# Vancomycin-resistant *Enterococcus* colonise the antibiotic-treated intestine by occupying distinct nutrient- and metabolite-defined intestinal niches

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## ABSTRACT

Antibiotic treatment significantly disrupts the gut microbiome and promotes vancomycin-resistant *Enterococcus* (VRE) intestinal colonisation. These disruptions lead to the intestine acting as a reservoir for VRE that seed difficult-to-treat infections. Patients would benefit from new treatments that prevent or treat VRE intestinal colonisation. However, the factors that drive VRE growth in the gut are poorly understood. We demonstrated that multiple antibiotics that promote VRE intestinal colonisation increased the concentration of a wide range of nutrients and decreased the concentration of a wide range of microbial metabolites. We demonstrated that specific short chain fatty acids and branched chain fatty acids found in healthy gut microbiomes inhibited VRE growth, however inhibition was incomplete at physiologically relevant concentrations. We also showed that VRE could use most nutrients increased in antibiotic-treated faecal microbiomes as carbon or nitrogen sources to support their growth, where growth was higher in the presence of oxygen. *Enterococcus faecium* and *Enterococcus faecalis* had some common and some distinct preferences for use of specific nutrients that were enriched in antibiotic-treated faecal microbiomes. Finally, we showed that *E. faecium* and *E. faecalis* occupied overlapping but distinct nutrient-defined intestinal niches that promoted high growth when cultured with each other and when cultured with carbapenem-resistant *Enterobacteriaceae* (another group of multidrug-resistant pathogens). Our results demonstrated that VRE occupy distinct intestinal niches in the antibiotic-treated intestine, defined by their abilities to utilise specific enriched nutrients and their abilities to grow with reduced concentrations of microbial metabolites.

41 **INTRODUCTION**

42

43 Vancomycin-resistant *Enterococcus* (VRE) are multidrug-resistant pathogens and a major
44 cause of nosocomial infections worldwide[1,2]. VRE pose a major therapeutic challenge as they
45 have intrinsic resistance to several commonly prescribed antibiotics, and have also acquired
46 resistance to many available antibiotics[3]. Vancomycin-resistant *Enterococcus faecium* is the
47 leading multidrug-resistant *Enterococcus* species in healthcare environments[4]. The World
48 Health Organisation has classified vancomycin-resistant *E. faecium* as a high-priority
49 pathogen for the development of new treatments[5].

50

51 The intestine is a major reservoir for VRE that seed difficult-to-treat invasive infections such
52 as bacteraemia, endocarditis, and urinary tract infections[5,6]. Patients exposed to broad-
53 spectrum antibiotics, that significantly disrupt the gut microbiome, are highly susceptible to
54 intestinal colonisation and domination by VRE[7-11]. Conversely, healthy individuals have intact
55 colonisation resistance, where their gut microbiomes protect them from VRE intestinal
56 colonisation. To develop targeted and effective therapies to restrict VRE intestinal colonisation
57 we must first understand what drives VRE colonisation in an antibiotic-disrupted gut
58 microbiome. Mechanisms of colonisation resistance that protect the host against VRE
59 intestinal colonisation are poorly understood. However, we previously demonstrated that
60 nutrient competition and inhibitory metabolite production by the healthy gut microbiota can
61 restrict the intestinal colonisation of other pathogens including carbapenem-resistant
62 *Enterobacteriaceae* (CRE) and *Clostridioides difficile*[12,13].

63

64 Pathogens must have access to nutrients that they can utilise to support their growth and
65 colonise the intestine. In a healthy gut microbiome, commensal microbes compete intensely
66 for nutrients, resulting in nutrient depletion[14]. However, antibiotic-induced killing of gut
67 commensals reduces nutrient competition, making the nutrients available to support pathogen
68 growth. We have a very poor understanding of VRE nutrient utilisation in antibiotic-treated gut

69 microbiomes. A previous study demonstrated that vancomycin-treated mice had increased
70 fructose in their caecal contents compared to antibiotic-naïve mice, and vancomycin-resistant
71 *E. faecium* could use fructose as a carbon source[10]. However, this study used a targeted
72 metabolomics approach that likely missed changes in other nutrients that were increased with
73 vancomycin treatment. Moreover, this study only tested one antibiotic, whereas there are
74 multiple broad-spectrum antibiotics that can promote VRE intestinal colonisation[7-11]. It is
75 important to understand how other broad-spectrum antibiotics that promote VRE intestinal
76 colonisation affect the availability of nutrients that support VRE growth.
77
78 Microbiome therapeutics designed to restrict VRE growth based on nutrient utilisation profiles
79 will not be effective unless they deplete all available carbon and nitrogen sources that support
80 VRE growth. Otherwise, VRE will switch to using other available nutrients to grow. We
81 previously demonstrated that broad-spectrum antibiotics that promote the intestinal
82 colonisation of CRE (another group of multidrug-resistant pathogens) caused an increase in
83 the concentration of a wide range of nutrients[12]. As our previous study demonstrated that
84 broad-spectrum antibiotics had a severe impact on the gut microbiota, in this study we
85 hypothesised that broad-spectrum antibiotics that promote VRE intestinal colonisation would
86 also increase the concentration of a wide range of nutrients in the intestine, increasing the
87 availability of many nutrients that could support VRE growth.
88
89 It is also not clear how VRE occupy intestinal niches in antibiotic-treated gut microbiomes. A
90 previous study by Caballero and colleagues proposed that vancomycin-resistant *E. faecium*
91 and carbapenem-resistant *Klebsiella pneumoniae* occupied distinct but overlapping intestinal
92 niches, as they showed that growth was not impaired when they colonised the mouse intestine
93 simultaneously[15]. Shared bacterial niches have also been observed in humans, as patients
94 can be co-colonised with both CRE and VRE simultaneously[16]. However, the mechanism of
95 how VRE and CRE occupied the antibiotic-treated intestine was not shown. Therefore, we
96 hypothesised that VRE and CRE occupy distinct but overlapping nutrient-defined intestinal

