

# 1 **Dissecting the collateral damage of antibiotics on gut microbes**

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23

24 **Abstract**

25 Antibiotics are used for fighting pathogens, but also target our commensal bacteria as a side  
26 effect, disturbing the gut microbiota composition and causing dysbiosis and disease<sup>1-3</sup>.  
27 Despite this well-known collateral damage, the activity spectrum of the different antibiotic  
28 classes on gut bacteria remains poorly characterized. Having monitored the activities of  
29 >1,000 marketed drugs on 38 representative species of the healthy human gut microbiome<sup>4</sup>,  
30 we here characterize further the 144 antibiotics therein, representing all major classes. We  
31 determined >800 Minimal Inhibitory Concentrations (MICs) and extended the antibiotic  
32 profiling to 10 additional species to validate these results and link to available data on  
33 antibiotic breakpoints for gut microbes. Antibiotic classes exhibited distinct inhibition spectra,  
34 including generation-dependent effects by quinolones and phylogeny-independence by  $\beta$ -  
35 lactams. Macrolides and tetracyclines, two prototypic classes of bacteriostatic protein  
36 synthesis inhibitors, inhibited almost all commensals tested. We established that both kill  
37 different subsets of prevalent commensal bacteria, and cause cell lysis in specific cases.  
38 This species-specific activity challenges the long-standing divide of antibiotics into  
39 bactericidal and bacteriostatic, and provides a possible explanation for the strong impact of  
40 macrolides on the gut microbiota composition in animals<sup>5-8</sup> and humans<sup>9-11</sup>. To mitigate the  
41 collateral damage of macrolides and tetracyclines on gut commensals, we exploited the fact  
42 that drug combinations have species-specific outcomes in bacteria<sup>12</sup> and sought marketed  
43 drugs, which could antagonize the activity of these antibiotics in abundant gut commensal  
44 species. By screening >1,000 drugs, we identified several such antidotes capable of  
45 protecting gut species from these antibiotics without compromising their activity against  
46 relevant pathogens. Altogether, this study broadens our understanding of antibiotic action on  
47 gut commensals, uncovers a previously unappreciated and broad bactericidal effect of  
48 prototypical bacteriostatic antibiotics on gut bacteria, and opens avenues for preventing the  
49 collateral damage caused by antibiotics on human gut commensals.

50 **MAIN TEXT**

51 Medication is emerging as major contributor for changes in the composition of the human gut  
52 microbiota<sup>4,13-15</sup>. Such severe and long-lasting changes are associated, and in some cases  
53 causatively linked, to dysbiosis and a wide range of diseases<sup>16</sup>. Although several non-  
54 antibiotic drugs may also have a previously unappreciated impact on the gut microbiome  
55 composition<sup>4,16,17</sup>, antibiotics, developed to have broad spectra and thereby target very  
56 diverse pathogens, are long known to take a heavy toll on our gut flora, causing a variety of  
57 gastrointestinal side-effects<sup>18</sup>, including *Clostridioides (former Clostridium) difficile* infections.  
58 Recently more attention has been given to this collateral damage of antibiotics on the gut  
59 microbiota and thereby on the host's wellbeing. *In vivo* studies highlight links between the  
60 long-term microbiota compositional changes and host dysbiosis, including the development  
61 of allergic, metabolic, immunological and inflammatory diseases<sup>5-8,10,11,19-21</sup>. While uncovering  
62 the direct effects of different antibiotics on our gut flora is critical to improve general health,  
63 technical difficulties hamper routine testing of antibiotic susceptibility in anaerobes<sup>22,23</sup>.  
64 Currently available data on bacterial susceptibility to antibiotics is focused on invasive  
65 pathogens and offers little to no resolution in the diversity of the human gut microbiota<sup>24</sup>.  
66 Information is missing even for the most prevalent and abundant gut species, or ones  
67 recently associated with dysbiosis and disease<sup>25,26</sup>. In addition, existing animal or cohort  
68 studies have used a handful of antibiotics or merge data from different antibiotic classes,  
69 precluding systematic and general conclusions on the matter.

70 We recently assessed the direct effect of ~1200 FDA-approved drugs on the growth  
71 of 38 prevalent and abundant or disease-associated human gut species under anaerobic  
72 conditions at a fixed concentration of 20  $\mu\text{M}$ <sup>4</sup>. This initial screen (referred to hereafter as  
73 "screen") included 144 antibiotics (Fig. 1a, Extended Data Fig. 1, Suppl. Table 1), with  
74 different classes having discernible effects on gut microbes (Fig. 1b). We validated these  
75 results by measuring 815 MICs (33 antibiotics and 2 antifungals for 17 species, 22 antibiotics  
76 for 10 additional species), using MIC gradient test strips (Fig. 1a, Extended Data Fig. 2,  
77 Suppl. Table 2 + 3). Despite differences in the experimental procedure, concordance

78 between data from the initial screen and MICs is very high: Specificity and sensitivity of 0.97  
79 (Extended Data Fig. 3a). The newly established MICs also correlate well with available data  
80 on antimicrobial susceptibility from databases such as EUCAST<sup>24</sup> or ChEMBL<sup>27</sup> ( $r_s=0.69$  and  
81  $r_s=0.64$ , respectively), despite differences in strains and media used (Extended Data Fig. 3b).  
82 Importantly, this new dataset considerably expands the available MICs, as much as by 80%  
83 for non-pathogenic bacteria (Fig. 1c, Extended Data Fig. 3c). Altogether, the initial screen  
84 and the new MIC dataset provide high-resolution information on the target spectrum of  
85 antibiotics on commensal gut microbes, which we explored further.

86 The antibiotics tested exhibited strong class-dependent effects (Fig. 1b, d).  
87 Consistent with literature, aminoglycosides hardly affected gut microbes under anaerobic  
88 conditions<sup>28</sup> and sulfonamides were inactive in the medium used for the screen<sup>4</sup>. Quinolones  
89 acted in a generation-dependent manner. First-generation variants were effective only on a  
90 narrow spectrum of microbes that included both commensal *E. coli* tested. Second- and  
91 third- generation quinolones increased the spectrum. Fourth-generation variants (developed  
92 to increase activity against anaerobes) inhibited all tested species, except for *Akkermansia*  
93 *muciniphila* (Fig. 1e, Extended Data Fig. 1, red box), a species associated with protection  
94 against different diseases and dysbiotic states<sup>29</sup>, and even positive responses to  
95 immunotherapy<sup>30</sup>. For  $\beta$ -lactams, resistance was patchy but distinct for different members  
96 and subclasses (Extended Data Fig. 2, 4a). For *Bacteroidetes*, we tested additional species  
97 and strains (in total 12 and 19, respectively) (Extended Data Fig. 4b, c), confirming that  $\beta$ -  
98 lactam sensitivity and phylogenetic relatedness are uncoupled (Extended Data Fig. 4d). This  
99 argues for resistance mechanisms being strain-specific and horizontally transferred.  
100 Macrolides showed a strong impact on gut commensals and inhibited all tested microbes  
101 (Fig. 1d), except for the opportunistic pathogen *C. difficile*, which was resistant to all tested  
102 macrolides and clindamycin (Extended data Fig. 2, red box). This is in line with the  
103 associated risk of *C. difficile* infection after macrolide/clindamycin treatment<sup>31</sup>. Finally, 8 of  
104 the 9 tested tetracyclines inhibited nearly all tested microbes, which is surprising in the light  
105 of the gut microbiota being considered as reservoir for tetracycline resistance genes<sup>32</sup>.



106 Concentration-resolved MICs confirmed the same drug class-dependent trends observed in  
107 the screen (Fig. 1d, f). In addition, MICs allow for comparisons with clinical breakpoints, i.e.  
108 MICs at which a species should be considered resistant or susceptible (Fig. 1f). Overall, the  
109 gut microbes in our assays (anaerobic growth, gut mimetic growth medium<sup>33</sup>) were slightly  
110 more resistant to most antibiotic classes than previously reported for pathogens (aerobic  
111 growth, Mueller-Hinton agar). Tetracyclines were the exception, inhibiting commensals at  
112 significantly lower concentrations than what is reported for pathogens (Fig. 1f). Thus,  
113 commensals might be considerably less resistant to tetracyclines than previously anticipated  
114 and suggested by the detection of tetracycline resistance elements in fecal metagenomes.

115         Recent *in-vivo* studies have shown that  $\beta$ -lactams and macrolides have a strong and  
116 long-lasting collateral impact on the gut microbiota composition and thereby on host health<sup>5-8</sup>.  
117 As  $\beta$ -lactams exhibited strain-specific effects (Extended Data Fig. 1, 2, 4) and are known to  
118 kill bacteria (bactericidal), they could *irrevocably* deplete *specific* members of the gut  
119 microbiota, thereby explaining their differential and long-lasting effects on the community  
120 composition. On the other hand, macrolides uniformly targeted all tested gut commensals  
121 (Fig. 1d) and are textbook bacteriostatic antibiotics, i.e. inhibit bacterial growth, but do not kill  
122 (at least at high numbers). In this case, the long-term community composition change is  
123 more difficult to rationalize, as all community members are inhibited, but should be able to  
124 regrow once drug is removed. Similarly, tetracyclines, another class of bacteriostatic  
125 antibiotics that acted on nearly all gut microbes we tested, have known gastro-intestinal side-  
126 effects<sup>18</sup>, which are indicative of gut microbiome dysbiosis. We thus wondered at which level  
127 macrolides and tetracyclines exert a differential effect on gut microbes. Although traditionally  
128 both clinical use<sup>34-37</sup> and basic research<sup>38,39</sup> heavily rely on this distinction between  
129 bactericidal and bacteriostatic antibiotics, there are reports of drugs changing their killing  
130 capacity depending on the organism, drug concentration or medium tested<sup>40,41</sup> (and  
131 increased evidence from meta-analyses that the distinction may have little relevance to  
132 clinical practice<sup>42,43</sup>). We therefore hypothesized that this bacteriostatic/bactericidal divide  
133 may be less rigid for gut commensals, which are more phylogenetically diverse than the few

134 pathogens usually tested, and hence provide a level where the effect of these drug classes  
135 on gut microbes becomes differential.

136         The standard way to determine whether an antibiotic has bactericidal or bacteriostatic  
137 activity is to calculate time-kill curves, where the bacterial survivors are counted on agar at  
138 various time-points after drug treatment. If, over a significant period of antibiotic treatment  
139 (ranging from 5 to 24 hours), the number of colony forming units (CFU)/ml of culture  
140 decreases by more than 99.9%, the antibiotic is considered bactericidal<sup>40</sup>. We assessed the  
141 survival of 12 abundant gut microbes over a 5-hour treatment of either a macrolide  
142 (erythromycin or azithromycin) or a tetracycline (doxycycline) at 5 x MIC (Fig 2a + b,  
143 Extended Data Fig. 5). About half of the tested species decreased in survival by >99.9%,  
144 pointing to these drugs being bactericidal to several abundant gut microbes. To confirm this  
145 further, we tested the viability of *B. vulgatus* and *E. coli* ED1a upon erythromycin,  
146 azithromycin or doxycycline treatments using live/dead staining. Microscopy and flow  
147 cytometry assessment of live/dead bacteria corroborated the initial observations (Fig. 2c,  
148 Extended Data Fig. 6). As tetracyclines are considered bona-fide bacteriostatic drugs in *E.*  
149 *coli*, we were surprised to see that doxycycline effectively killed the commensal *E. coli* ED1a  
150 (Fig. 2a). We verified that these effects held also in the presence of oxygen (Extended Data  
151 Fig. 7a) and confirmed that doxycycline has a stronger bactericidal action on this natural  
152 isolate than on the domesticated *E. coli* K-12 lab strain, BW25113 (Extended Data Fig. 7b).  
153 In parallel, we excluded that the differences in killing capacity were confounded by growth  
154 rate, growth phase or MIC of the bacterial species tested (Extended Data Fig. 8). We also  
155 noticed that *B. vulgatus* and *B. uniformis* cultures decreased density in the presence of  
156 erythromycin (Fig. 2d). We confirmed by time-lapse microscopy that this was due to lysis.  
157 Erythromycin caused cell shape defects, including blebbing, cytoplasmic shrinkage, and  
158 ultimately cell lysis in both *B. vulgatus* and *B. uniformis* (Figure 2e, Movies 1-4). Altogether,  
159 this selective bactericidal activity of macrolides and tetracyclines on specific gut commensals  
160 could provide an explanation for the strong effects these drug classes have on the gut  
161 microbiota composition of human individuals. The gut microbes killed from the drug would be

162 inadvertently removed from community, whereas the ones being only inhibited could recover  
163 when the therapy stops.

164         Knowing that drug combinations often have species-specific outcomes<sup>12</sup>, we  
165 reasoned that we could identify drugs that selectively antagonize the effect of antibiotics on  
166 gut microbes, while retaining activity against pathogens. Therefore, we screened the  
167 Prestwick library to identify antagonizing compounds to erythromycin or doxycycline on two  
168 abundant and prevalent gut microbes, *B. vulgatus* and *B. uniformis* (Fig. 3a, Extended Data  
169 Fig. 9). Of the 19 identified hits (Fig. 3b, Suppl. Table 4), we tested the 14 candidates with  
170 the strongest activity in a concentration-dependent manner (Extended Data Fig. 10a). Nine  
171 retained antagonistic activity over a broader concentration range, which we confirmed by  
172 checkerboard assays (Fig. 3c). The antidotes that showed the strongest antagonisms were  
173 the anticoagulant drug dicumarol, and two non-steroidal anti-inflammatory drugs, tolfenamic  
174 acid and diflunisal. While dicumarol rescued *B. vulgatus* from erythromycin and diflunisal  
175 from doxycycline, tolfenamic acid was able to protect *B. vulgatus* from both drugs. In  
176 addition, these interactions were able to at least partially rescue the killing of *B. vulgatus* by  
177 erythromycin and doxycycline (Extended Data Fig. 10b). We then probed two of these drugs  
178 for their ability to protect other abundant gut commensals and confirmed that both dicumarol  
179 and tolfenamic acid were able to counteract erythromycin on several species (Fig. 3d,  
180 Extended Data Fig. 11). In contrast, both drugs did not affect the potency of erythromycin on  
181 *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecium*, pathogens  
182 against which erythromycin is active/prescribed (Fig. 3e, Extended Data Fig. 12a). For  
183 example, tolfenamic acid and dicumarol at concentration ranges of 5-40  $\mu$ M could rescue the  
184 growth of five out of seven tested abundant gut commensal species at clinically relevant  
185 erythromycin concentrations (Fig. 3f, Extended Data Fig. 12b). Altogether, our data provides  
186 a proof-of-principle for identifying antidotes that specifically mask the collateral damage of  
187 antibiotics on commensals. This concept would need to be further validated in the future in  
188 animal models. Antidotes may also need to be modified to late (colon)-release or non-

189 absorbable formulations to ensure they reach the gut and to minimize adverse effects from  
190 their primary action.

