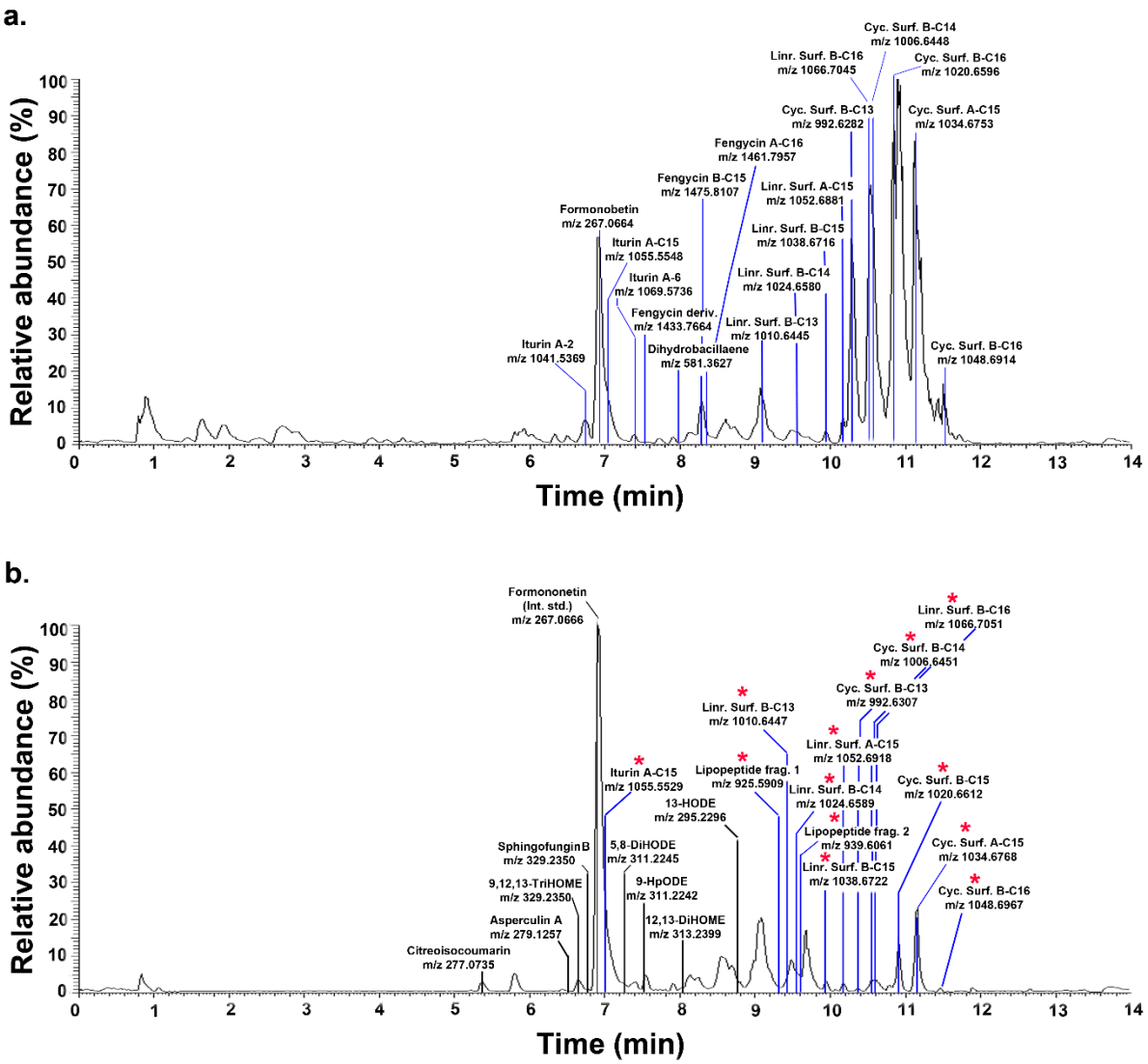
Supplementary figure 1. Time correlated **(a)** PCA, and **(b)** PLS-DA score plots representing the extracellular metabolite profiles for *Bacillus* cultures subjected to oxylipin 12,13-DiHOME treatment and control sets based on the non-untargeted UHPLC-LTQ-Orbitrap-MS data acquired in negative ion mode, and **(c)** the corresponding heat map based on the PLD-DA model displaying the relative abundance of the significantly discriminant metabolites between oxylipin treated and control sets.