97 niches, where VRE share the utilisation of some nutrients with CRE, but also has its own
98 distinct nutrient utilisation abilities.
99
100 In the healthy gut microbiome, nutrient utilisation results in the production of microbial
101 metabolites, some of which can inhibit pathogen growth[14]. Therefore, VRE must be able to
102 grow in the presence of microbial metabolites to successfully colonise the intestine. We
103 previously demonstrated that broad-spectrum antibiotics that promote CRE intestinal
104 colonisation decreased the concentration of a wide range of metabolites in the gut microbiome,
105 and several of these metabolites highly inhibited CRE growth[12]. Another study from our group
106 demonstrated that antibiotic treatment promoted *C. difficile* growth and decreased the
107 concentration of valerate in the gut microbiome, and that valerate inhibited *C. difficile* growth[13].
108 However, we do not understand how microbial metabolites may impact VRE growth in the
109 intestine. Therefore, we also wanted to determine whether microbial metabolites that are found
110 in healthy gut microbiomes and decreased with antibiotic treatment could inhibit VRE growth.
111
112 VRE are high-priority pathogens that are included in the "ESKAPE" group of pathogens, and
113 urgently require new treatments, including new therapies to prevent VRE intestinal
114 colonisation[17]. These therapies would remove a reservoir of VRE, thereby significantly
115 reducing the subsequent development of VRE invasive infections and patient-to-patient VRE
116 transmission[18]. Therefore, this study aimed to measure the nutrient and metabolite
117 landscapes encountered by VRE in a gut microbiome treated with multiple different antibiotics
118 that are known to promote VRE intestinal colonisation. Moreover, we wanted to define the
119 niche that VRE occupied in an antibiotic-treated gut microbiome, how distinct these niches
120 were between *E. faecium* and *E. faecalis*, and how different these niches were compared to
121 niches occupied by other multidrug-resistant pathogens. The results from this study will inform
122 the rational design of a new microbiome therapeutic that can occupy the same niche as VRE
123 to displace them from the intestine.
124

125 **RESULTS**

126

127 **Antibiotics alter the nutrient and metabolite niches in the intestine in a manner that**
128 **supported VRE growth**

129

130 We performed *ex vivo* faecal culture experiments to determine how antibiotics that promote
131 VRE intestinal colonisation killed gut commensals, altered nutrient availability and altered the
132 presence of microbial metabolites. In these experiments, faeces from healthy human donors
133 were cultured in a distal gut growth medium that was supplemented with water or an antibiotic
134 known to promote VRE intestinal colonisation: metronidazole (MTZ), clindamycin (CLI),
135 vancomycin (VAN), ceftriaxone (CRO), or piperacillin/tazobactam (TZP), at concentrations
136 mimicking those found in human faeces[19-24]. Samples were analysed by 16S rRNA gene
137 sequencing to measure changes in bacterial taxa and by $^{1}$H-NMR spectroscopy to measure
138 changes in nutrients and metabolites.

139

140 Faecal cultures treated with antibiotics that promote VRE intestinal colonisation had a
141 significant decrease in many bacterial families, including *Ruminococcaceae* and
142 *Bifidobacteriaceae*, (which were decreased in all antibiotic-treated groups; **Fig. 1a**). MTZ
143 caused a decrease in 16 families, but also caused an increase in *Enterobacteriaceae*. CLI
144 caused a decrease in *Bifidobacteriaceae* and *Ruminococcaceae*. VAN caused a decrease in
145 9 families, but also caused an increase in 3 families. CRO caused a decrease in 8 families.
146 TZP caused a decrease in 11 families.

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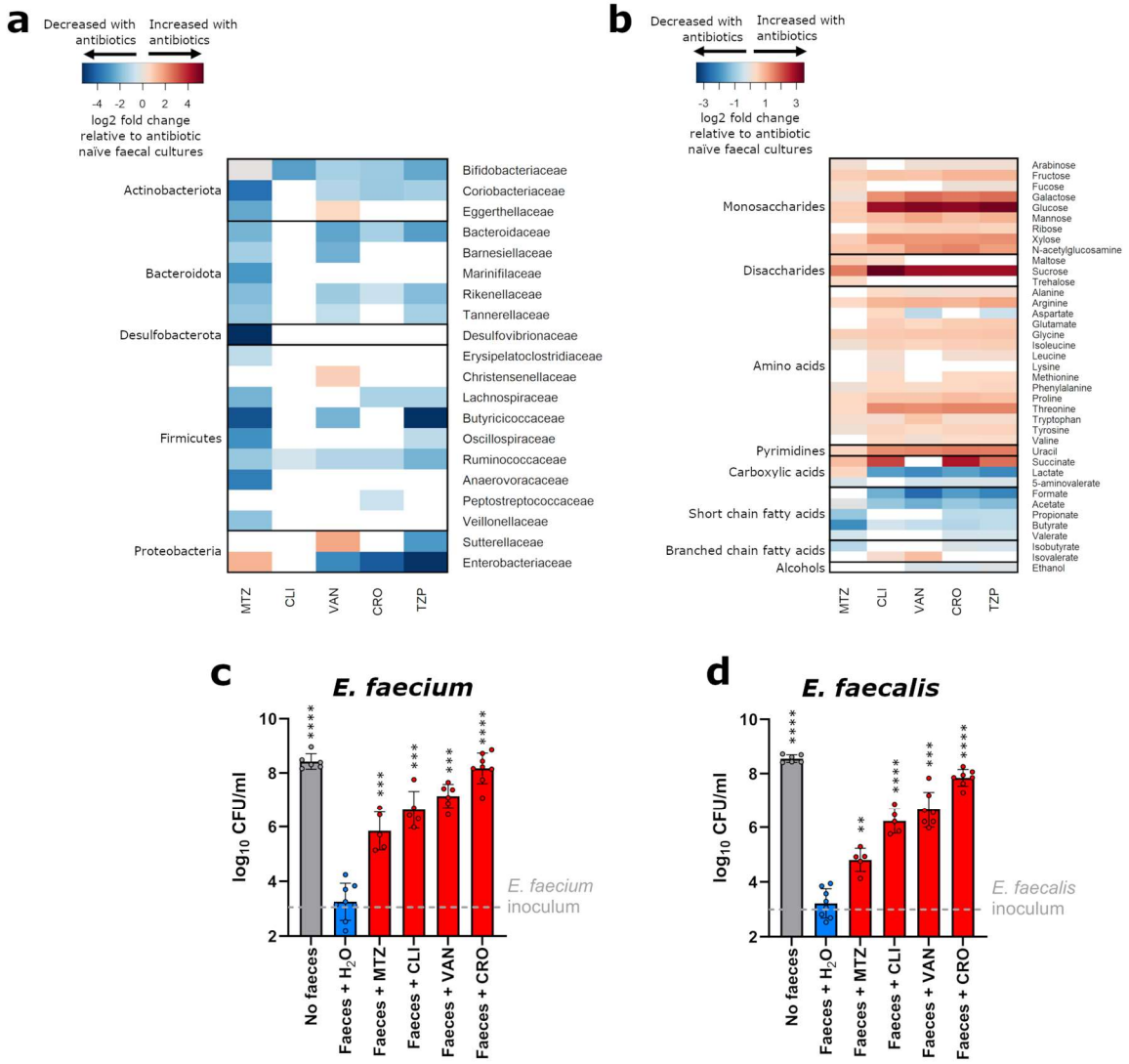