191 In summary, our study provides a high-resolution map of the collateral damage of  
192 antibiotics on 50 different resident gut microbes down to the level of individual drugs, species  
193 and partially even strains. We challenge the universal divide of antibiotics into bacteriostatic  
194 and bactericidal across bacteria, as this breaks down when tested beyond model organisms.  
195 Antibiotics with preferential killing of some species may be the most detrimental to our gut  
196 flora, although the first studies in a few healthy individuals point to the gut microbiota having  
197 some resilience against specific antibiotic regimens<sup>44</sup>. Understanding the underlying  
198 mechanisms for this selective killing might open up ways for the development of new  
199 antimicrobials, but also strategies for controlled microbiome modulation<sup>15</sup>. Finally, we provide  
200 a proof-of-concept that species-specificity of drug combinations<sup>12</sup> can be exploited to identify  
201 antidotes that selectively protect the gut microbiota from the adverse effects of systemic  
202 antibiotic therapy. This new approach adds to proposed and existing strategies of gut  
203 microbiota protection against antibiotics, such as co-administration of activated charcoal<sup>45</sup>,  $\beta$ -  
204 lactamases<sup>46</sup>, probiotics or (autologous) fecal transplants<sup>47</sup>. Overall, our results suggest that  
205 interactions of antibiotics and commensals merit deeper exploration, as our current  
206 knowledge of the mode(s) of action of antibiotics in model pathogens is not necessarily  
207 transferable to commensals.

## 208 **METHODS**

### 209 **Growth conditions**

210 All experiments from this study were performed in an anaerobic chamber (Coy Laboratory  
211 Products Inc) (2% H<sub>2</sub>, 12% CO<sub>2</sub>, 86% N<sub>2</sub>) and all materials and solutions used for these  
212 experiments were pre-reduced for at least 24 h before use unless specified otherwise.  
213 Bacteria used in this study were typically pre-cultured for two overnights: Cells were cultured  
214 in 5 ml modified Gifu Anaerobic Medium broth (MGAM) (HyServe GmbH & Co.KG, Germany,  
215 produced by Nissui Pharmaceuticals) and grown at 37°C overnight. The next day, cells were  
216 diluted 1/100 in 5 ml MGAM medium and grown at 37°C for a second overnight before  
217 starting the experiments.

218

### 219 **Quantitative assay for minimum inhibitory concentration determination with MICs test** 220 **strips**

221 MICs test strips were purchased from Liofilchem or Oxoid (Suppl. Table 2). All MICs were  
222 measured under anaerobic growth conditions inside a Coy anaerobic chamber. Bacteria  
223 were precultured in MGAM for two overnights and cultures were diluted to OD<sub>578</sub> = 0.5. 50 µl  
224 of the diluted culture were spread on a MGAM agar plate and allowed to dry for 15 min. The  
225 MIC test strip was placed on the agar with sterile tweezers, allowing the part with the lowest  
226 concentration touch the agar first. Plates were incubated at 37°C inside the anaerobic  
227 chamber, at least overnight and longer depending on the species-specific growth  
228 requirements. After formation of a symmetrical inhibition ellipse, plates were taken out of the  
229 chamber and imaged under controlled lighting conditions (splmager S&P Robotics Inc.) using  
230 an 18 megapixel Canon Rebel T3i (Canon Inc. USA). MICs were directly determined from  
231 the strip scale at the point where the edge of the inhibition ellipse intersects the MIC test  
232 strip. All MICs were determined in duplicates. In cases of an eight-fold difference between  
233 the two values, a third replicate was done. In all cases, this resulted in a clear outlier (> 8-fold  
234 different from other two MICs) that was removed from the dataset.

235

## 236 **MIC comparison to ChEMBL and EUCAST databases**

237 Previously known MICs were extracted from the ChEMBL database (version 24)<sup>27</sup> and  
238 EUCAST (obtained on May 14, 2018)<sup>24</sup>. Antibiotics from these two datasets were mapped to  
239 our dataset by name. Species were mapped using NCBI Taxonomy Identifiers and species  
240 names. For MICs from ChEMBL, a keyword-based approach was used to exclude  
241 experiments on species with mutations, deletions, insertions, etc. The EUCAST database  
242 contains a large number of reported MICs for each compound–species pair. We collapsed  
243 these to a single value by calculating the median MIC.

244 Estimates on the abundance and prevalence of species in the healthy human gut  
245 microbiome were calculated using mOTUs v2<sup>48</sup> as follows: Relative species abundances  
246 were determined in 727 shotgun metagenomic samples from donors in the control groups of  
247 multiple studies from various countries and continents<sup>49-53</sup>. Prior to taxonomic profiling,  
248 metagenomes were quality controlled using the MOCAT2 -rtf procedure<sup>54</sup>, which removed  
249 reads with  $\geq 95\%$  sequence identity and an alignment length of  $\geq 45$ bp to the human genome  
250 hg19. Taxonomic profiles were then created using mOTUs version 2.1.0<sup>48</sup> with parameters -l  
251 75 ; -g 2; and -c. Afterwards relative abundances below  $10^{-4}$  were set to zero and species  
252 with nonzero abundance in  $< 5$  samples discarded. For the retained 1,350 species,  
253 prevalence was defined as the percentage of samples with nonzero abundance; a  
254 prevalence cut-off of 1% was chosen to classify species into “rare” and “common” species.  
255 For all species in the MIC dataset, we manually assessed their status as pathogenic or non-  
256 pathogenic species using encyclopaedic and literature knowledge. Pathogenic species that  
257 occur in more than 1% of healthy people (i.e. are designated as “common”) were classified  
258 as “potentially pathogenic species” that can, for example, cause diseases in  
259 immunocompromised patients.

260

## 261 **Killing curves and survival assay**

262 Cells were precultured as described in the *growth conditions* section before being diluted to  
263 an  $OD_{578}=0.01$  and grown for 2 h at 37°C (unless specified otherwise). Next, cells were

264 diluted 1/2 in MGAM containing a 10-fold MIC of erythromycin, azithromycin or doxycycline  
265 (final antibiotic concentration is 5-fold MIC) and incubated in the presence of the antibiotic for  
266 5 h at 37°C. At several time-points (0, 1h, 2h, 3h, 4h, 5h), 100 µl of cells were serial-diluted in  
267 PBS ( $10^{-1}$  to  $10^{-8}$  dilutions) and plated on MGAM-Agar plates for CFU counting. When no  
268 cells were detected using this method, a bigger volume of culture (up to 2 ml) was plated to  
269 be able to detect CFUs. Agar plates were incubated overnight at 37°C and colonies were  
270 counted the next day, either manually, for low CFU numbers, or using the *Analyze Particles*  
271 tool from ImageJ<sup>55</sup>.

272

### 273 **Live/dead staining**

274 Cells were precultured as described in the *growth conditions* section before being diluted to  
275 an  $OD_{578}=0.01$  and grown for 2 h at 37°C. Cells were next diluted 1/2 in MGAM containing  
276 10-fold MIC of erythromycin, azithromycin or doxycycline (final concentration is 5-fold the  
277 MIC) and incubated in the presence of the antibiotic for 5 h at 37°C. Then, cells were  
278 live/dead stained using the *LIVE/DEAD BacLight Bacterial viability and counting kit* (#L34856  
279 Molecular Probes, ThermoFisher) according to the manufacturer's protocol before and after  
280 antibiotic treatment.

281

### 282 **Flow cytometry**

283 Stained cells were counted using a BD LSRFortessa<sup>TM</sup> flow cytometer. The forward and side  
284 scatter signals (488 nm) as well as the green and red fluorescent signals (488-530/30A filter  
285 and 561-610/20A filter, respectively) were acquired. The FSC/SSC detectors were set to  
286 logarithmic scale. The flow rate varied between 12 µl/min and 60 µl/min depending on the  
287 concentration of each sample, and the analysis was stopped when 10,000 target events  
288 were measured. Graphs were generated using the FlowJo V10.3 software (Treestar).

289

### 290 **Microscopy**



291 For live/dead imaging, stained cells were washed twice in 0.85% NaCl before being spotted  
292 on 0.85% NaCl +1% agarose pads between a glass slide and a coverslip. For time-lapse  
293 imaging, cells were precultured as described in the *growth conditions* section. Cells were  
294 then diluted to an  $OD_{578}=0.01$  and grown for 3 h at 37°C before being spotted on MGAM  
295 +1% agarose pads, supplemented or not with 15 µg/ml erythromycin (5-fold MIC) between a  
296 glass slide and a coverslip. Slides were sealed with valap (to avoid/delay oxygen  
297 permeation) and taken outside of the anaerobic chamber for imaging. In these conditions,  
298 untreated bacteria kept growing rapidly (Movie 1 + 3). The imaging was performed using a  
299 Nikon Eclipse Ti inverted microscope, equipped with a Nikon DS-Qi2 camera, a Nikon Plan  
300 Apo Lambda 60X oil Ph3 DM phase contrast objective and a Nikon HC mCherry filter set (Ex  
301 562/40; DM 593; BA 641/75) to detect propidium iodide fluorescence. Images were acquired  
302 with the NIS-Elements AR4.50.00 software and processed with Fiji v.2.0.0-rc-68/1.52h<sup>56</sup>.

303

#### 304 **Growth curves**

305 Cells were precultured as described in the *growth conditions* section. Then, cells were diluted  
306 to an  $OD_{578}=0.01$  in a 96-well plate sealed with a breathable membrane (Breathe-Easy®)  
307 and grown for 2 h. Next, erythromycin was added to the culture to a final concentration of 15  
308 µg/ml (5-fold MIC) and growth curves were acquired for 20 h using a microplate  
309 spectrophotometer (EON, Biotek) by measuring the  $OD_{578}$  every hour after 30 sec of linear  
310 shaking.

311

#### 312 **Screen for microbiome-protective antibiotic antagonism**

313 *Preparation of screening plates.* The Prestwick Chemical Library was purchased from  
314 Prestwick Chemical Inc. and drugs were re-arrayed, diluted and stored in 96 well format as  
315 described before<sup>4</sup>. We prepared drug plates (2 x drug concentration) in MGAM medium and  
316 stored them at -30°C. For each experiment, drug plates were thawed, supplemented with the  
317 respective antibiotic solution (freshly prepared in MGAM) and pre-reduced in the anaerobic



318 chamber overnight. All rearranging and aliquoting steps were done using the Biomek FXP  
319 (Beckman Coulter) system.

320 *Inoculation and screening conditions.* Strains were grown twice overnight, the second  
321 overnight culture was diluted in MGAM to reach  $OD_{578\text{ nm}} 0.04$  (4 x the desired starting OD).  
322 25  $\mu\text{l}$  of the diluted cultures were used to inoculate wells containing 50  $\mu\text{l}$  of 2x concentrated  
323 Prestwick drug and 25  $\mu\text{l}$  of the 4x concentrated antibiotic using the semi-automated, 96-well  
324 multi-channel pipette ep*Motion*96 (Eppendorf). Each well contained 1% DMSO, 20  $\mu\text{M}$  of the  
325 Prestwick drug and a species-specific antibiotic concentration that was just inhibitory for the  
326 respective strain (0.625  $\mu\text{M}$  for erythromycin, 0.04  $\mu\text{M}$  doxycycline for *B. uniformis* and 0.08  
327  $\mu\text{M}$  doxycycline for *B. vulgatus*). Plates were sealed with breathable membranes (Breathe-  
328 Easy®) and  $OD_{578}$  was measured hourly after 30 sec of linear shaking with a microplate  
329 spectrophotometer (EON, Biotek) and an automated microplate stacker (Biostack 4, Biotek)  
330 fitted inside a custom-made incubator (EMBL Mechanical Workshop). Growth curves were  
331 collected up to 24 h. For each antibiotic, each species was screen in biological duplicates. All  
332 experiments included control wells of unperturbed growth (32 wells per run) and control wells  
333 for growth in the presence of the antibiotic only (8 wells per plate).

334 *Analysis pipeline and hit calling.* All growth curves within a plate were truncated at the  
335 transition time from exponential to stationary phase and converted to normalized AUCs using  
336 in-run control wells (no drug) as described before<sup>4</sup>. We then calculated z-scores based on  
337 these normalized AUCs, removed replicates with 8-fold differences in z-scores to eliminate  
338 noise effects, computed mean z- scores across the two replicates and selected combinations  
339 with mean z-scores > 3. This selection included 19 potential antibiotic antagonists and we  
340 followed up on 14 of them (7 potential erythromycin and 7 potential doxycycline antagonists  
341 in either *B. vulgatus* or *B. uniformis* – see Extended Data Fig. 9) in independent experiments.

342 *Validation of microbiome-protective antagonists.* First, we kept the erythromycin/doxycycline  
343 concentration constant (0.625  $\mu\text{M}$  for erythromycin, 0.078  $\mu\text{M}$  (*B. vulgatus*)/ 0.039  $\mu\text{M}$  (*B.*  
344 *uniformis*) for doxycycline) and tested concentration gradients of the potential antagonists  
345 with ranges depending on the antagonist's solubility. Compounds were purchased from

346 independent vendors (Suppl. Table 5) and dissolved at 100x starting concentration in DMSO.  
347 Eight 2-fold serial dilutions were prepared in 96-well plates with each row containing a  
348 different antagonist, sufficient control DMSO wells and wells with just the respective antibiotic  
349 ('antibiotic-only' control). These master plates were diluted in MGAM medium (50  $\mu$ l) to 2 x  
350 assay concentration and 25  $\mu$ l freshly prepared antibiotic solution (4x test concentration) was  
351 added. Plates were pre-reduced overnight in an anaerobic chamber and inoculated with 25  
352  $\mu$ l of overnight cultures (prepared as described under *Growth conditions*) to reach a starting  
353 OD<sub>578</sub> of 0.01 and 1% DMSO concentration. Growth was monitored hourly for 24 h after 30  
354 sec of linear shaking (as described for the screen<sup>4</sup>). Experiments were performed in  
355 biological triplicates. For analysis, growth curves were converted into normalized AUCs (see  
356 above). We accounted for residual growth in the presence of the antibiotic by subtracting the  
357 median normalized AUCs of the 'antibiotic-only' control per plate. We computed medians  
358 across triplicates and considered a normalized AUC > 0.25 as concentration-dependent  
359 growth rescue by the antagonist.