Supplementary figure 2. Time correlated **(c)** PCA, and **(d)** PLS-DA score plots depicting marked variations between the metabolite profiles of cyclic surfactin A-C15 treated, and control *Aspergillus* culture extracts based on the non-untargeted UHPLC-LTQ-Orbitrap-MS datasets acquired in negative ion mode, and **(c)** the corresponding heat map based on the PLD-DA model displaying the relative abundance of the significantly discriminant metabolites between the surfactin treated and control culture extracts.



Supplementary figure 3. The UHPLC-LTQ-Orbitrap-MS/MS chromatograms representing the QC (quality control) mixtures for **(a)** the late-log phase *Aspergillus* (A_d; donor species) culture extracts used in the metabolite cross feeding to *Bacillus*, and (b) the *Bacillus* (B_r; receiver species) culture extracts after MCF-1 ($A_d \rightarrow B_r$). Here, the cross-fed metabolites of *Aspergillus* origin which were re-extracted from *Bacillus* cultures are highlighted with red asterisks (*) in the chromatogram b. Only the annotated metabolites are shown while the non-identified metabolites are not indicated in the chromatogram.



Supplementary figure 4. The UHPLC-LTQ-Orbitrap-MS/MS chromatograms representing the QC (quality control) mixtures for **(a)** the late-log phase *Bacillus* (B_d; donor species) culture extracts used in the metabolite cross feeding to *Aspergillus*, and (b) the *Aspergillus* (A_r; receiver species) culture extracts after MCF-2 ($B_d \rightarrow A_r$). Here, the cross-fed metabolites of *Bacillus* origin which were re-extracted from *Aspergillus* cultures are highlighted with red asterisks (*) in the chromatogram b. Only the annotated metabolites are shown while the non-identified metabolites are not indicated in the chromatogram.

Supplementary tables

Supplementary table 1. List of the significantly discriminant metabolites (VIP>0.7 and p<0.05) and their chromatographic & spectral characteristics based on the LC-MS analysis obtained for MCF-1 treated ($A_d \rightarrow B_r$) and control sets of *Bacillus*.