**<u>Figure 1:</u> Antibiotic treatment significantly reduced gut commensals, increased nutrients, decreased microbial metabolites, and increased VRE growth. (a)** Heatmap illustrating the log2 fold change (antibiotic-treated relative to antibiotic-naïve) in bacterial taxa that were significantly decreased (shown in blue) or increased (shown in red) in faecal cultures following treatment with antibiotics that promote VRE intestinal colonisation. Taxa that were not significantly changed with antibiotic treatment were not plotted (shown in white). n = 12 healthy human faecal donors, Wilcoxon signed rank test (two-sided) of log-transformed abundances with Benjamini Hochberg false discovery rate (FDR) correction, $p < 0.05$. **(b)** Heatmap illustrating the log2 fold change (antibiotic-treated relative to antibiotic-naïve) in nutrients and metabolites that were significantly decreased (shown in blue) or increased (shown in red) in faecal cultures following treatment with antibiotics that promote VRE intestinal colonisation. Nutrients and metabolites that were not significantly changed with antibiotic treatment were not plotted (shown in white). n = 12 healthy human faecal donors, Wilcoxon signed rank test (two-sided) with Benjamini Hochberg FDR correction, $p < 0.05$. **(c-d)** Antibiotic treatment significantly promoted the growth of vancomycin-resistant *E. faecium* and *E. faecalis* in faecal microbiomes. *E. faecium* or *E. faecalis* were spiked into faecal cultures at $10^3$ CFU/ml (horizontal dashed line). n = 5-8 healthy human faecal donors, with data shown as mean ± SD. Antibiotic-treated faecal culture counts were compared to water-treated faecal culture counts using a mixed effects model of log transformed CFU/ml with Dunn's multiple comparison. No faeces counts were compared to water-treated faecal culture counts using an unpaired t-test (two-sided) of log transformed CFU/ml. ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$. MTZ = metronidazole, CLI = clindamycin, VAN = vancomycin, CRO = ceftriaxone, TZP = piperacillin/tazobactam.

149 Antibiotic treatment also significantly altered the abundances of nutrients and metabolites that
150 would be encountered by VRE in an antibiotic treated faecal microbiome. Antibiotic treatment
151 resulted in a significant increase in a wide range of nutrients, including monosaccharides,
152 disaccharides, amino acids, uracil, and succinate (**Fig. 1b**). Monosaccharides increased with
153 antibiotics included arabinose, fructose, fucose, galactose, glucose, mannose, ribose, xylose,
154 and N-acetylglucosamine. Disaccharides increased with antibiotics included maltose, sucrose,
155 and trehalose. Amino acids increased with antibiotics included alanine, arginine, aspartate,
156 glutamate, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine,
157 tryptophan, tyrosine, and valine. Antibiotic treatment also resulted in a decrease in a wide
158 range of microbial metabolites, including short chain fatty acids (SCFAs; formate, acetate,
159 propionate, butyrate, and valerate), carboxylic acids (lactate and 5-aminovalerate),
160 isobutyrate, and ethanol (**Fig. 1b**).
161
162 Next, we performed another set of faecal culture experiments to confirm whether vancomycin-
163 resistant *E. faecium* and *E. faecalis* were able to grow in these antibiotic-treated faecal
164 microbiomes that had increased nutrient concentrations and decreased metabolite
165 concentrations. First, MIC assays confirmed that both strains were resistant to all the
166 antibiotics tested (**Table S1**). *E. faecium* and *E. faecalis* were also cultured in the presence of
167 the antibiotics at concentrations found in human faeces. Both *E. faecium* and *E. faecalis* were
168 able to grow axenically in faecal concentrations of MTZ, CRO, CLI, and VAN, however these
169 strains were killed with faecal concentrations of TZP (**Fig. S1**). Therefore, TZP was not tested
170 further in the faecal culture experiments.
171
172 Faecal culture experiments were performed by spiking vancomycin-resistant *E. faecium* or *E.*
173 *faecalis* into antibiotic-treated faecal cultures as described above. We demonstrated that MTZ,
174 CLI, VAN, and CRO-treated faecal cultures promoted the growth of *E. faecium* and *E. faecalis*,
175 while water-treated faecal cultures restricted *E. faecium* and *E. faecalis* growth (**Fig. 1c,d**).
176