360 *Checkerboard assays for anaerobic commensals.* Validated antagonists were further  
361 investigated in 8x8 checkerboard assays, where both antibiotics and antagonists were  
362 titrated against each other. Such assays were first performed for the commensals that were  
363 originally screened (i. e. *B. vulgatus* and *B. uniformis* – 4 replicates) and later expanded  
364 towards six further gut microbes (*B. caccae*, *B. fragilis* NT, *B. ovatus*, *B. thetaiotaomicron*, *P.*  
365 *copri*, *P. distasonis* – 2 replicates). For vertical gradients, 2-fold serial dilutions of the  
366 antagonists were prepared first in 100x in DMSO and diluted in MGAM as described above  
367 (section 'Validation of microbiome-protective antagonists'). Horizontal antibiotic dilution  
368 series were freshly prepared in MGAM at 4x final concentration in equidistant concentration  
369 steps. Both, vertical and horizontal dilution series were combined (50  $\mu$ l of the antagonist  
370 gradients (2x) and 25  $\mu$ l of the antibiotic gradients (4x)) in 96 well plates. Plates were pre-  
371 reduced under anaerobic conditions overnight, inoculated with 25  $\mu$ l of diluted overnight  
372 culture (at 4x starting OD) and sealed with breathable membrane (Breathe-Easy®). Bacterial  
373 growth was monitored once per hour for 24 h after 30 sec linear shaking (Eon + Biostack 4,

374 Biotek) under anaerobic conditions. Growth curves were converted into normalized AUCs as  
375 described using in-plate controls to define unperturbed growth.

376 *Checkerboard assays for pathogens under aerobic conditions.* For three pathogens (*S.*  
377 *aureus* DSM20231 ATCC 12600 and *E. faecium* ATCC19434) 8x8 checkerboard assays  
378 were performed in transparent 384 well plates (Greiner BioOne GmbH), with each well  
379 containing a total volume of 30  $\mu$ l in total for *S. aureus* and 55  $\mu$ l for *E. faecium*. *S. aureus*  
380 strains were grown in tryptic soy broth (TSB, Sigma Aldrich), *E. faecium* in BHI medium  
381 (Sigma Aldrich). Drugs were arrayed in 2-fold serial dilutions for the checkerboards. Cell  
382 were inoculated at initial  $OD_{595nm} \sim 0.01$  from an overnight culture. Plates were sealed with  
383 breathable membranes (Breathe-Easy), incubated at 37°C (Cytomat 2, Thermo Scientific)  
384 with continuous shaking and  $OD_{595nm}$  was measured every 30 min for 16 h in a Filtermax F5  
385 multimode plate reader (Molecular Devices). For *S. pneumoniae* D39, we only tested  
386 concentration gradients of the potential antagonists in a constant antibiotic concentration (0.2  
387  $\mu$ M erythromycin) in BHI medium. All experiments were done at least in 2 biological  
388 replicates and 2 technical replicates. Wells in which there was significant condensation were  
389 removed and background due to medium was subtracted. Growth curves were trimmed at  
390 the transition to stationary phase (9 h for *S. aureus*, 12 h for *E. faecium*). AUCs were  
391 calculated and normalised by the median of the internal no-drug control wells ( $n = 6$ ).  
392 Interactions were quantified according to the Bliss interaction model<sup>57</sup>. Interactions were  
393 called antagonistic if the median of all the interaction scores for a given checkerboard was  
394 above 0.05, synergistic if the value was below -0.05 and neutral if lying between these two  
395 cut-offs.

396

### 397 **Phylogenetic analysis/phylogenetic tree construction**

398 In order to generate a phylogenetic tree for the different isolates, the nucleotide sequences  
399 for a set of universally occurring, protein coding, single copy phylogenetic marker genes<sup>48,58</sup>  
400 were extracted from reference genomes or genome assemblies using fetchMG<sup>58</sup>  
401 (<https://motu-tool.org/fetchMG.html>). Within the framework of the ete3 toolkit<sup>59</sup>,

402 ClustalOmega<sup>60</sup> was used to create sequence alignments for each marker gene  
403 independently and all columns with more than 10% gaps were removed. The individual  
404 alignments were concatenated and finally, a phylogenetic tree was inferred from the  
405 combined alignment using IQTree<sup>61</sup>.

406

407 **Data availability**

408 Data is available upon request.

409

410 **Code availability**

411 Code is available upon request.

412

413

## 414 REFERENCES

- 415 1 Blaser, M. J. Antibiotic use and its consequences for the normal microbiome. *Science*  
416 **352**, 544-545, doi:10.1126/science.aad9358 (2016).
- 417 2 Modi, S. R., Collins, J. J. & Relman, D. A. Antibiotics and the gut microbiota. *J Clin*  
418 *Invest* **124**, 4212-4218, doi:10.1172/JCI72333 (2014).
- 419 3 Becattini, S., Taur, Y. & Pamer, E. G. Antibiotic-Induced Changes in the Intestinal  
420 Microbiota and Disease. *Trends Mol Med* **22**, 458-478,  
421 doi:10.1016/j.molmed.2016.04.003 (2016).
- 422 4 Maier, L. *et al.* Extensive impact of non-antibiotic drugs on human gut bacteria.  
423 *Nature* **555**, 623-628, doi:10.1038/nature25979 (2018).
- 424 5 Cho, I. *et al.* Antibiotics in early life alter the murine colonic microbiome and adiposity.  
425 *Nature* **488**, 621-626, doi:10.1038/nature11400 (2012).
- 426 6 Cox, L. M. *et al.* Altering the intestinal microbiota during a critical developmental  
427 window has lasting metabolic consequences. *Cell* **158**, 705-721,  
428 doi:10.1016/j.cell.2014.05.052 (2014).
- 429 7 Nobel, Y. R. *et al.* Metabolic and metagenomic outcomes from early-life pulsed  
430 antibiotic treatment. *Nat Commun* **6**, 7486, doi:10.1038/ncomms8486 (2015).
- 431 8 Ruiz, V. E. *et al.* A single early-in-life macrolide course has lasting effects on murine  
432 microbial network topology and immunity. *Nat Commun* **8**, 518, doi:10.1038/s41467-  
433 017-00531-6 (2017).
- 434 9 Hirsch, A. G. *et al.* Early-life antibiotic use and subsequent diagnosis of food allergy  
435 and allergic diseases. *Clin Exp Allergy* **47**, 236-244, doi:10.1111/cea.12807 (2017).
- 436 10 Korpela, K. *et al.* Intestinal microbiome is related to lifetime antibiotic use in Finnish  
437 pre-school children. *Nat Commun* **7**, 10410, doi:10.1038/ncomms10410 (2016).
- 438 11 Parker, E. P. K. *et al.* Changes in the intestinal microbiota following the administration  
439 of azithromycin in a randomised placebo-controlled trial among infants in south India.  
440 *Sci Rep* **7**, 9168, doi:10.1038/s41598-017-06862-0 (2017).
- 441 12 Brochado, A. R. *et al.* Species-specific activity of antibacterial drug combinations.  
442 *Nature* **559**, 259-263, doi:10.1038/s41586-018-0278-9 (2018).
- 443 13 Falony, G. *et al.* Population-level analysis of gut microbiome variation. *Science* **352**,  
444 560-564, doi:10.1126/science.aad3503 (2016).
- 445 14 Rothschild, D. *et al.* Environment dominates over host genetics in shaping human gut  
446 microbiota. *Nature* **555**, 210-215, doi:10.1038/nature25973 (2018).
- 447 15 Schmidt, T. S. B., Raes, J. & Bork, P. The Human Gut Microbiome: From Association  
448 to Modulation. *Cell* **172**, 1198-1215, doi:10.1016/j.cell.2018.02.044 (2018).
- 449 16 Jackson, M. A. *et al.* Gut microbiota associations with common diseases and  
450 prescription medications in a population-based cohort. *Nat Commun* **9**, 2655,  
451 doi:10.1038/s41467-018-05184-7 (2018).
- 452 17 Maier, L. & Typas, A. Systematically investigating the impact of medication on the gut  
453 microbiome. *Curr Opin Microbiol* **39**, 128-135, doi:10.1016/j.mib.2017.11.001 (2017).
- 454 18 Kuhn, M., Letunic, I., Jensen, L. J. & Bork, P. The SIDER database of drugs and side  
455 effects. *Nucleic Acids Res* **44**, D1075-1079, doi:10.1093/nar/gkv1075 (2016).
- 456 19 Arrieta, M. C. *et al.* Early infancy microbial and metabolic alterations affect risk of  
457 childhood asthma. *Sci Transl Med* **7**, 307ra152, doi:10.1126/scitranslmed.aab2271  
458 (2015).
- 459 20 Dethlefsen, L. & Relman, D. A. Incomplete recovery and individualized responses of  
460 the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad*  
461 *Sci U S A* **108 Suppl 1**, 4554-4561, doi:10.1073/pnas.1000087107 (2011).
- 462 21 Saari, A., Virta, L. J., Sankilampi, U., Dunkel, L. & Saxen, H. Antibiotic exposure in  
463 infancy and risk of being overweight in the first 24 months of life. *Pediatrics* **135**, 617-  
464 626, doi:10.1542/peds.2014-3407 (2015).
- 465 22 Gajdacs, M., Spengler, G. & Urban, E. Identification and Antimicrobial Susceptibility  
466 Testing of Anaerobic Bacteria: Rubik's Cube of Clinical Microbiology? *Antibiotics*  
467 (*Basel*) **6**, doi:10.3390/antibiotics6040025 (2017).

- 468 23 Nagy, E., Boyanova, L., Justesen, U. S. & Infections, E. S. G. o. A. How to isolate,  
469 identify and determine antimicrobial susceptibility of anaerobic bacteria in routine  
470 laboratories. *Clin Microbiol Infect* **24**, 1139-1148, doi:10.1016/j.cmi.2018.02.008  
471 (2018).
- 472 24 The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables  
473 for interpretation of MICs and zone diameters, v., 2019.  
474 [http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/). .
- 475 25 Bullman, S. *et al.* Analysis of Fusobacterium persistence and antibiotic response in  
476 colorectal cancer. *Science* **358**, 1443-1448, doi:10.1126/science.aal5240 (2017).
- 477 26 Manfredo Vieira, S. *et al.* Translocation of a gut pathobiont drives autoimmunity in  
478 mice and humans. *Science* **359**, 1156-1161, doi:10.1126/science.aar7201 (2018).
- 479 27 Gaulton, A. *et al.* The ChEMBL database in 2017. *Nucleic Acids Res* **45**, D945-D954,  
480 doi:10.1093/nar/gkw1074 (2017).
- 481 28 Bryan, L. E., Kowand, S. K. & Van Den Elzen, H. M. Mechanism of aminoglycoside  
482 antibiotic resistance in anaerobic bacteria: Clostridium perfringens and Bacteroides  
483 fragilis. *Antimicrob Agents Chemother* **15**, 7-13, doi:10.1128/aac.15.1.7 (1979).
- 484 29 Cani, P. D. & de Vos, W. M. Next-Generation Beneficial Microbes: The Case of  
485 Akkermansia muciniphila. *Front Microbiol* **8**, 1765, doi:10.3389/fmicb.2017.01765  
486 (2017).
- 487 30 Routy, B. *et al.* Gut microbiome influences efficacy of PD-1-based immunotherapy  
488 against epithelial tumors. *Science* **359**, 91-97, doi:10.1126/science.aan3706 (2018).
- 489 31 Slimings, C. & Riley, T. V. Antibiotics and hospital-acquired Clostridium difficile  
490 infection: update of systematic review and meta-analysis. *J Antimicrob Chemother*  
491 **69**, 881-891, doi:10.1093/jac/dkt477 (2014).
- 492 32 Baron, S., Diene, S. & Rolain, J.-M. Human microbiomes and antibiotic resistance.  
493 *Human Microbiome Journal* (2018).
- 494 33 Tramontano, M. *et al.* Nutritional preferences of human gut bacteria reveal their  
495 metabolic idiosyncrasies. *Nat Microbiol* **3**, 514-522, doi:10.1038/s41564-018-0123-9  
496 (2018).
- 497 34 Cohen J, O. S. M., Powderly W G, editors. . *Infectious diseases.2 vol. 3rd ed. St.*  
498 *Louis, Mo.; London: Mosby.* (2010.).
- 499 35 Habib, G. *et al.* 2015 ESC Guidelines for the management of infective endocarditis:  
500 The Task Force for the Management of Infective Endocarditis of the European  
501 Society of Cardiology (ESC). Endorsed by: European Association for Cardio-Thoracic  
502 Surgery (EACTS), the European Association of Nuclear Medicine (EANM). *Eur Heart*  
503 *J* **36**, 3075-3128, doi:10.1093/eurheartj/ehv319 (2015).
- 504 36 Kasper DL, F. A., Hauser SL, Longo DL. *Harrison's Principles of Internal Medicine,*  
505 *18e, 2012 McGraw-Hill.*
- 506 37 Liang, S. Y. & Kumar, A. Empiric antimicrobial therapy in severe sepsis and septic  
507 shock: optimizing pathogen clearance. *Curr Infect Dis Rep* **17**, 493,  
508 doi:10.1007/s11908-015-0493-6 (2015).
- 509 38 Lobritz, M. A. *et al.* Antibiotic efficacy is linked to bacterial cellular respiration. *Proc*  
510 *Natl Acad Sci U S A* **112**, 8173-8180, doi:10.1073/pnas.1509743112 (2015).
- 511 39 Ocampo, P. S. *et al.* Antagonism between bacteriostatic and bactericidal antibiotics is  
512 prevalent. *Antimicrob Agents Chemother* **58**, 4573-4582, doi:10.1128/AAC.02463-14  
513 (2014).
- 514 40 French, G. L. Bactericidal agents in the treatment of MRSA infections--the potential  
515 role of daptomycin. *J Antimicrob Chemother* **58**, 1107-1117, doi:10.1093/jac/dkl393  
516 (2006).
- 517 41 Jelic, D. & Antolovic, R. From Erythromycin to Azithromycin and New Potential  
518 Ribosome-Binding Antimicrobials. *Antibiotics (Basel)* **5**,  
519 doi:10.3390/antibiotics5030029 (2016).
- 520 42 Nemeth, J., Oesch, G. & Kuster, S. P. Bacteriostatic versus bactericidal antibiotics for  
521 patients with serious bacterial infections: systematic review and meta-analysis. *J*  
522 *Antimicrob Chemother* **70**, 382-395, doi:10.1093/jac/dku379 (2015).