S. No.	Metabolites	RT	m/z (-ESI)	m/z (+ESI)	Mol. Wt.	Mass Fragmentation	Elemental composition	Δm (ppm)	References
Cross-fed and re-extracted metabolites of *Aspergillus* origin									
1	9,12,13-TriHOME	6.62	329.23	ND	330	(-) 329>311, **293**, 229, 211, 171	$C_{18}H_{34}O_5$	0.07	[2]
2	Sphingofungin B	6.79	388.27	ND	389	(-) 388>**370**, 346>352, 328>**310**, 293, 285, 267	$C_{20}H_{39}O_6N$	0.74	[3]
3	12, 13-DiHOME	8.06	313.24	ND	314	(-) 313>**295**, 283, 267, 183	$C_{18}H_{34}O_4$	1.27	[2], Std. comp.
Endogenous metabolites of *Bacillus* origin									
4	Iturin A-C14	6.77	1041.54	1043.55	1042	(-) 1041>1023, **1011**>994, **993**>977, 922, **822**	$C_{48}H_{74}O_{14}N_{12}$	-0.34	[4, 5]
5	Iturin A-C15	7.01	1055.55	1057.57	1056	(-) 1056>1037, **1025**, 1019>**1008**>1009, **990**, 965	$C_{49}H_{76}O_{14}N_{12}$	-0.48	[5]
6	Iturin A-C16	7.36	1069.57	1071.58	1070	(-) 1069>**1052**, 1040>**1022**>**1005**	$C_{50}H_{78}O_{14}N_{12}$	-0.95	[6]
7	Fengycin A-C14	7.79	1433.75	1435.78	1435	(-) 1434>**1390**>1373, **1346**>**1328**, 1199, 1144, 1102	$C_{70}H_{106}O_{20}N_{12}$	-0.80	[7]
8	Dihydrobacillaene	7.96	581.36	ND	582	(-) 537, 519, **358**>**340**, 330, 300> 322, 312, 254, **226**	$C_{34}H_{50}O_6N_2$	-1.44	PubChem
9	Fengycin B-C15	8.24	1475.80	1477.82	1477	(-) 1476>1458, **1432**>1414, **1388**>1370, 1326, **1176**,	$C_{73}H_{112}O_{20}N_{12}$	-1.31	[7]
10	Fengycin C-C16	8.37	1461.78	1463.81	1463	(-) 1462>1433, **1417**>1400, **1374**>1356, **1144**, 1102	$C_{72}H_{110}O_{20}N_{12}$	0.28	[7]
11	Linear surfactin B-C13	9.29	1010.64	1012.66	1012	(-) 1011>**993**>**975**>957, **818**, 775	$C_{50}H_{89}O_{14}N_7$	-0.68	Theoretical
12	Linear surfactin B-C14	9.52	1024.65	1026.67	1026	(-) 1025>1008, **989**, 971, 819>776, **664**, 452	$C_{51}H_{91}O_{14}N_7$	-0.93	Theoretical
13	Linear surfactin B-C15	9.90	1038.67	1040.69	1040	(-) 1039>**1021**>**1003**>984, **818**, 776, 696, 678, 452	$C_{52}H_{93}O_{14}N_7$	-3.79	Theoretical
14	Linear surfactin A-C15	10.11	1052.68	1054.70	1054	(-) 1053>**1035**>**1017**>999, 888, **818**	$C_{53}H_{95}O_{14}N_7$	-5.70	Theoretical
15	Cyclic surfactin B-C13	10.22	992.63	994.64	994	(+) 995>977, 881, **685**>667, 554, **441**>413, **328**	$C_{50}H_{87}O_{13}N_7$	-0.83	[8]
16	Linear surfactin B-C16	10.43	1066.70	1068.72	1068	(-) 1067>**1049**>**1031**>1013, **819**, 776	$C_{54}H_{97}O_{14}N_7$	-4.85	Theoretical
17	Cyclic surfactin B-C14	10.46	1006.64	1008.66	1008	(-) 1007>**989**>971, 927, 776, **665**>**452**, 339/ (+) 1009>991, **685**>667, 554, **441**>227, **199**	$C_{51}H_{89}O_{13}N_7$	-2.50	[9]
18	Cyclic surfactin B-C15	10.82	1020.66	1022.67	1022	(-) 1021>**1003**>**985**, 941, 776, 678>**422**	$C_{52}H_{91}O_{13}N_7$	-2.45	[8]
19	Cyclic surfactin A-C15	11.12	1034.67	1036.69	1036	(-) 1035>**1017**>**999**>355, 790, **692**, 466	$C_{53}H_{93}O_{13}N_7$	-5.86	Std. comp.
20	Cyclic surfactin B-C16	11.41	1048.69	1050.70	1050	(+) 1051>1033, 938, **685**>667, 554, **441**>413, **328**	$C_{54}H_{95}O_{13}N_7$	-1.66	[10]
Non-identified metabolites (N.I)									
21	N.I. 1 (*Aspergillus* origin)	6.22	420.15	ND	421	(-) 420>**269**>223, **187**, 144, 124	NA	NA	NA
22	N. I. 2	3.07	295.18	297.19	296	(-) 295>251, **207**>**179**, 151>**164**, 136	NA	NA	NA
23	N. I. 3	5.70	377.18	ND	278	(-) 377>359, **320**>303, 277, **206**>**192**>**178**	NA	NA	NA
24	N. I. 4	6.34	316.21	ND	317	(-) 316>**286**, 272>**242**>**226**, 170	NA	NA	NA
25	N. I. 5	6.51	609.29	ND	610	(-) 609>**563**, 541>519, **495**>427, **408**, 385	NA	NA	NA
26	N.I. 6 (*Bacillus* origin)	7.39	588.28	* 612.27	589	(-) 588>552, 433, 305, **186**>167, **142**>**124**	NA	NA	NA
27	N.I. 7 (*Bacillus* origin)	7.23	741.40	743.41	742	(-) 741>679, 559, 518>500, 460, 434, **356**>**338**, 242	NA	NA	NA
28	N. I. 8	8.13	438.26	ND	439	(-) 438>297, **241**>**223**>**81**	NA	NA	NA

- **RT:** Retention time; **Δm:** mass error; * [M + Na^+ adduct]; **ND:** not detected; **NA:** not applicable; **Std. comp:** verified with standard compound.
- Bold numbers in the mass fragmentation column indicates the major peaks.