177 We demonstrated that antibiotic treatment killed gut commensals and promoted VRE growth.
178 This reduction in gut commensal abundance resulted in an increase in the concentration of
179 nutrients (including monosaccharides, disaccharides, amino acids, uracil, and succinate) and
180 a decrease in the concentration of microbial metabolites (including SCFAs, lactate, 5-
181 aminovalerate, isobutyrate, and ethanol).
182
183 **Vancomycin treatment caused an increase in nutrients and a decrease in metabolites**
184 **in mouse faeces and promoted *E. faecium* growth**
185
186 We used a mouse model to demonstrate that antibiotic treatment alters nutrient availability
187 and metabolite production *in vivo*. Mice were treated with vancomycin as it has been
188 previously demonstrated to promote VRE intestinal colonisation in both humans and
189 mice[7,8,10,11]. Mice were colonised with *E. faecium* to confirm that vancomycin disrupts
190 colonisation resistance and creates an intestinal environment that promotes *E. faecium*
191 growth.
192
193 We measured changes in nutrient availability and metabolite production in the faeces of
194 vancomycin-treated mice compared to water-treated mice using $^{1}$H-NMR spectroscopy.
195 Faeces from vancomycin-treated mice had an increase in the concentration of several
196 nutrients compared to water-treated mice, including arabinose, glucose, trehalose, arginine,
197 aspartate, glycine, phenylalanine, and tyrosine (**Fig. 2a**). Moreover, faeces from vancomycin-
198 treated mice had a decrease in the concentration of several metabolites compared to water-
199 treated mice, including propionate, butyrate, isobutyrate, lactate, and ethanol (**Fig. 2b**).
200 Fucose and alanine concentrations were decreased in the faeces of vancomycin-treated mice
201 compared to water-treated mice (**Fig. S2**).
202

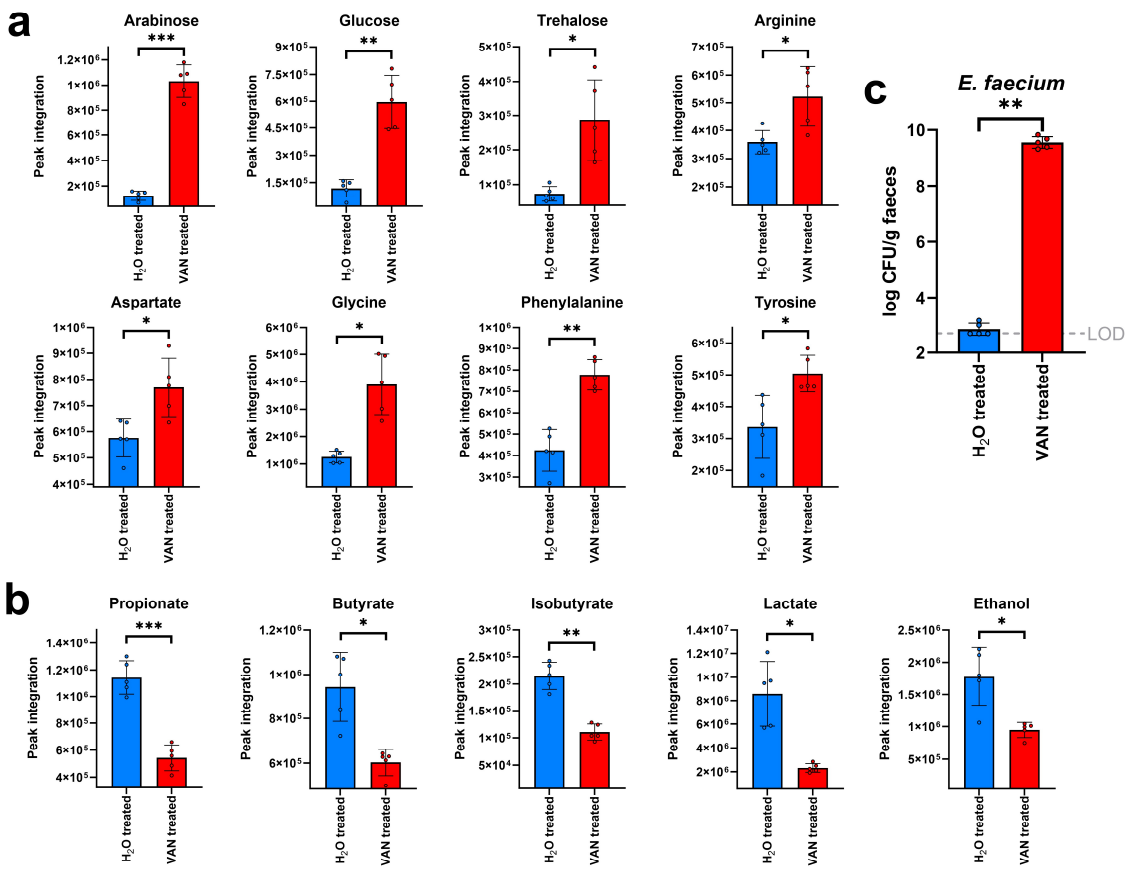


**Figure 2: Nutrients were increased and metabolites were decreased in the faeces of vancomycin-treated mice. (a)** Nutrients that were increased in the faeces of VAN-treated mice compared to $H_2O$-treated mice. **(b)** Metabolites that were decreased in the faeces of VAN-treated mice compared to $H_2O$-treated mice. Comparison of nutrients and metabolites in the VAN-treated faeces to the $H_2O$-treated faeces used an unpaired t-test (two-sided) with Benjamini Hochberg FDR. **(c)** Vancomycin-resistant *E. faecium* counts were high in the faeces from vancomycin-treated mice and nearly undetectable in the faeces of water-treated mice. Comparisons of the *E. faecium* plate counts were made using a Mann–Whitney U test (two-sided) of log10-transformed counts. n = 5 mice per group from one independent experiment, with data shown as mean ± SD. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. VAN = vancomycin, $H_2O$ = water, LOD = limit of detection.

Vancomycin-treated and water-treated mice were gavaged with vancomycin-resistant *E. faecium*, and *E. faecium* growth was quantified in mouse faecal samples (**Fig. 2c**). Mice treated with vancomycin had high *E. faecium* counts in their faeces, while mice treated with water had low *E. faecium* counts at or below the limit of detection.