- 523 43 Wald-Dickler, N., Holtom, P. & Spellberg, B. Busting the Myth of "Static vs Cidal": A  
524 Systemic Literature Review. *Clin Infect Dis* **66**, 1470-1474, doi:10.1093/cid/cix1127  
525 (2018).
- 526 44 Palleja, A. *et al.* Recovery of gut microbiota of healthy adults following antibiotic  
527 exposure. *Nat Microbiol* **3**, 1255-1265, doi:10.1038/s41564-018-0257-9 (2018).
- 528 45 de Gunzburg, J. *et al.* Protection of the Human Gut Microbiome From Antibiotics. *The*  
529 *Journal of Infectious Diseases* **217**, 628-636, doi:10.1093/infdis/jix604 (2017).
- 530 46 Stiefel, U., Nerandzic, M. M., Koski, P. & Donskey, C. J. Orally administered beta-  
531 lactamase enzymes represent a novel strategy to prevent colonization by *Clostridium*  
532 *difficile*. *J Antimicrob Chemother* **62**, 1105-1108, doi:10.1093/jac/dkn298 (2008).
- 533 47 Suez, J. *et al.* Post-Antibiotic Gut Mucosal Microbiome Reconstitution Is Impaired by  
534 Probiotics and Improved by Autologous FMT. *Cell* **174**, 1406-1423 e1416,  
535 doi:10.1016/j.cell.2018.08.047 (2018).
- 536 48 Milanese, A. *et al.* Microbial abundance, activity and population genomic profiling with  
537 mOTUs2. *Nat Commun* **10**, 1014, doi:10.1038/s41467-019-08844-4 (2019).
- 538 49 Feng, Q. *et al.* Gut microbiome development along the colorectal adenoma-  
539 carcinoma sequence. *Nat Commun* **6**, 6528, doi:10.1038/ncomms7528 (2015).
- 540 50 Vogtmann, E. *et al.* Colorectal Cancer and the Human Gut Microbiome:  
541 Reproducibility with Whole-Genome Shotgun Sequencing. *PLoS One* **11**, e0155362,  
542 doi:10.1371/journal.pone.0155362 (2016).
- 543 51 Wirbel, J. *et al.* Meta-analysis of fecal metagenomes reveals global microbial  
544 signatures that are specific for colorectal cancer. *Nat Med* **25**, 679-689,  
545 doi:10.1038/s41591-019-0406-6 (2019).
- 546 52 Yu, J. *et al.* Metagenomic analysis of faecal microbiome as a tool towards targeted  
547 non-invasive biomarkers for colorectal cancer. *Gut* **66**, 70-78, doi:10.1136/gutjnl-  
548 2015-309800 (2017).
- 549 53 Zeller, G. *et al.* Potential of fecal microbiota for early-stage detection of colorectal  
550 cancer. *Mol Syst Biol* **10**, 766, doi:10.15252/msb.20145645 (2014).
- 551 54 Kultima, J. R. *et al.* MOCAT2: a metagenomic assembly, annotation and profiling  
552 framework. *Bioinformatics* **32**, 2520-2523, doi:10.1093/bioinformatics/btw183 (2016).
- 553 55 Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of  
554 image analysis. *Nat Methods* **9**, 671-675 (2012).
- 555 56 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat*  
556 *Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).
- 557 57 CI, B. The toxicity of poisons applied jointly. *Ann Appl Biol* **26**, 585-615 (1939).
- 558 58 Sunagawa, S. *et al.* Metagenomic species profiling using universal phylogenetic  
559 marker genes. *Nat Methods* **10**, 1196-1199, doi:10.1038/nmeth.2693 (2013).
- 560 59 Huerta-Cepas, J., Serra, F. & Bork, P. ETE 3: Reconstruction, Analysis, and  
561 Visualization of Phylogenomic Data. *Mol Biol Evol* **33**, 1635-1638,  
562 doi:10.1093/molbev/msw046 (2016).
- 563 60 Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence  
564 alignments using Clustal Omega. *Mol Syst Biol* **7**, 539, doi:10.1038/msb.2011.75  
565 (2011).
- 566 61 Nguyen, L. T., Schmidt, H. A., von Haeseler, A. & Minh, B. Q. IQ-TREE: a fast and  
567 effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol*  
568 *Biol Evol* **32**, 268-274, doi:10.1093/molbev/msu300 (2015).

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571

572 **SUPPLEMENTARY INFORMATION**

573 **Supplementary tables**

574 **Supplementary Table 1**

575 Annotation and classification of antibiotics

576 **Supplementary Table 2**

577 MICs strips used in this study

578 **Supplementary Table 3**

579 MICs as determined by MIC strips for selected antibiotics

580 **Supplementary Table 4**

581 Hitlist: Screen for microbiome-protective antagonists to erythromycin/tetracycline

582 **Supplementary Table 5**

583 Compounds used in this study

584 **Supplementary movies**

585 **Movie 1**

586 Time-lapse of *B. vulgatus* growing on MGAM-Agarose 1% pad.

587 **Movie 2**

588 Time-lapse of *B. vulgatus* growing on MGAM-Agarose 1% pad containing 5-fold MIC of  
589 erythromycin.

590 **Movie 3**

591 Time-lapse of *B. uniformis* growing on MGAM-Agarose 1% pad.

592 **Movie 4**

593 Time-lapse of *B. uniformis* growing on MGAM-Agarose 1% pad containing 5-fold MIC of  
594 erythromycin.

595

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605

606

#### 607 **Author contributions**

608 This study was conceived by KRP, PB and AT; designed by LM, CVG, MP and AT; and  
609 supervised by LM and AT. Experiments were conducted by LM, CVG and MP. TB and EEA  
610 contributed to MIC measurements and EC to checkerboard analyses. Data preprocessing,  
611 curation and comparisons to existing databases were performed by JW, MK, AM, UL, SKF  
612 and GZ. Data interpretation was performed by LM, CVG, JW, GZ and AT. LM, CVG and AT  
613 wrote the manuscript with feedback from all authors; LM, CVG, JW and MK designed figures  
614 with inputs from GZ and AT. All authors approved the final version for publication.

615

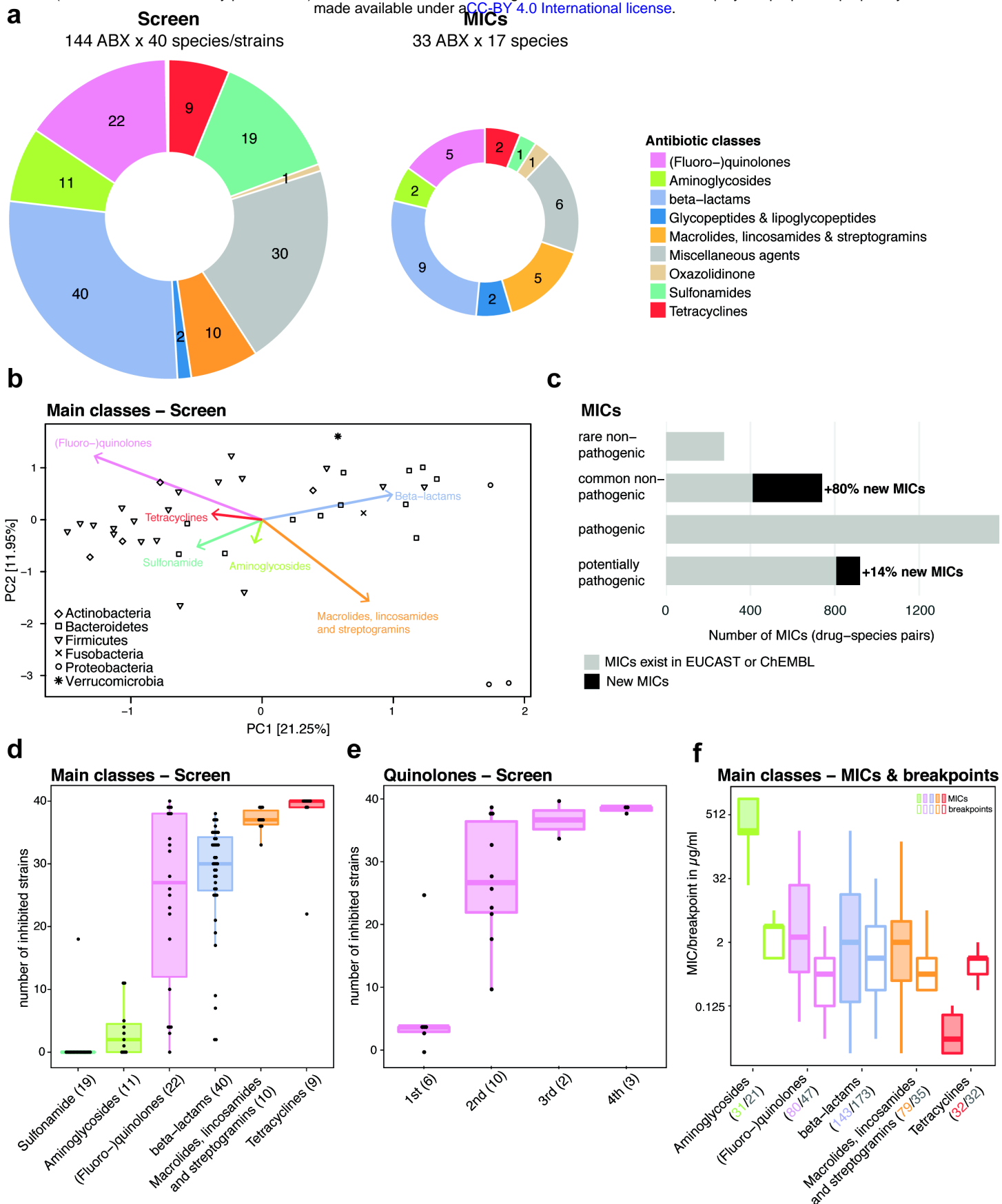
#### 616 **Author information**

617 EMBL has filed a patent application on using the antidotes identified in this study for  
618 prevention and/or treatment of dysbiosis and for microbiome protection (European patent  
619 application number EP19216548.8). LM, CVG, EC and AT are listed as inventors.

620 Correspondence and requests for materials should be addressed to [typas@embl.de](mailto:typas@embl.de) or  
621 [l.maier@uni-tuebingen.de](mailto:l.maier@uni-tuebingen.de).

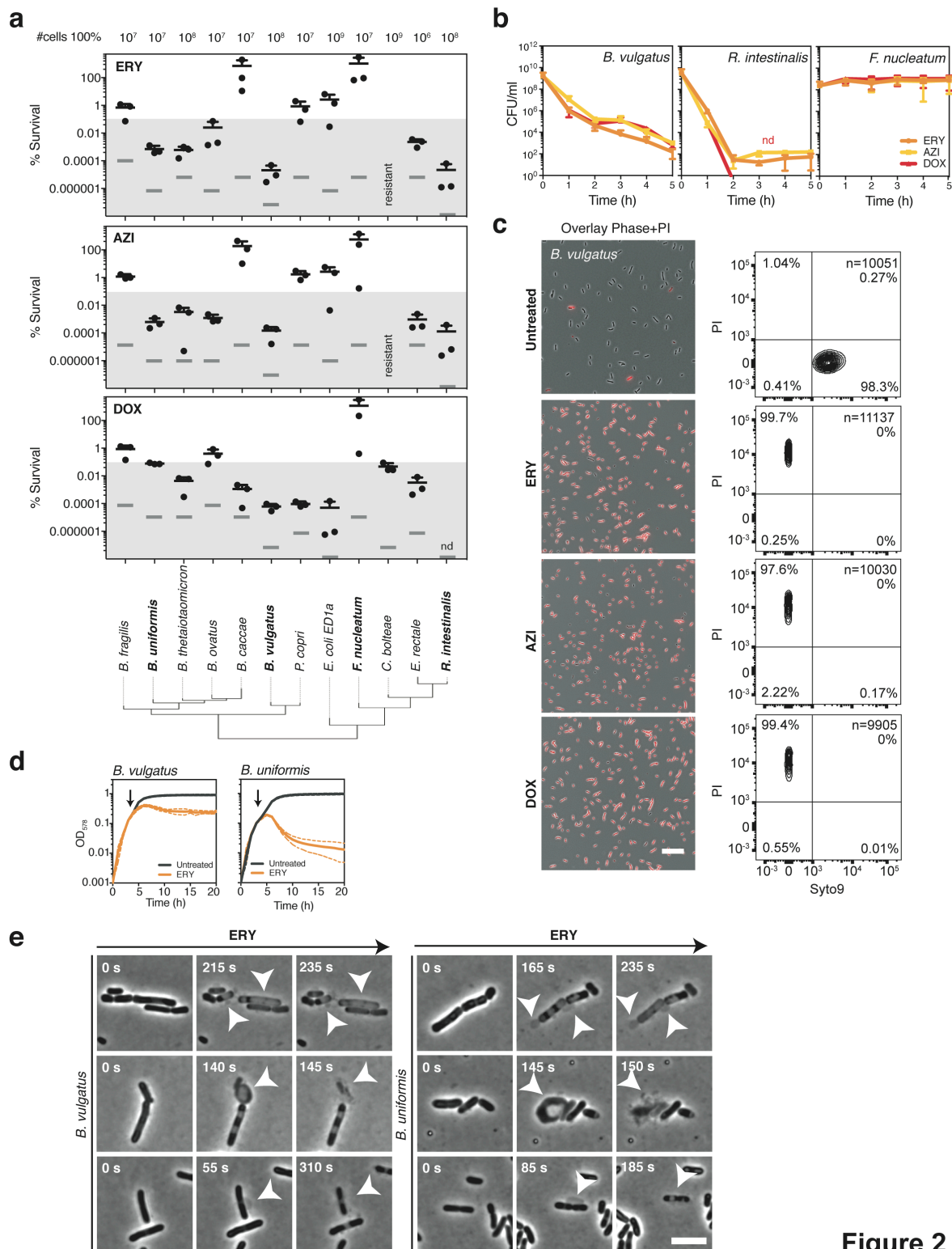
622

623



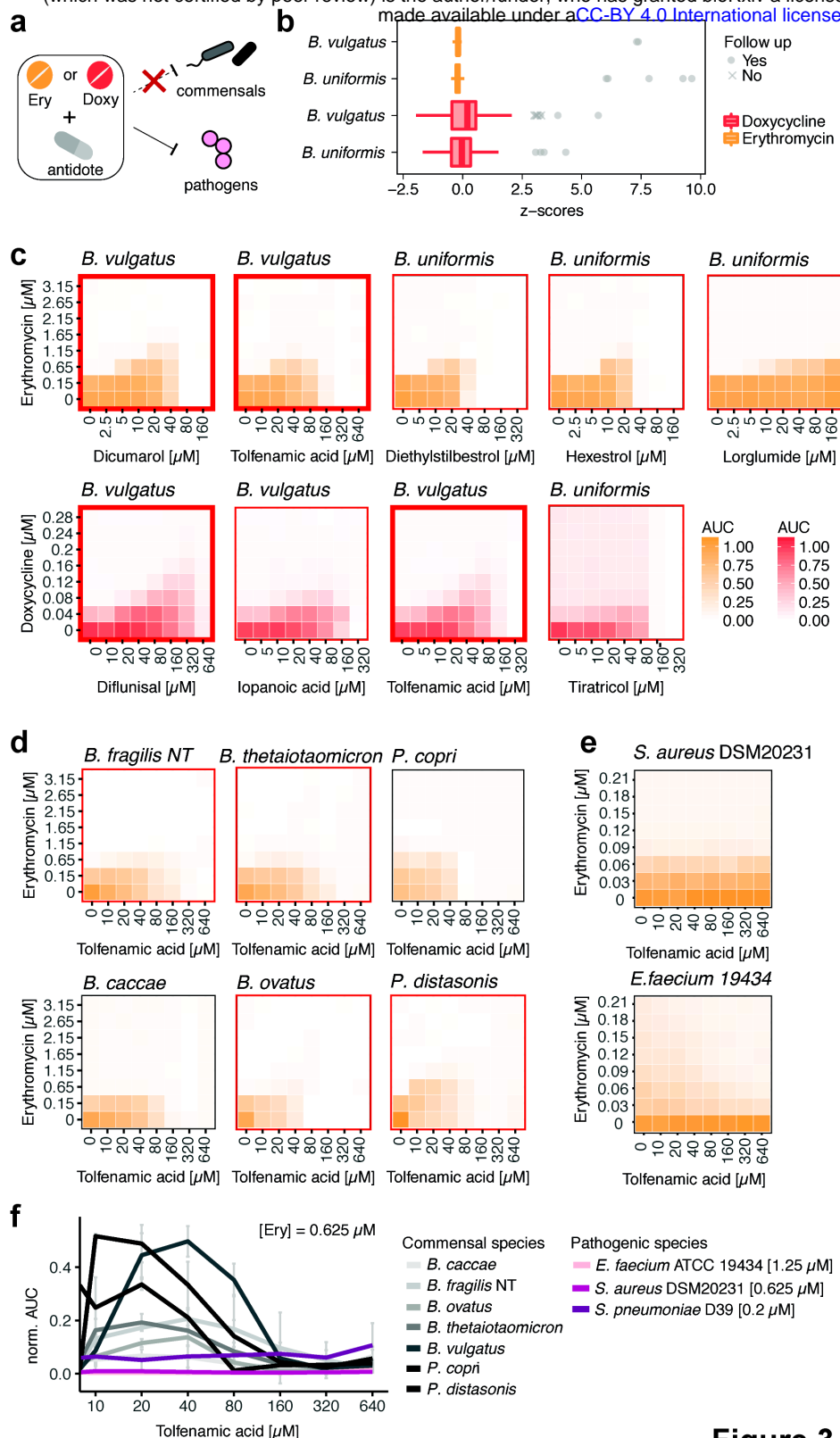
**Figure 1 –Activity spectrum of antibiotic classes on human gut commensals.**