- (-)/ (+) in the mass fragmentation column indicates the ESI modes in which the data were recorded for respective peaks.

Supplementary table 2. List of the significantly discriminant metabolites (VIP>0.7 and p<0.05) and their chromatographic & spectral characteristics based on the LC-MS analysis obtained for MCF-2 treated ($B_d \rightarrow A_r$) and control sets of *Aspergillus*.

S. No.	Candidate metabolites	RT	m/z (-ESI)	m/z (+ESI)	Mol. Wt.	Mass Fragmentation	Elemental composition	Δm (ppm)	References
Endogenous metabolites of *Aspergillus* origin									
1	Citreoisocoumarin	5.30	277.07	279.09	278.00	(-)277>233, **219**>191>147, **123**	$C_{14}H_{14}O_6$	6.24	[11]
2	Asperaculin A	6.51	279.13	ND	280.00	(-)279>235, **205**>187, 177, **149**>**106**	$C_{18}H_{20}O_5$	3.02	[11]
3	9,12,13-TriHOME	6.60	329.24	ND	330.00	(-)311> **293**, 229, 211>153, 127, **171**	$C_{18}H_{34}O_5$	8.50	[2]
4	Sphingofungin B	6.76	388.27	390.28	389.00	Fragmentation not observed	$C_{20}H_{39}O_6N$	7.67	[3]
5	5,8-DiHODE	7.22	311.22	ND	312.00	(-)311>**293**>275, **249**>257, **231**, 177	$C_{18}H_{32}O_4$	1.75	[2], Std. comp.
6	9-HpODE	7.35	311.22	ND	312.00	(-)311>**293**, 267>275, **249**>231, 205, 156, **113**	$C_{18}H_{32}O_4$	1.31	[12]
7	12,13-DiHOME	8.05	313.24	ND	314.00	(-)313>298, **295**>281, **277**>**259**, 249	$C_{18}H_{34}O_4$	1.41	[2], Std. comp.
8	13-HODE	8.94	295.23	ND	296.00	(-)295>**277**, 251>259, **233**>**191**, 179	$C_{18}H_{32}O_3$	9.51	[12]
Cross-fed and re-extracted metabolite of *Bacillus* origin									
9	Iturin A-C15	7.00	1055.56	ND	1057.00	Fragmentation not observed	$C_{49}H_{76}O_{14}N_{12}$	2.65	[5]
10	Lipopeptide frag. 1	9.31	925.59	927.60	926.00	(-)926>**907**, 795, 697> **890**, 697, 663, 452, 339>434, **240**	ND	NA	NA
11	Linear surfactin B-C13	9.32	1010.65	1012.65	1012.00	(-)1011>**993**>**975**>956, **819**, 776, 669, 452	$C_{50}H_{89}O_{14}N_7$	3.74	Theoretical
12	Linear surfactin B-C14	9.56	1024.66	1026.67	1026.00	(-)1025>**1008**, 989>836, **819**, 683>777, 683	$C_{51}H_{91}O_{14}N_7$	2.17	Theoretical
13	Lipopeptide frag. 2	9.56	939.61	941.62	941.00	(-)940>922, **809**, 711>**692**, 512, 452, 339>**434**, 240, 222	ND	NA	NA
14	Linear surfactin B-C15	9.94	1038.68	1040.69	1040	(-)1039>**1021**>**1003**>985, **819**, 777, 696, 452	$C_{52}H_{93}O_{14}N_7$	1.30	Theoretical
15	Linear surfactin A-C15	10.17	1052.69	1054.70	1054.00	(-)1053>**1035**>**1017**>**999**, 887, **818**, 776, 711, 693, 452	$C_{53}H_{95}O_{14}N_7$	0.91	Theoretical
16	Cyclic Surfactin B-C13	10.29	992.63	ND	994.00	(-)993>**975**>956, **913**, 794>776, **650**, 452	$C_{50}H_{87}O_{13}N_7$	2.27	[8]
17	Cyclic Surfactin B-C14	10.53	1006.65	1008.66	1008.00	(-)1007>**989**>971, 777, **665**>**452**, 339	$C_{51}H_{89}O_{13}N_7$	1.49	[9]
18	Linear surfactin B-C16	10.55	1066.71	ND	1068.00	Fragmentation not observed	$C_{54}H_{97}O_{14}N_7$	3.96	Theoretical
19	Cyclic Surfactin B-C15	10.88	1020.66	1022.67	1022.00	(-)1021>**1003**>985, 777, 679, 452	$C_{52}H_{91}O_{13}N_7$	2.58	[8]
20	Cyclic Surfactin A-C15	11.18	1034.68	1036.69	1036.00	(-)1035>**1017**>999, 777, **693**>452, **339**	$C_{53}H_{93}O_{13}N_7$	2.40	Std. comp.
21	Cyclic surfactin B-C16	11.29	1048.69	1050.70	1050.00	Fragmentation not observed	$C_{54}H_{95}O_{13}N_7$	5.14	[10]
Non-identified metabolites (N.I)									
22	N.I. 1	8.30	491.27	493.28	492.00	(-)473, **447**, 400, 344, 326, 262, 227>283, 194, 179>164	NA	NA	NA
23	N.I. 2	8.54	487.34	ND	488.00	(-)487>**469**, 427, 397>425, 407, 383>392, 337	NA	NA	NA