In summary, the results from our mouse experiment were supportive of the results we observed in our *ex vivo* experiments. The faeces from vancomycin-treated mice had an increased concentration of several nutrients (including monosaccharides, disaccharides, and

212 amino acids) and a decrease in several microbial metabolites (including SCFAs, BCFAs,
213 carboxylic acids, and ethanol). We also confirmed that vancomycin-treated mice had disrupted
214 colonisation resistance that promoted vancomycin-resistant *E. faecium* intestinal colonisation.
215

216 **Specific microbial metabolites that were decreased in antibiotic-treated faecal**
217 **microbiomes showed moderate inhibition of VRE growth at physiologically relevant**
218 **concentrations**

219

220 We demonstrated that antibiotics that promote VRE intestinal colonisation reduced the
221 concentration of microbial metabolites in faecal microbiomes (**Figs. 1-2**). Previous work
222 demonstrated that microbial metabolites can inhibit the growth of pathogens in the intestine,
223 including CRE and *C. difficile*[12,13,25]. Therefore, we were interested in whether microbial
224 metabolites that were decreased in concentration by antibiotics could also inhibit VRE growth.
225

226 First, we tested whether a mixture of metabolites (containing formate, acetate, propionate,
227 butyrate, valerate, isobutyrate, isovalerate, lactate, 5-aminovalerate, ethanol) were able to
228 inhibit *E. faecium* and *E. faecalis* growth. The mixture was tested at three concentrations that
229 represented the average, low, and high concentrations of these metabolites measured in
230 healthy human faeces[12]. The mixtures were also tested at pH 6, 6.5, and 7 to mimic the pH
231 range of the healthy large intestine[25,26]. The metabolite mixture was inhibitory to *E. faecium*
232 and *E. faecalis* growth at the high metabolite concentration at pH 6 (**Fig. 3**).
233

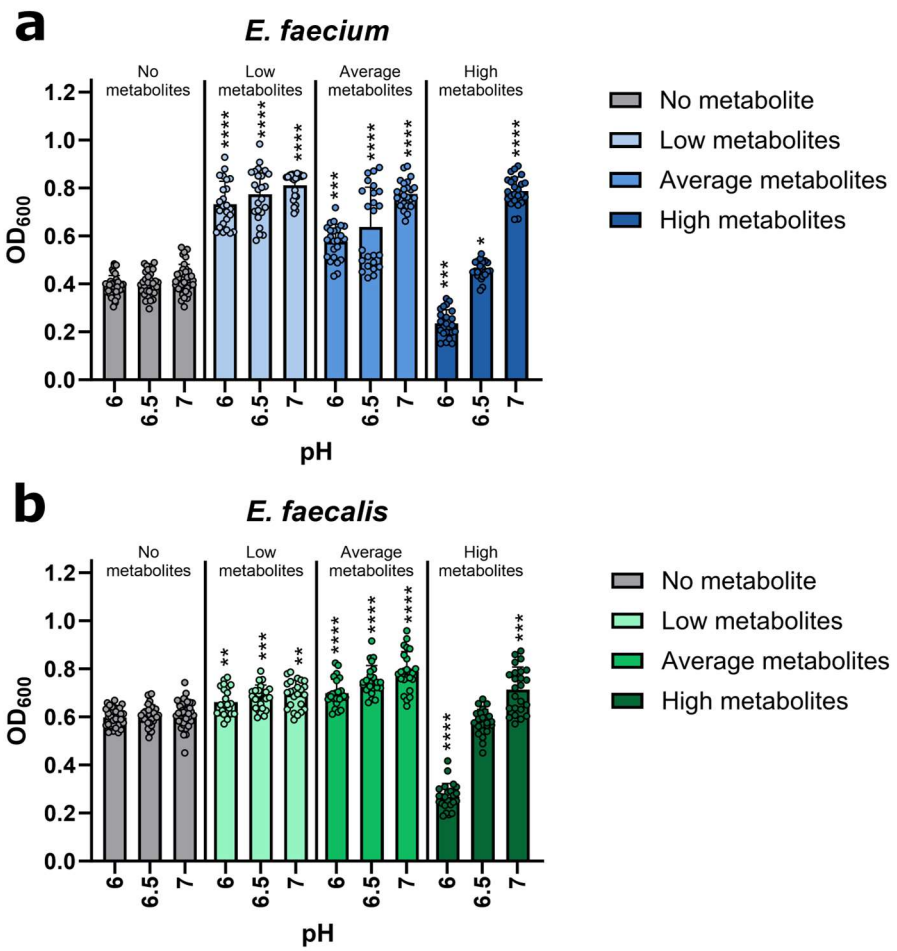


**Figure 3: VRE growth was inhibited at pH 6.0 by a mixture of microbial metabolites at high concentrations.** Vancomycin-resistant *E. faecium* and *E. faecalis* were grown in tryptic soy broth supplemented with a metabolite mixture mimicking the low, average, or high concentrations measured in human faeces, or unsupplemented ("No metabolite" control). Cultures were incubated at pH 6.0, 6.5, and 7.0 to mimic the pH of the healthy large intestine. Data shown as mean ± SD, with 24-36 replicates from 3 independent experiments. Kruskal-Wallis with Dunn's multiple comparison test comparing the No metabolite control to each concentration of the metabolite mixture at the same pH. * $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$. $OD_{600}$ = optical density at 600 nm.

234

235 Next, *E. faecium* and *E. faecalis* growth was measured in the presence of each individual
236 metabolite. Individual metabolites were tested at concentrations that mimicked the average,
237 low, and high concentrations of these metabolites measured in healthy human faeces at pH
238 6. *E. faecium* was moderately inhibited by propionate, butyrate, and valerate at the high
239 concentration, and propionate also inhibited *E. faecium* at the average concentration (**Fig. 4a**).
240 The other metabolites (formate, acetate, isobutyrate, isovalerate, lactate, 5-aminovalerate,
241 and ethanol) were not inhibitory at these concentrations (**Fig. S3**). *E. faecalis* was moderately
242 inhibited by propionate and isovalerate at the high concentration, and propionate also inhibited

243 *E. faecalis* at the average concentration (**Fig. 4b**). The other metabolites (formate, acetate,
244 butyrate, valerate, isobutyrate, and lactate) were not inhibitory at these concentrations (**Fig.**
245 **S4**). *E. faecalis* had a small, but significant increase in growth when supplemented with 5-
246 aminovalerate at low, average, and high concentrations and when supplemented with ethanol
247 at the low concentration measured in healthy human faeces (**Fig. S4**).
248