**a.** Overview of antibiotics tested in initial screen at 20  $\mu\text{M}$  concentration<sup>4</sup> and validated by MIC determination in this study. **b.** Principal component analysis based on AUCs from the initial screen on the effects of antibiotics on gut commensals. Antibiotic classes drive some separation at the phylum level, e.g. beta-lactams separate Bacteroidetes and macrolides/lincosamides/streptogramins separate Proteobacteria. **c.** Comparison of MICs for MICs available from public databases. Species are classified as “common” or “rare” if they are present in the gut microbiome of more or less than 1% of 727 healthy individuals, respectively (see Methods). **d.** For the main antibiotic classes from the screen, the numbers of inhibited strains are shown (N as in **a**). 40 strains tested in total at a 20  $\mu\text{M}$  antibiotic concentration. Boxes span the IQR and whiskers extend to the most extreme data points up to a max of 1.5 times the IQR. **e.** Number of inhibited strains per (fluoro-)quinolone drug generation. Number of tested drugs per generation is indicated in brackets on x-axis labeling. Boxplots as in panel **d**. **f.** MICs of drug-species pairs for the main antibiotic classes measured in this study are depicted next to EUCAST clinical (susceptibility) breakpoints for pathogens. Numbers of drug-species pairs (MICs; colored) and of antibiotic per class (EUCAST clinical breakpoints; grey) are shown in brackets. Boxplots as in panel **d**, y-axis is log<sub>2</sub> scale.



**Figure 2 - Macrolides and tetracyclines kill human gut commensal species**

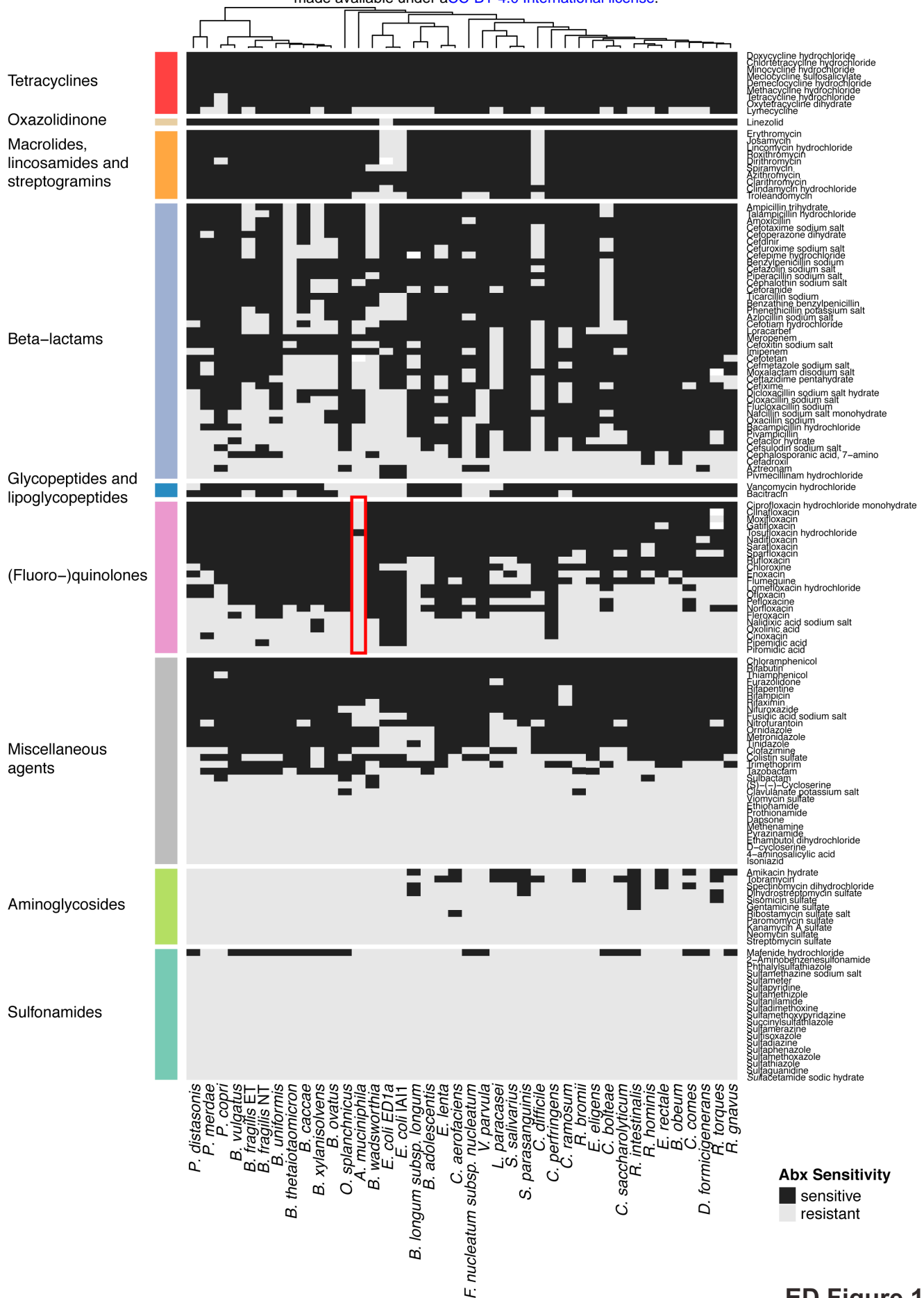
**a.** The survival of 12 abundant gut microbe species was measured after a 5-hour treatment with a 5-fold MIC of erythromycin (ERY), azithromycin (AZI) or doxycycline (DOX). The survival was assessed by counting CFUs/ml before and after antibiotic treatment. The number of CFUs/ml before treatment was set as 100%. The detection limit for each experiment (gray bar) and the bactericidal threshold (shaded area) are indicated. Species are plotted according to phylogeny (IQTree, Methods) and in bold are noted the species that are used in later panels. The graph shows the mean±SD of 3 independent experiments. **b.** Time-kill curves of *B. vulgatus*, *R. intestinalis* and *F. nucleatum* after antibiotic treatments. Survival was assessed by CFU counting over a 5 hour-treatment of ERY, AZI or DOX. This graph shows the mean±SD of 3 independent experiments. Nd: non-detectable. Time-kill curves for the other tested gut microbes can be found in Extended Data Fig. 5. **c.** Live/dead staining of macrolide or tetracycline-treated *B. vulgatus*. The left panel shows an overlay of phase contrast and fluorescence microscopy images of propidium iodide (PI)-stained *B. vulgatus* before and 5 hours after ERY, AZI or DOX treatment. Cultures were concentrated before imaging; the scale bar is 10  $\mu$ m. The right panel shows the corresponding quantification of live/dead-stained cells by flow cytometry with Syto9 on the x-axis (live cells) and PI on the y-axis (dead cells). Both the total number of measured events (n) and the percentage of cells found in each quadrant are indicated. **d.** Erythromycin induces lysis of *B. vulgatus* and *B. uniformis*. *B. vulgatus* and *B. uniformis* were grown for 3 hours before addition (yellow) or not (black) of 15  $\mu$ g/ml ERY treatment (5-fold MIC; yellow) as indicated by the arrow. Growth curves were acquired for 20 hours. This graph shows the mean±SD (dotted line) of 3 independent experiments. **e.** Erythromycin induces blebbing, cytoplasmic shrinkage and lysis in *B. vulgatus* and *B. uniformis*. Phase contrast movies of *B. vulgatus* and *B. uniformis* were acquired after ERY treatment (5-fold MIC). Here shown 3 frames of 3 images per strain (time indicated in the upper left corner; t=0 when drug added). White arrows indicate blebs, cytoplasmic shrinkage and bacterial lysis; the scale bar is 5  $\mu$ m. Movies are available in Supplementary Material (Movies 1-4).



**Figure 3**

**Figure 3 – Antidotes for selective protection of prevalent and abundant gut commensal species from macrolides and tetracyclines.**

**a.** Schematic illustration of the screen concept: searching for antidote compounds that antagonize the antibacterial effect of erythromycin or doxycycline on commensal but not on pathogenic bacteria. **b.** Z-scores on bacterial growth (based on areas under the curve (AUCs)) for combinatorial drug exposure with antibiotic (ERY or DOX) and FDA-approved drug. Compounds that successfully rescued *B. vulgatus* and/or *B. uniformis* growth in the presence of the antibiotic (z-score > 3) are indicated in gray. The strongest hits (circles) were validated further in concentration-dependent assays (Extended Data Fig. 10a). For each antibiotic and each strain, ~1200 drugs were tested in two replicates. Boxplots are defined as in Figure 1d. **c.** For 9 of the validated antagonists, 8 x 8 checkerboard assays were performed to determine concentration ranges of the antagonistic interaction. Heat maps depict bacterial growth based on normalized median of AUCs of 4 replicates. All interactions were antagonistic, and pairs tested further in other commensal species are in bold. **d.** Checkerboard assays confirm the ability of tolfenamic acid to protect further gut commensals from growth inhibition by erythromycin. Heat map as in **c**, but for 2 replicates. Antagonistic interactions are framed in red. **e.** Checkerboard of tolfenamic acid with erythromycin reveal neutral interactions in *S. aureus* and *E. faecium* (aerobic conditions). Heat maps as in **c**, based on at least two independent experiments with two technical replicates each. **f.** Tolfenamic acid concentration-dependent rescue of commensal growth at clinical relevant erythromycin concentrations based on AUCs (anaerobic conditions). Erythromycin still retains its activity against pertinent pathogens such as *S. aureus*, *E. faecium* and *S. pneumoniae* (aerobic conditions). 0.625  $\mu\text{M}$  correspond to ~0.5  $\mu\text{g/ml}$  erythromycin, which is in the range of the MIC breakpoints for *Staphylococcus* (1  $\mu\text{g/ml}$ ), *S. pneumoniae* (0.25  $\mu\text{g/ml}$ ) and *Streptococci* groups A, B, C & G (0.25  $\mu\text{g/ml}$ ). Error bars depict standard deviation.