RT: Retention time; **Δm:** mass error

- Bold numbers in the mass fragmentation column indicates the major peaks.
- **ND:** not detected
- **NA:** not applicable

Supplementary methods

Supplementary method 1. List of components used in the common minimal medium (CMM) used in the study.

Components	Concentration
$NaNO_3$	2 mg/mL
NH_4NO_3	8 mg/mL
K_2HPO_4	1 mg/mL
KH_2PO_4	4 mg/mL
Na_2HPO_4	14.3 mg/mL
$MgSO_4$	0.26 mg/mL
$CaCl_2$	0.01 mg/mL
KCl	0.5 mg/mL
FeSO4	0.015 mg/mL
EDTA	0.015 mg/mL
Sucrose (Carbon source)/ filter sterilized	30 mg/mL
pH	6.5

Supplementary method 2. Biofilm quantification assay for *Bacillus* culture

The biofilm quantification assay was adopted from the method described previously by **O'Toole (2011)**. The same batch of freshly grown seed culture was centrifuged, twice-washed (1X PBS), and resuspended in CMM to attain the predetermined cell density prior to its inoculation (0.1%) in 1.5 mL CMM in each well of the 12-well plate (Corning Inc. NY, USA). Both the treated (MCF-1) and control sets were incubated under the same conditions as with the corresponding flask cultures. Biofilm formation was examined at every 12 h alongside flask cultures with three biological replicates. First, the medium from each culture well was gently removed without disturbing the biofilm followed by PBS (1X) washing three times. Then, 1.5 mL of crystal violet (0.1%) solution was added to each well followed by 15 min incubation at room temperature. Crystal violet was pipetted out and the culture wells were gently washed three times with distilled water. The culture plates were dried under the hot air oven (65 °C) for 3 h and 1.5 mL of acetic acid (30%) was added to dissolve the embedded CV in biofilm for next 15 min. Solubilized CV was transferred to another microtiter plate and the absorbance was recorded at 550 nm with appropriate blanks.

Supplementary method 3. LC-MS instrumentation for the analysis of metabolite extracts

Metabolite extracts were analyzed using an ultrahigh performance liquid chromatography – linear trap quadrupole – orbitrap – tandem mass spectrometry (UHPLC-LTQ-Orbitrap-MS/MS) system fitted with a vanquish binary pump (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Reverse phase chromatographic separation of analytes (injection volume; 5 µL) was achieved using a C18 column (100 mm × 2.1 mm, 1.7 µm particle size), Phenomenex Kinetex, Torrance, CA, USA. The chromatographic solvent system was composed of water (A) and acetonitrile (B), each containing 0.1% formic acid (FA). The solvent gradients were run at a constant flow rate of 0.3 mL/min during the 14 min run program (5% solvent B for 1 min, 100% solvent B for 9 min, maintained for 1 min, and again 5% solvent B in last 4 min). Mass spectrometry (MS) system included LTQ-Orbitrap-Velos pro with an ion-tap (IT) and HESI-II probe. Probe heater temperature was set at 300 °C, while the capillary temperature and voltages were fixed at 350 °C and 2.5 kV (-ESI)/ 3.7 kV (+ESI), respectively. Non-targeted metabolite profiling was performed in both negative and positive ESI modes across a m/z range of 150-1500.

Supplementary references

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