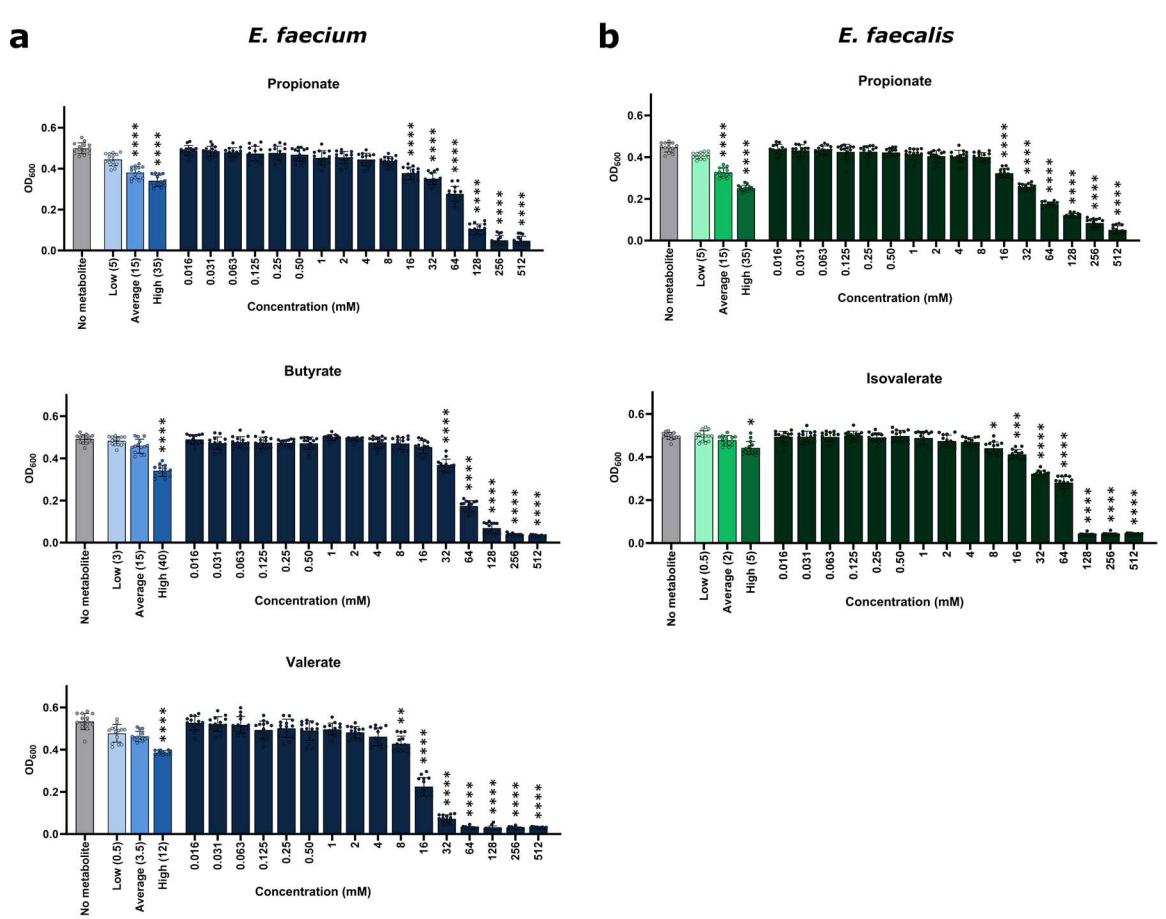


**Figure 4: Some individual microbial metabolites moderately inhibited VRE growth.** (**a**) Vancomycin-resistant *E. faecium* and (**b**) vancomycin-resistant *E. faecalis* were grown in tryptic soy broth (pH 6) supplemented with an individual metabolite at low, average or high concentrations measured in healthy human faeces, concentrations spanning a defined range (0.016-512 mM), or unsupplemented (no metabolite control). Growth of each isolate was measured with 14 replicates in two independent experiments. Kruskal-Wallis test with Dunn's multiple comparison test (each metabolite was compared to the no metabolite control). * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001. Data are presented as mean values ± SD. Optical density at 600 nm, $OD_{600}$.

249

250 We compared the inhibition of each individual metabolite at the same concentrations which
251 also included higher (supraphysiological) concentrations which could be considered for future
252 therapeutic drug designs. We measured *E. faecium* and *E. faecalis* growth at 2-fold increasing

253 concentrations of metabolites that ranged from 0.016 to 512 mM at pH 6 (**Figs. 4, S3, S4**). For
254 *E. faecium* the minimum concentration that caused a significant decrease in growth was 8 mM
255 for valerate, 16 mM for propionate, 32 mM for butyrate, isovalerate, and formate, 128 mM for
256 isobutyrate and 5-aminovalerate, 256 mM for acetate and lactate, and 512 mM for ethanol.
257 For *E. faecalis* the minimum concentration that caused a significant decrease in growth was 8
258 mM for isovalerate and lactate, 16 mM for propionate and formate, 32 mM for valerate, 128
259 mM for butyrate and isobutyrate, and 256 mM for acetate. *E. faecalis* was not inhibited by 5-
260 aminovalerate and ethanol. The half maximal inhibitory concentrations ($IC_{50}$) for each
261 metabolite for *E. faecium* and *E. faecalis* are shown in **Table S2**.
262
263 Overall, we observed significant inhibition of vancomycin-resistant *E. faecium* and *E. faecalis*
264 by specific microbial metabolites. However, at physiologically relevant concentrations
265 (measured in human faeces) these metabolites were not sufficient to fully inhibit VRE growth.
266 The $IC_{50}$ values of all the microbial metabolites exceeded the high faecal concentrations
267 measured in healthy faeces, although the valerate $IC_{50}$ for *E. faecium* and the propionate $IC_{50}$
268 for *E. faecalis* were close to the maximum concentrations measured in healthy faeces.
269
270 **Nutrients enriched in antibiotic-treated faecal microbiomes supported VRE growth**
271
272 We demonstrated that antibiotics that promote VRE intestinal colonisation increased the
273 concentration of nutrients in faecal microbiomes (**Figs. 1-2**). Therefore, next we measured
274 whether the VRE isolates were able to grow in a mixture of nutrients that were enriched with
275 antibiotic treatment. Growth was measured in the presence and absence of oxygen as
276 previous studies have demonstrated that antibiotic treatment increases oxygen availability in
277 the intestine, which could impact nutrient utilisation by pathogens[12,27]. Therefore, we wanted
278 to determine whether the presence of oxygen also impacted nutrient utilisation for VRE.
279

280 Vancomycin-resistant *E. faecium* and *E. faecalis* were cultured in a minimal growth medium
281 supplemented with a mixture of nutrients that were increased in antibiotic-treated faecal
282 microbiomes. VRE growth was supported by this mixture of nutrients under both anaerobic
283 and aerobic conditions (**Fig. 5**). Growth was higher for *E. faecium* and *E. faecalis* under
284 aerobic conditions compared to anaerobic conditions.