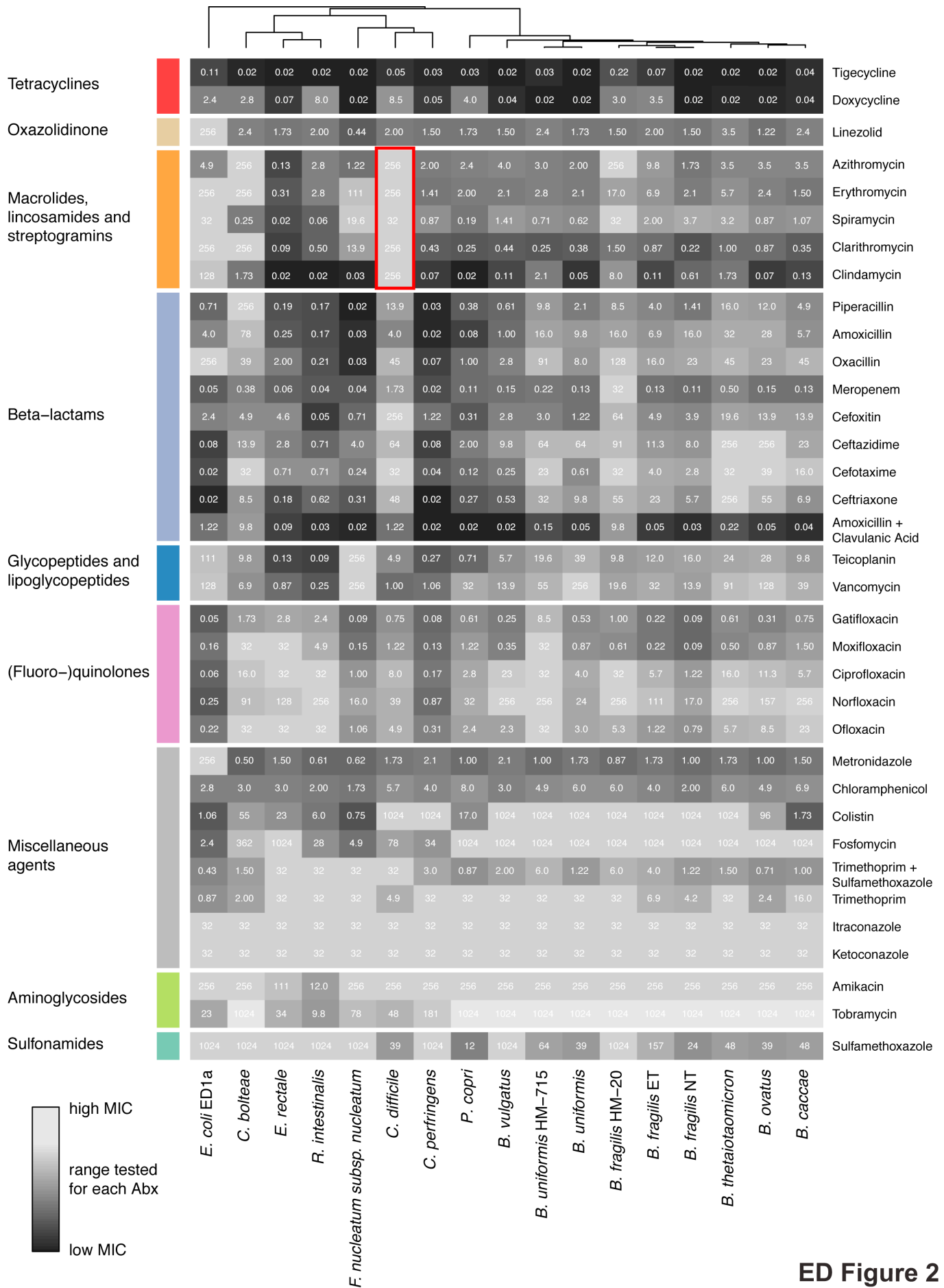


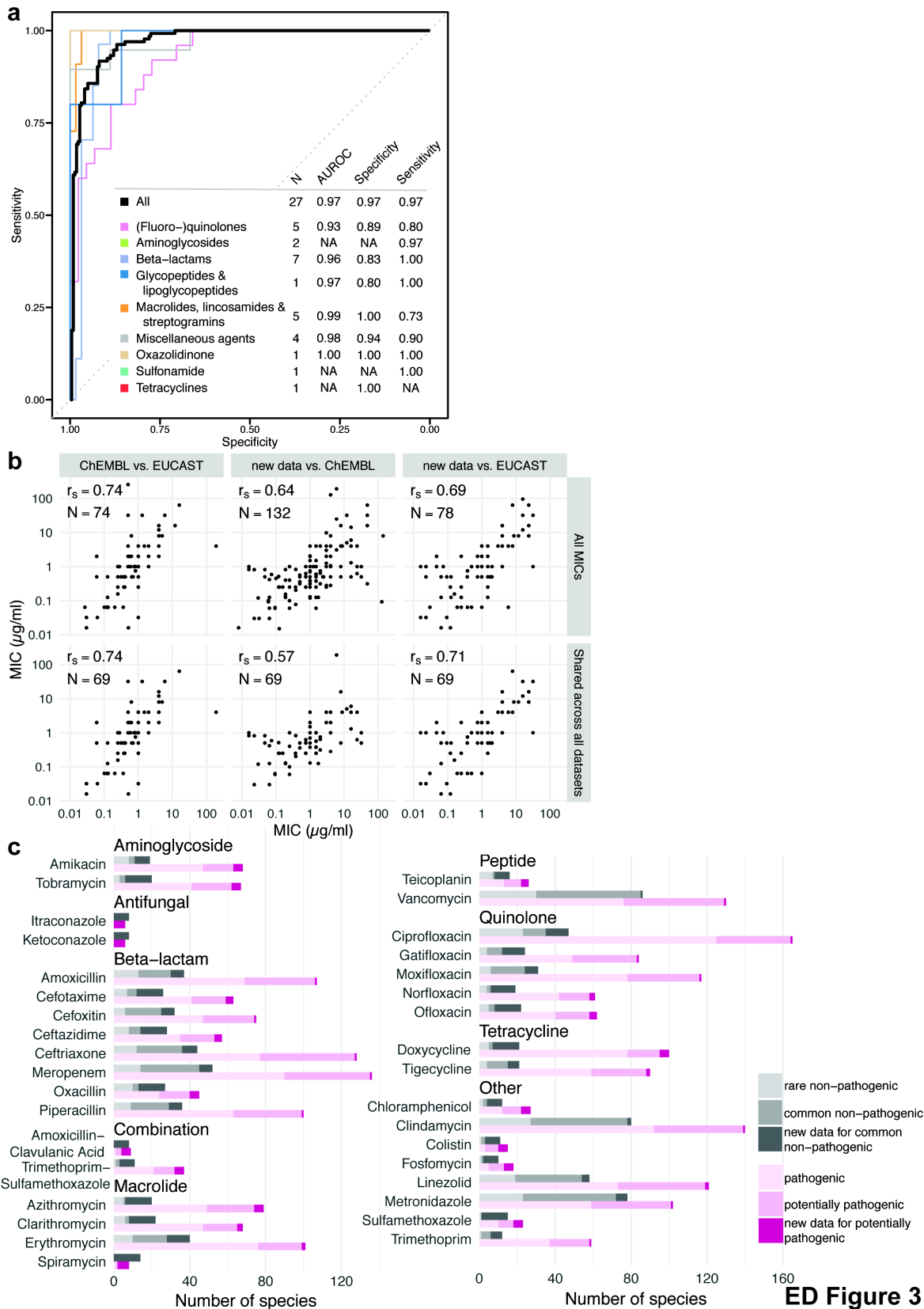
ED Figure 1

**Extended Data Figure 1 – Effects of 144 antibiotics on 40 human gut commensals**

Heat map according to sensitivity or resistance of each strain to the respective antibiotic at a concentration of 20 µM. Antibiotics are grouped according to drug classes and species are clustered according to their responses across the 144 antibiotics tested. Data is replotted from<sup>4</sup>. *Akkermansia muciniphila* (Muc, DSM22959, type strain) is resistant to nearly all quinolone antibiotics (red box). We consolidated this finding by MIC determination for Ciprofloxacin (>32 µg/ml), Gatifloxacin (>32 µg/ml), Moxifloxacin (>32 µg/ml), Norfloxacin (>256 µg/ml) and Ofloxacin (>32 µg/ml).







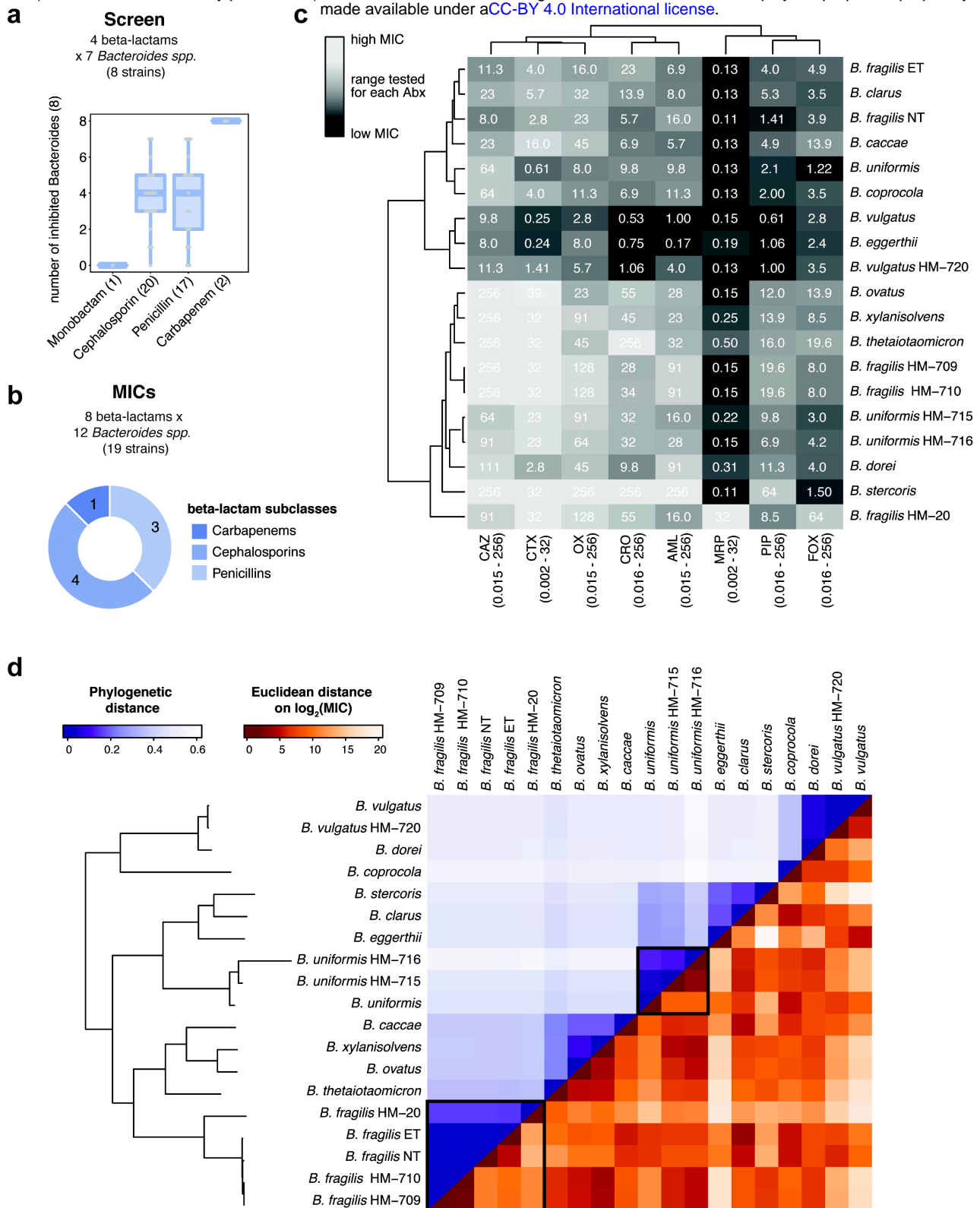
**ED Figure 3**

**Extended Data Figure 3 – MIC dataset validates antibiotic sensitivity profiles from the screen dataset and is consistent with publically available MICs.**

**a.** Receiver operating characteristic (ROC) curve analysis was performed to evaluate sensitivity and specificity of the screen<sup>4</sup> using the MIC dataset. Results from the screen were considered as validated if MICs were below/above the 20 µM antibiotic concentration that was tested in the screen (allowing a two-fold error margin). N is the number of antibiotics that we tested both in the screen and determined MICs for, AUROC is the area under the characteristic ROC. TN denotes true negatives, FP false positives, TP true positives, FN false negatives.

**b.** Comparison including Spearman correlation coefficients of the MICs from this study to MICs from the ChEMBL<sup>27</sup> and EUCAST<sup>24</sup> databases. Panels in the upper row: comparison between all MICs that are shared between the two indicated datasets. Panels in the lower row: comparison of the 69 MICs that are shared across all three datasets. Despite experimental differences, our MICs correlate well with available EUCAST/ChEMBL data.

**c.** Number of the sum of new (this study) and already available MICs (EUCAST/ ChEMBL) per drug according to antibiotic class and prevalence/virulence of the bacterial species. The new dataset expands MICs across the board and specifically fills the knowledge gap on non-pathogenic species.



**ED Figure 4**

**Extended Data Figure 4 –  $\beta$ -lactam antibiotic resistance profiles do not recapitulate phylogenetic relationship between *Bacteroides* spp.**

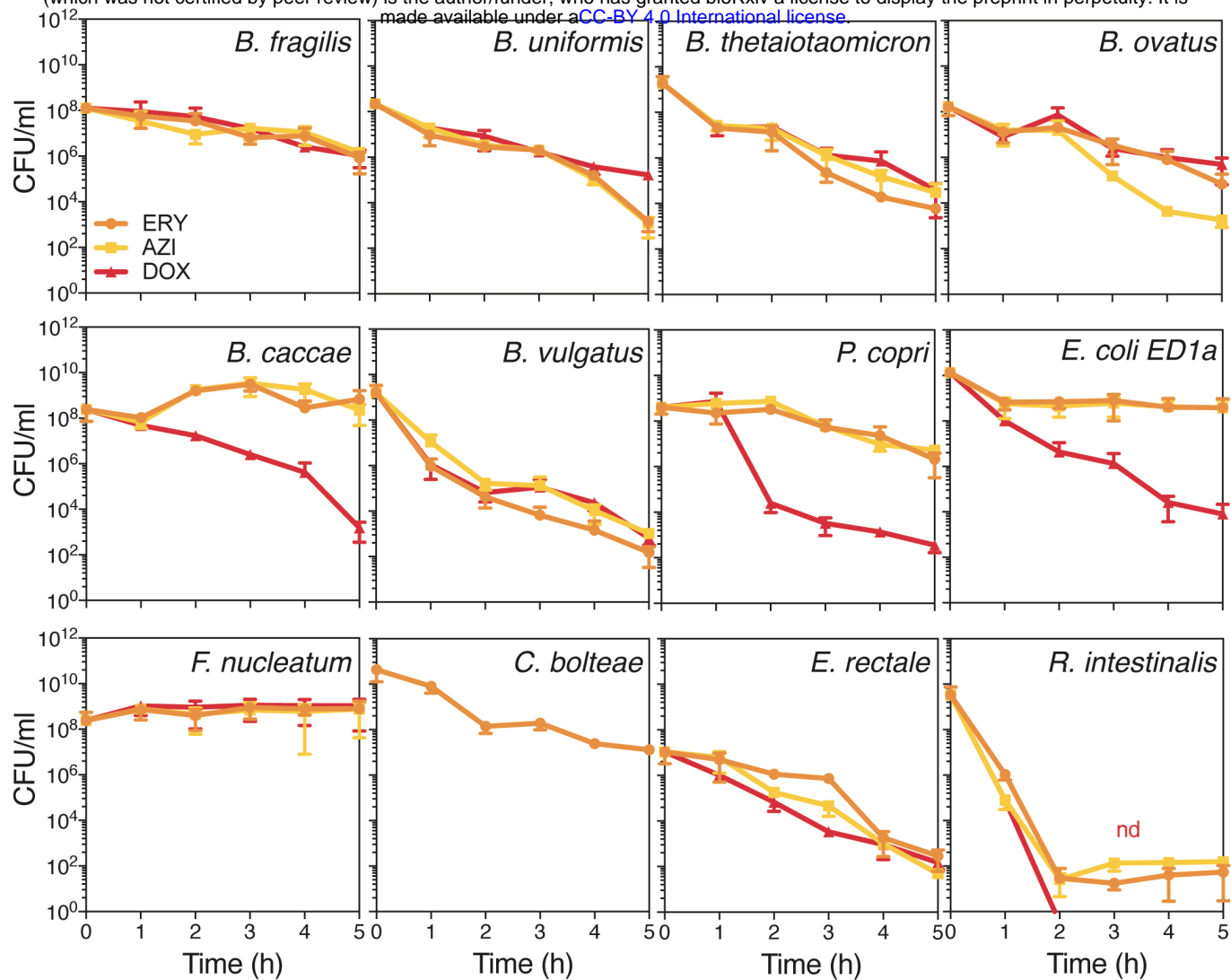
**a.** Number of inhibited *Bacteroides* spp. (out of 8 tested) at 20  $\mu$ M per  $\beta$ -lactam subclass, based on the initial screen<sup>4</sup>. Number of drugs per class tested are shown in parenthesis. Boxes plotted as in Figure 1d.

**b.** Overview of the number of drugs tested per  $\beta$ -lactam subclasses on *Bacteroides* spp.; compared to ED Figure 2, 10 additional strains were tested: *B. eggerthii*, *B. clarus*, *B. coprocola*, *B. vulgatus* HM-720, *B. xylanisolvans*, *B. fragilis* HM-709, *B. fragilis* HM-710, *B. uniformis* HM-716, *B. dorei* and *B. stercoris*.

**c.** MIC heat map for 8  $\beta$ -lactam antibiotics on 19 *Bacteroides* spp. Strains are clustered according to resistance profiles across all  $\beta$ -lactam antibiotics, drugs are clustered according to their effects on *Bacteroides* spp.; compared to ED Figure 2, 10 additional strains were tested: *B. eggerthii*, *B. clarus*, *B. coprocola*, *B. vulgatus* HM-720, *B. xylanisolvans*, *B. fragilis* HM-709, *B. fragilis* HM-710, *B. uniformis* HM-716, *B. dorei* and *B. stercoris*. Heat map gradients are adjusted to the antibiotic concentration ranges tested with lighter color depicting resistance and darker color depicting sensitivity.

**d.** Heat map of phylogenetic relationship between *Bacteroides* spp. (upper triangular matrix) ordered by phylogeny and their resistance profiles across  $\beta$ -lactam antibiotics (lower triangular matrix). Colors represent the pairwise phylogenetic distance and the Euclidean distance on the log<sub>2</sub> transformed MICs for  $\beta$ -lactams (panel c). Examples of strains from the same species (*B. fragilis* / *B. uniformis*) that respond differently to  $\beta$ -lactam antibiotics, are highlighted.

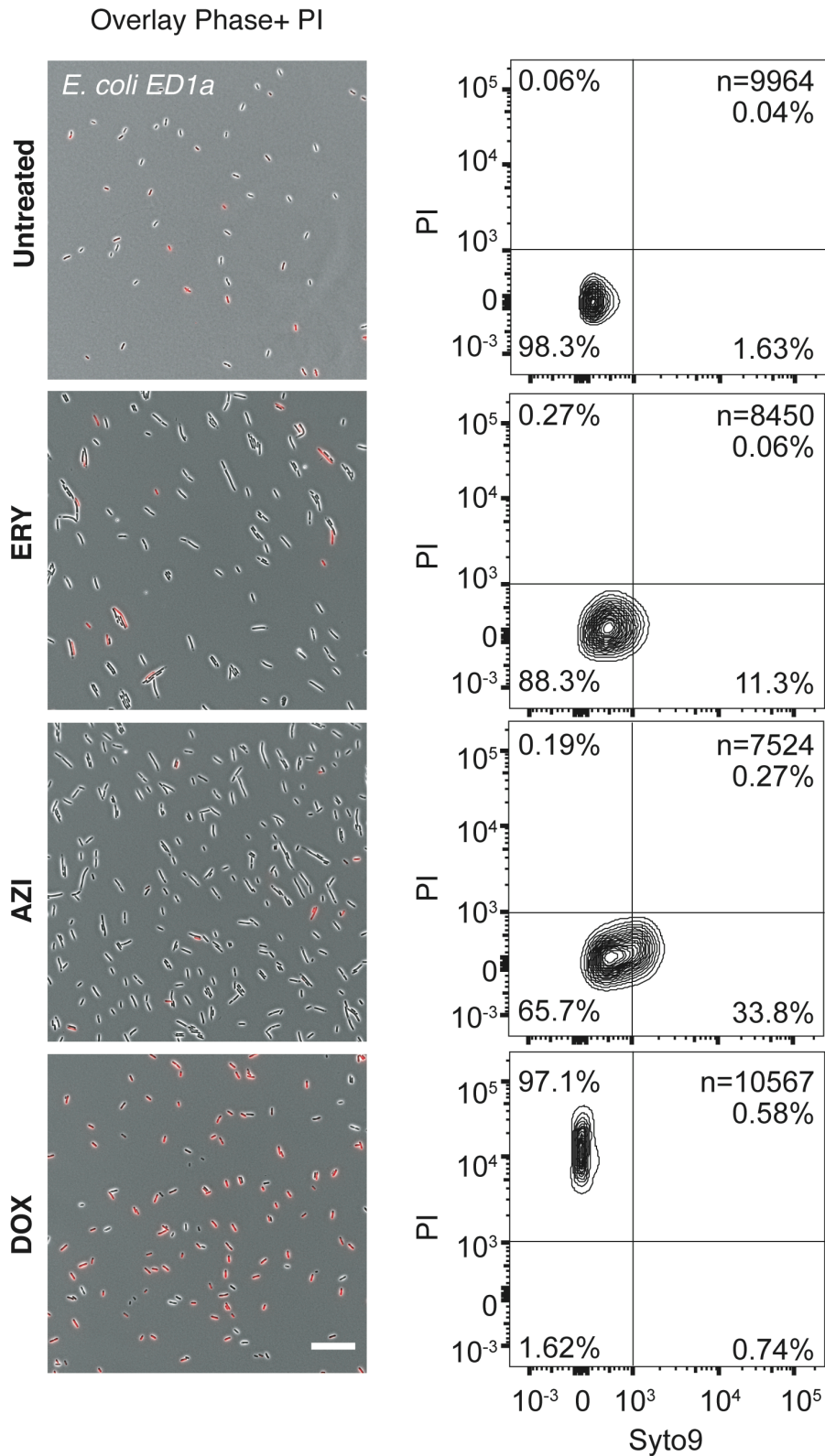




## ED Figure 5

### Extended Data Figure 5 - Time-kill curves of 12 abundant gut microbes after treatment with macrolides and tetracyclines.

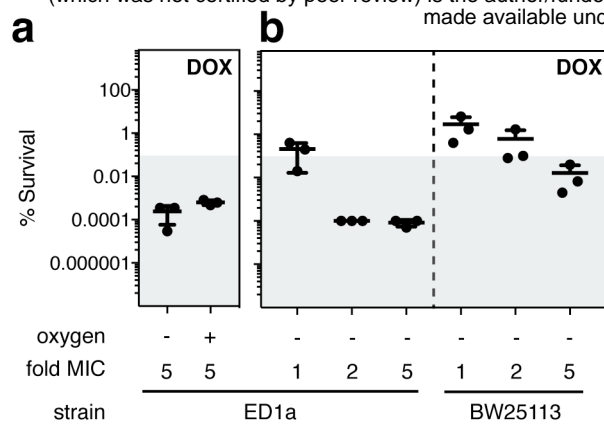
Survival of 12 abundant gut microbes was assessed by CFU counting over a 5 hour-treatment of either ERY, AZI or DOX. This graph shows the mean±SD of 3 independent experiments.



## ED Figure 6

### Extended Data Figure 6 - Live/dead staining of macrolide or tetracycline-treated *E. coli* ED1a.

The left panel shows an overlay of phase contrast and fluorescence microscopy images of propidium iodide (PI)-stained *E. coli* ED1a before and 5 hours after ERY, AZI or DOX treatments. The number of cells on each frame has no meaning, as cultures were concentrated before imaging; the scale bar is 10  $\mu$ m. The right panel shows the corresponding quantification of live/dead-stained cells by flow cytometry with Syto9 on the x-axis (live cells) and PI on the y-axis (dead cells). Both the total number of measured events (n) and the percentage of cells found in each quadrant are indicated on the graphs.

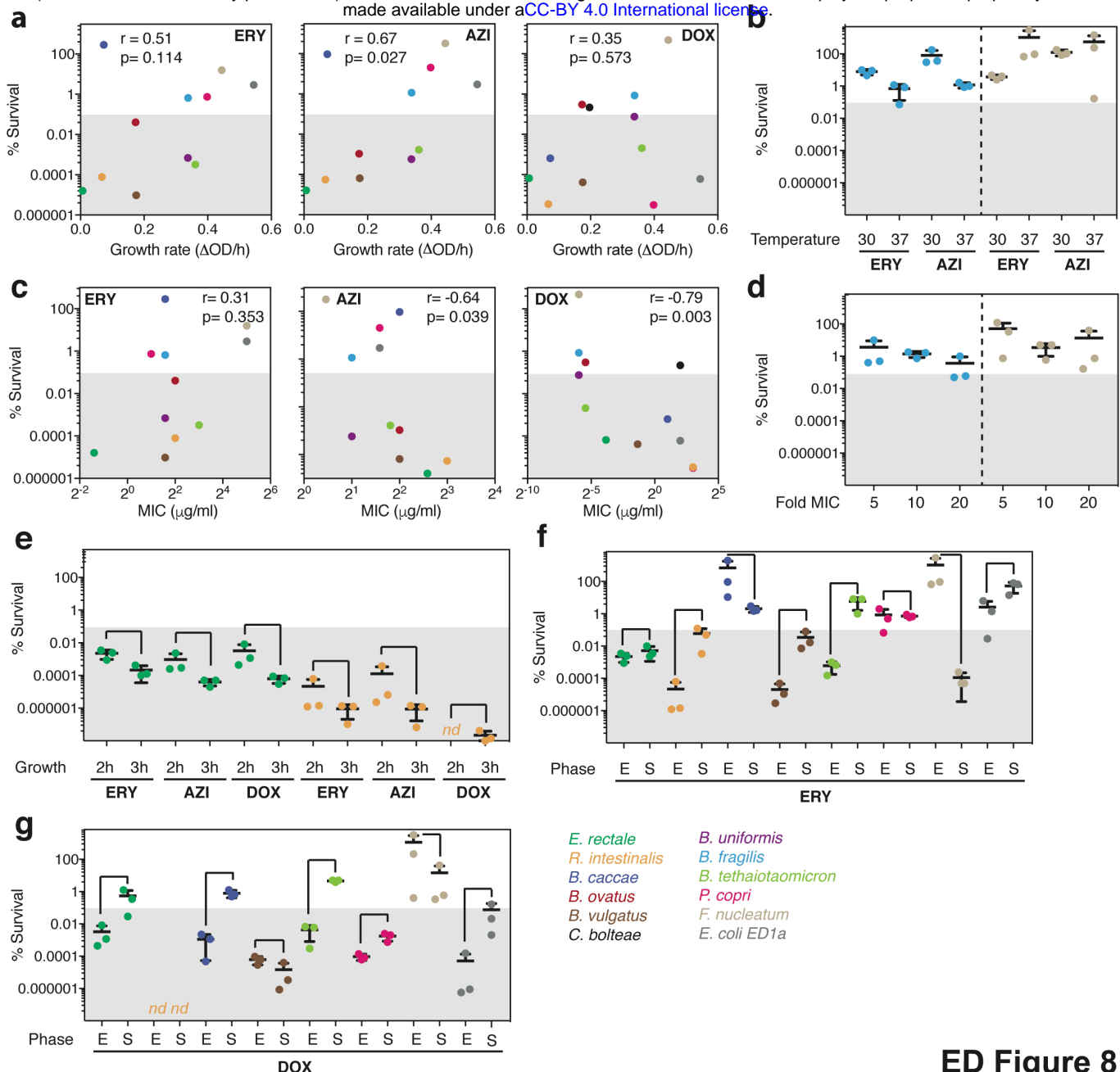


## ED Figure 7

### Extended Data Figure 7 - Effect of oxygen and strain specificity on survival after doxycycline treatment

**a.** The survival of *E. coli* ED1a was assessed after a 5-hour treatment with 5-fold MIC of DOX in the presence or absence of oxygen. Killing was similarly effective in both conditions.

**b.** The survival of *E. coli* ED1a and *E. coli* BW25113 were assessed after a 5-hour treatment with 1, 2 and 5-fold MIC of DOX in MGAM medium in anaerobic conditions. The lab strain is more resistant to killing with doxycycline becoming boarder-line bactericidal at higher MICs.



## ED Figure 8

### Extended Data Figure 8 – Assessing potential confounding factors for the killing capacities of erythromycin, azithromycin and doxycycline

**a.** Scatter plot of individual bacterial growth rates and percentage survival after a 5-hour treatment with 5-fold MIC of ERY, AZI or DOX treatments.  $r$  indicates the Spearman correlation coefficient. Tested species are color-coded here and in all panel thereafter as indicated in the bottom of this figure. Positive correlations for macrolides were tested further in **b** to check if changing growth rate in same species affects percentage killed.

**b.** The survival of *B. fragilis* (blue) and *F. nucleatum* (beige) were assessed after a 5-hour macrolide treatment (5-fold MIC of ERY and AZI) at either 30°C (slow growth) or 37°C (fast growth) to test the effect of slowing down growth on survival. No significant change observed. This graph shows the mean $\pm$ SD of three independent experiments.

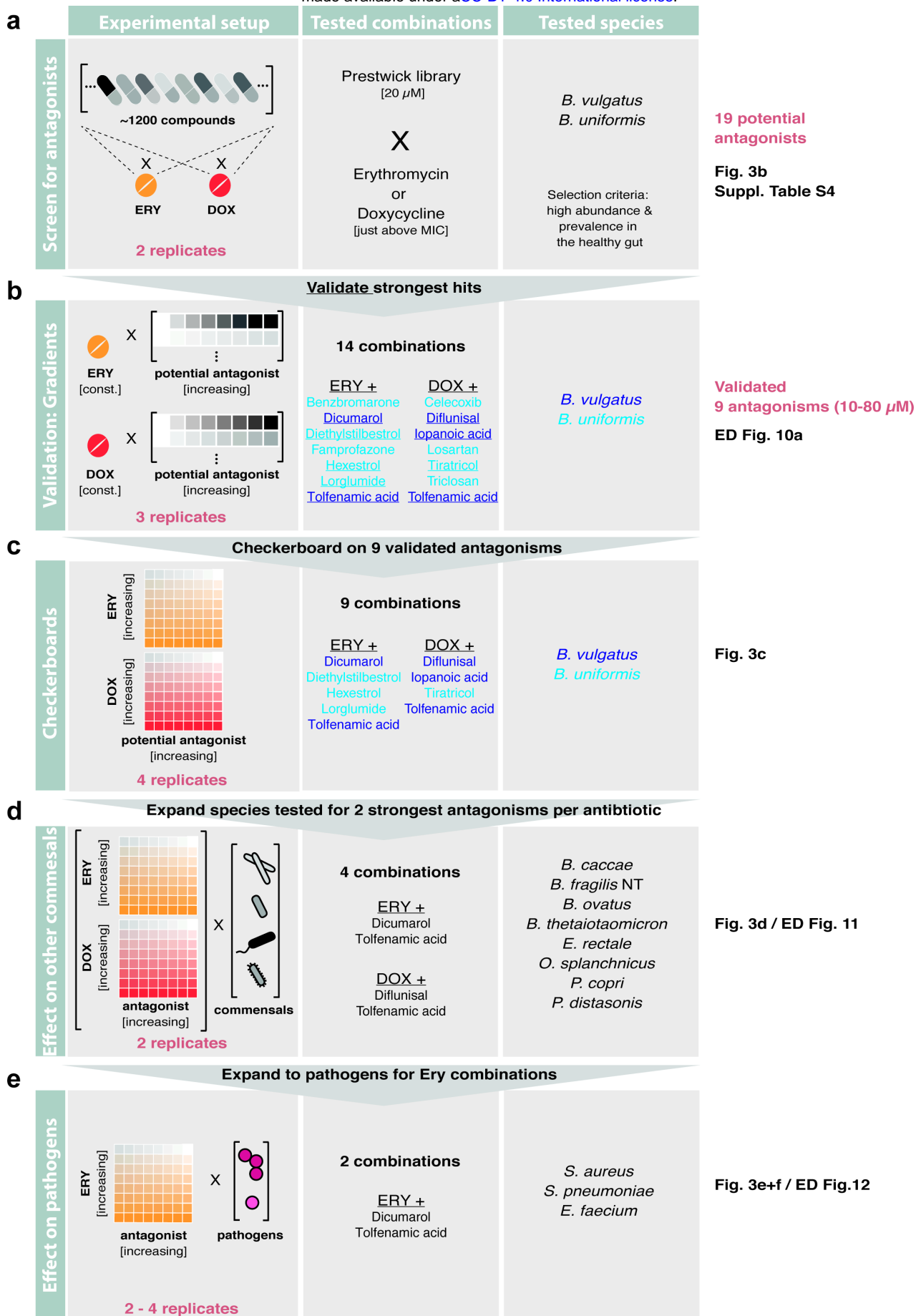
**c.** Scatter plot of MICs and percentage survival after a 5-hour treatment with 5-fold MIC of ERY, AZI or DOX treatments.  $r$  indicates the Spearman correlation coefficient. Doxycycline exhibited a strong and significant anti-correlation, that is that species which were more sensitive to doxycycline (lower MIC) were not killed when they were treated with 5-fold MIC concentrations. Thus, we tested further whether increasing the drug concentration in some of those sensitive strains decreased the % of survival (panel **d**).