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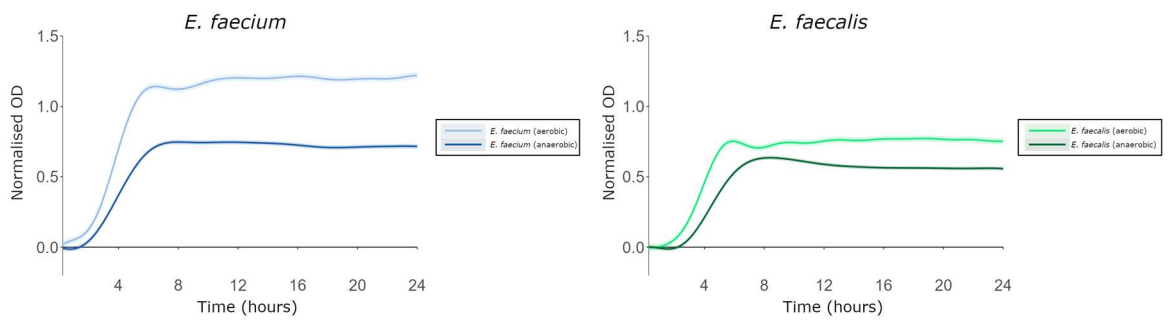



**Figure 5: VRE used nutrients enriched in antibiotic-treated faecal microbiome as sole carbon and nitrogen sources.** AMiGA-predicted growth curves for vancomycin-resistant *E. faecium* and *E. faecalis* cultured in minimal medium supplemented with a mixture of nutrients under anaerobic or aerobic conditions. The bold lines show the predicted mean of growth while the shaded bands show the predicted 95% credible intervals. N = 24 replicates in 3 independent experiments for each strain under each condition, OD = optical density.

286

287 Next, we measured whether an antibiotic-naïve faecal microbiota was able to deplete nutrients
288 (that we demonstrated were increased in antibiotic-treated faecal microbiomes) to restrict VRE
289 growth. First, faecal microbiotas were cultured in a minimal medium supplemented with a
290 mixture of the nutrients of interest. Next, the spent supernatants were adjusted to pH 7 and
291 supplemented with water or glucose. Vancomycin-resistant *E. faecium* or *E. faecalis* were
292 inoculated into the spent faecal supernatants and cultured overnight. As a positive control, *E.*
293 *faecium* and *E. faecalis* growth was also measured in *E. faecium* or *E. faecalis* spent
294 supernatant supplemented with water or glucose.

295

296 We demonstrated that VRE growth was suppressed in spent faecal supernatants and spent
297 VRE supernatants supplemented with water, compared to growth in a minimal medium
298 supplemented with the nutrient mixture (**Fig. 6**). We also demonstrated that glucose
299 supplementation of spent faecal supernatants and spent VRE supernatants caused an

300 increase in growth that was comparable to growth achieved in the nutrient-supplemented
301 minimal medium. These results support the hypothesis that nutrient depletion was responsible
302 for suppressing VRE growth in a healthy gut microbiome.
303