**d.** The survival of *B. fragilis* (blue) and *F. nucleatum* (beige) were assessed after a 5-hour treatment with increasing concentrations of DOX (5, 10 or 20- fold of MIC) to test whether higher concentrations of DOX induced more killing. This seemed not be the case. This graph shows the mean $\pm$ SD of three independent experiments.

**e.** To evaluate whether outgrowth of stationary phase and homogeneity of population affected our results, we selected two slow-growing strains, *E. rectale* and *R. intestinalis* and grew for 2 or 3 hours after being diluted from an overnight culture to an of OD<sub>578</sub> 0.01. Both strains were then treated for 5 hours with 5-fold MIC of ERY, AZI or DOX and their survival was assessed to test the impact of the growth phase on the percentage survival. Although slight differences were observed and 3h grown cultures were killed more effectively (presumably because more cells had exited stationary phase and were growing exponentially by then), the general trends remained the same. If anything, this means that we are underestimating the killing for slow-growers, since we performed all other experiments with 2 hours outgrowth. This graph shows the mean $\pm$ SD of three independent experiments.

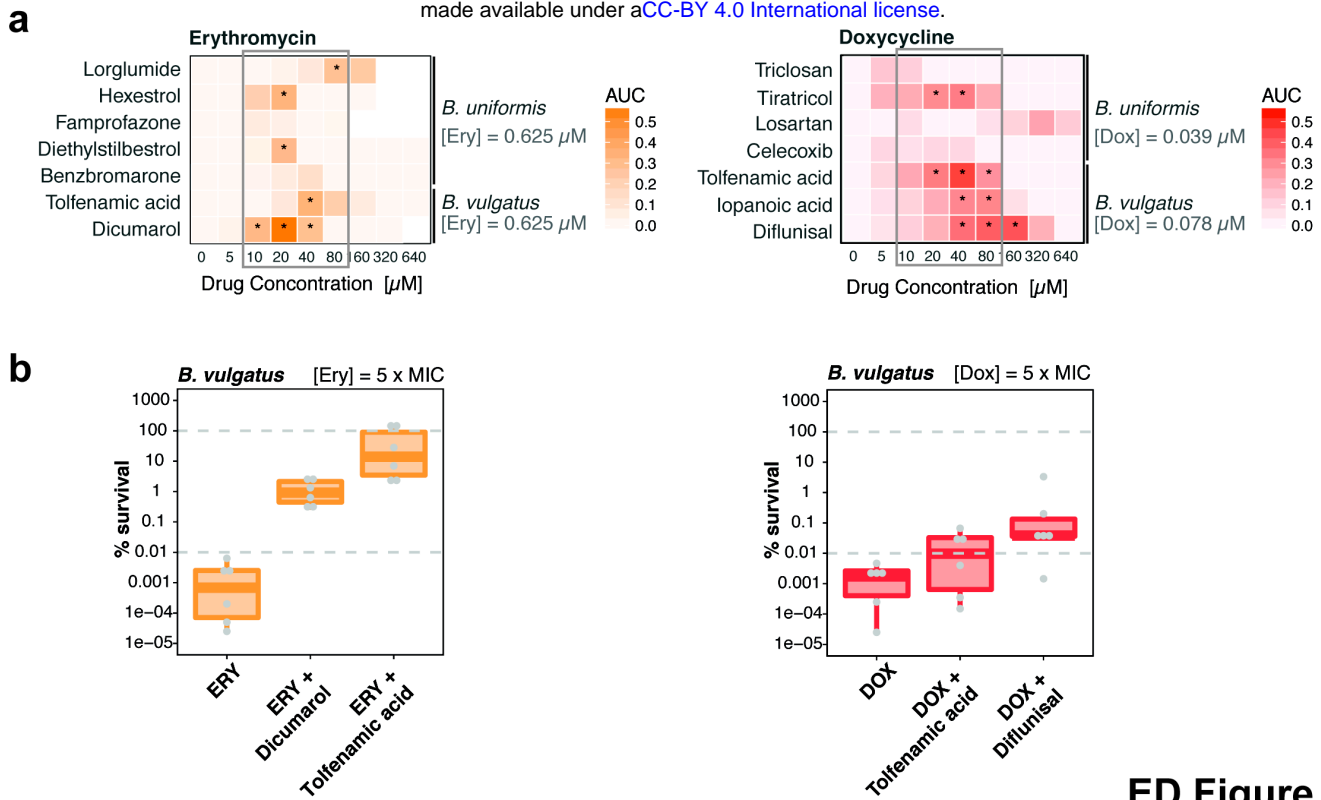
**f.** The survival of 8 selected gut microbes was measured after treating cells in exponential phase (E – 2 hours after dilution from an overnight culture) or in stationary phase (S – overnight growth) with 5-fold MIC of ERY for 5 hours to test the impact of the growth phase on the percentage survival. As expected, survival is higher in stationary phase for half of the strains, but in some cases stationary phase cells were as or more sensitive than exponentially growing cells – this is the case for *B. caccae* and *F. nucleatum*. This graph shows the mean $\pm$ SD of three independent experiments.

**g.** Same as in **f** but with DOX. Similar effects observed as in **f**, with more than half of strains becoming more resistant in stationary phase.



**ED Figure 9**

Extended Data Figure 9 – Schematic overview of screen for microbiome-protective antibiotic antagonisms  
Workflow with decision process on which antagonist to move on to next evaluation step.

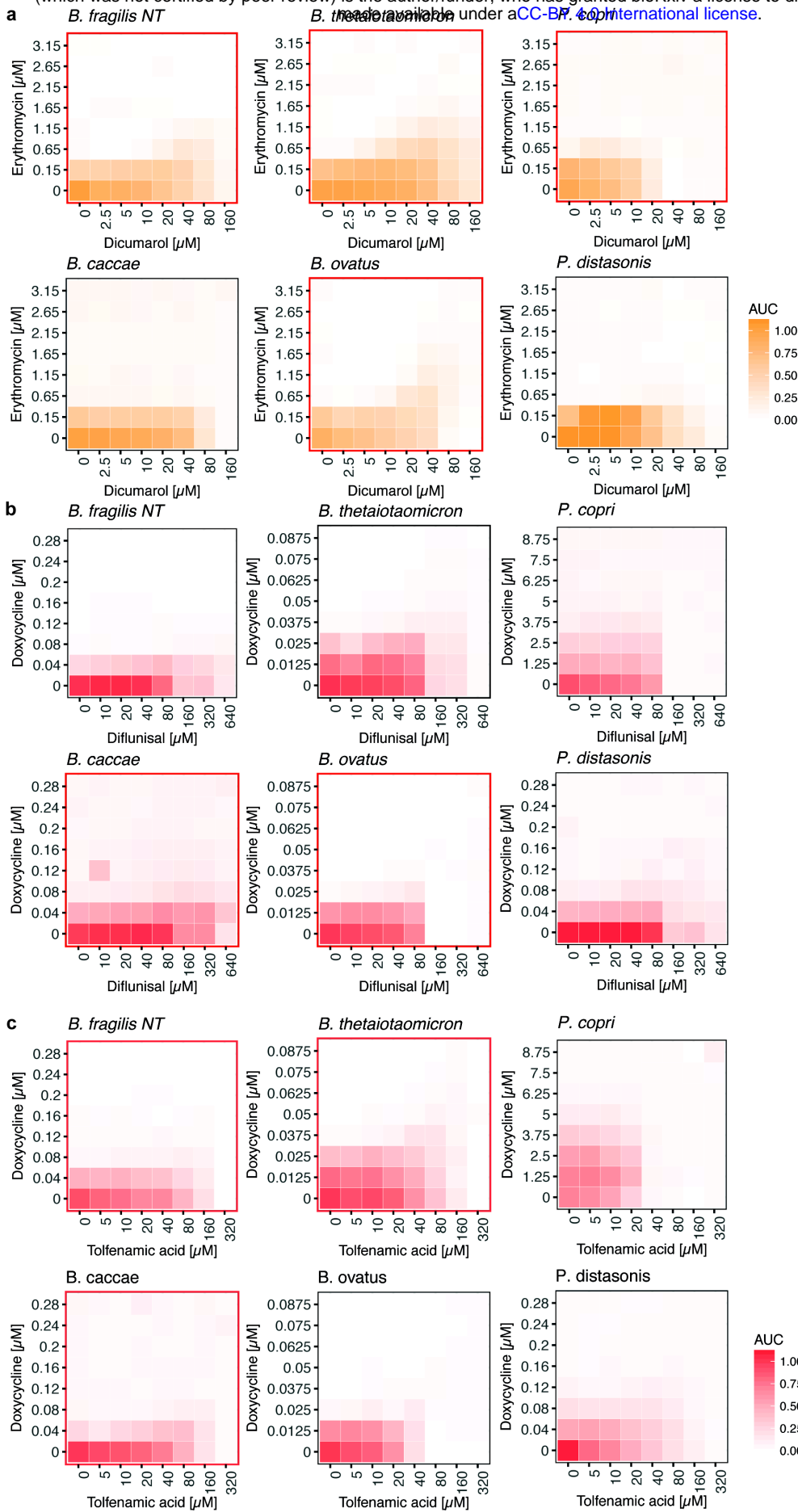


**ED Figure 10**

**Extended Data Figure 10 – Validation of potential microbiome-protective antagonists**

**a.** Validation of the strongest antagonists in independent experiments. Erythromycin and doxycycline concentrations were kept constant ([ERY]=0.625 μM, [DOX] = 0.039 / 0.078 μM) and concentration ranges were tested for antagonist. Asterisks indicate that at least 25% of the bacterial growth (compared to no drug controls) could be rescued by the antagonist at a given concentration. Heat map depicts median AUCs across triplicates.

**b.** Percentage of surviving *B. vulgatus* cells were determined after 5 h incubation with either erythromycin (3.25μM) or doxycycline (0.4 μM) alone or in presence of the antagonist dicumarol (20 μM), tolfenamic acid (40 μM) or diflunisal (80 μM). Data is based on 3 independent experiments. Boxplots are plotted as in Figure 1d.

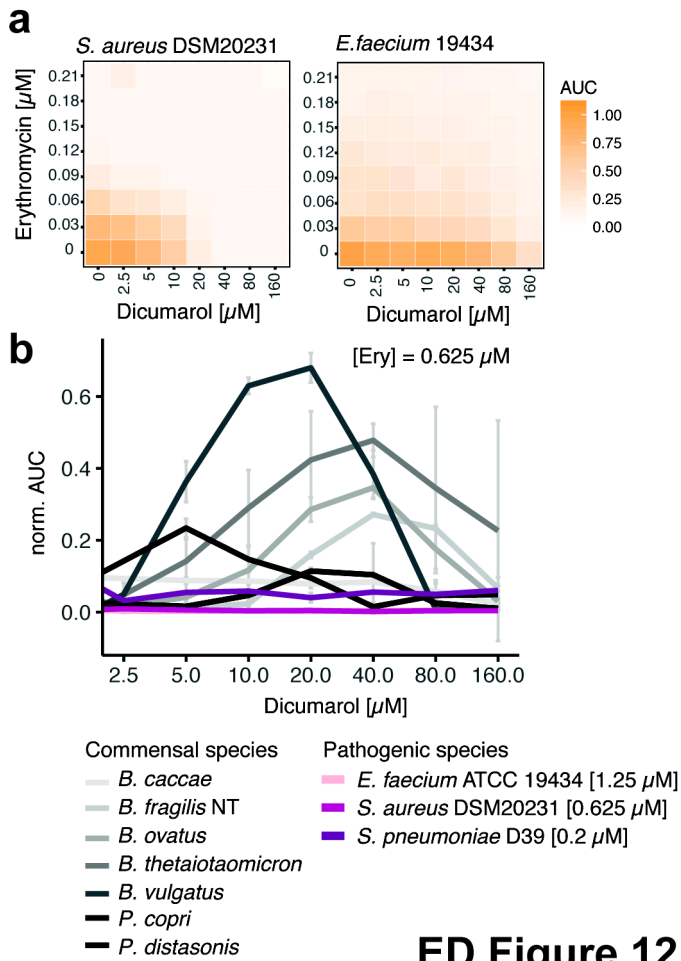


**ED Figure 11**

**Extended Data Figure 11 – Effect of antidotes on further gut commensals**

8 x 8 checkerboard assays to investigate if antidote is also protective for additional gut commensals for the following combinations: erythromycin and dicumarol (a), doxycycline and diflunisal (b) and doxycycline and tolfenamic acid (c). Heat map depicts bacterial growth based on median AUCs from two independent replicates. Red contours indicate antagonistic drug interactions.





**Extended Data Figure 12– Effect of the antidote dicumarol on pathogens, relatively to commensal species.**

**a.** Checkerboard assays for the drug combinations erythromycin-tolfenamic acid and erythromycin-dicumarol on the pathogens *S. aureus* (two different strains) and *E. faecium*. Heat map depict median normalized AUCs of checkerboard assays (at least three independent replicates).

**b.** Dicumarol rescues commensal growth (based on median AUCs, N=2) at clinical relevant erythromycin concentrations in a concentration-dependent manner. Erythromycin still retains its activity against pertinent pathogens such as *S. aureus*, *E. faecium* and *S. pneumoniae* and is even slightly more active (synergy) for *E. faecium* (based on median AUCs, N=3). Error bars depict standard deviation.