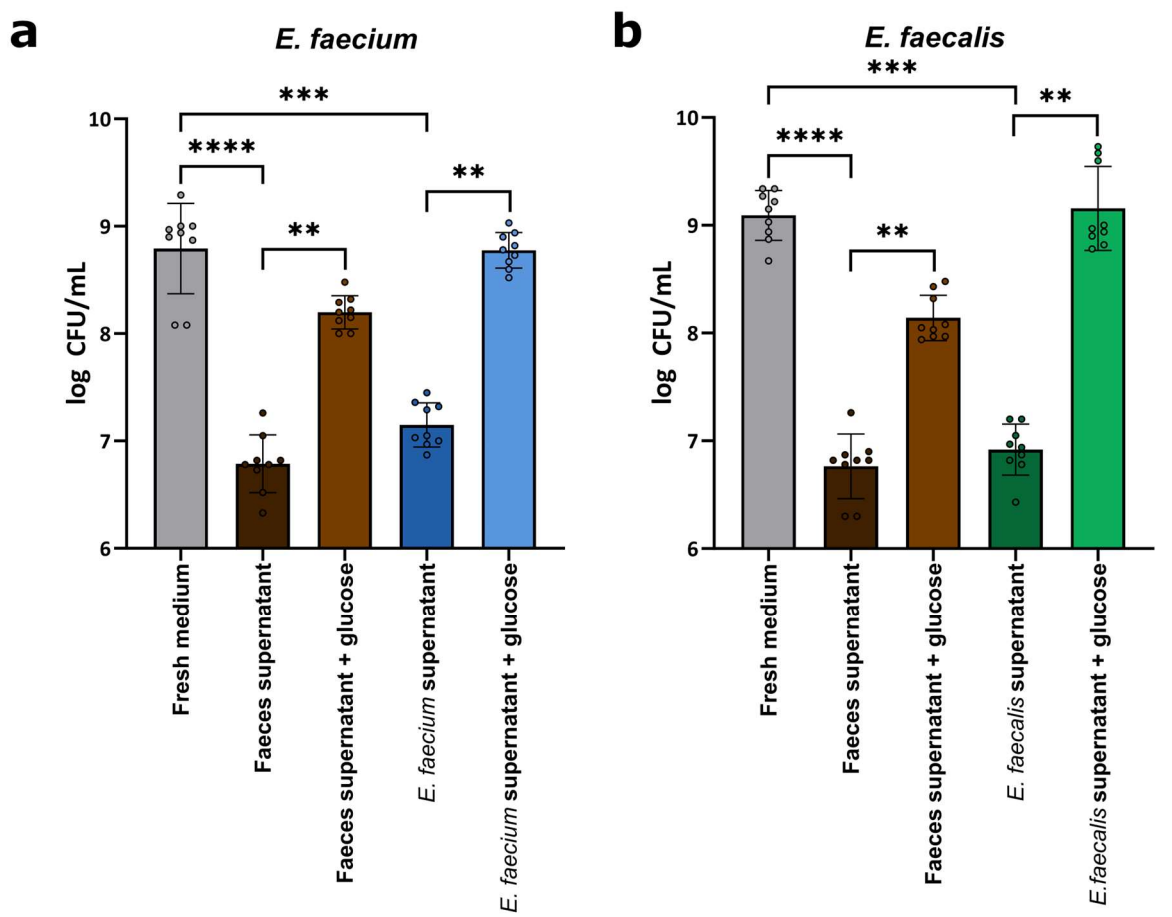
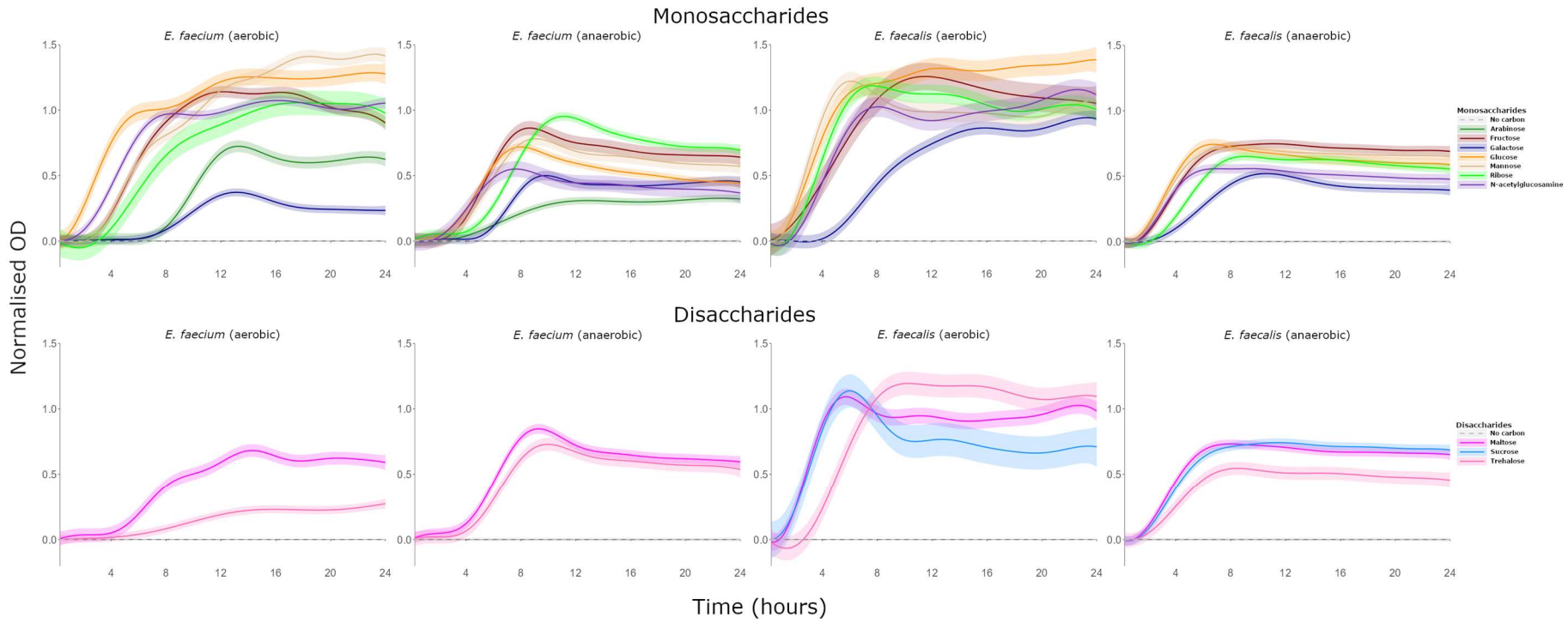



**Figure 6: Faecal culture supernatants suppressed VRE growth through nutrient depletion.** Minimal medium was supplemented with a mixture of nutrients that were increased with antibiotics and inoculated with either fresh healthy human faeces (n = 3 donors for 9 replicates from 3 independent experiments), a single VRE strain (*E. faecium* in panel **a** or *E. faecalis* in panel **b**), or left uninoculated (n = 9 replicates from 3 independent experiments). Spent supernatants were filter sterilised and then inoculated with a single VRE strain. As a control, spent supernatants were also supplemented with 0.5% glucose to confirm nutrient depletion. VRE growth was measured after overnight incubation using plate counts. Comparisons between growth in the spent supernatants and fresh medium were assessed using a Kruskal-Wallis with Dunn's multiple comparison test. Comparison of unsupplemented vs glucose-supplemented supernatants was assessed through a Wilcoxon signed rank test. ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001. Data are presented as mean values ± SD.

304

305 **Many nutrients enriched in antibiotic-treated faecal microbiomes acted as sole carbon**
306 **or nitrogen sources to support VRE growth**
307
308 We measured VRE growth on individual carbon and nitrogen sources that were enriched in
309 antibiotic-treated faecal microbiomes to determine which specific nutrients were capable of
310 supporting VRE growth. This information could help inform the design of new microbiome
311 therapeutics composed of gut commensals that can outcompete the VRE strains for use of
312 these specific nutrients. We also wanted to determine whether vancomycin-resistant *E.*
313 *faecium* and *E. faecalis* had any differences in the nutrients that they could utilise, as this may
314 indicate whether these strains could share the same intestinal niches.
315
316 First, vancomycin-resistant *E. faecium* and *E. faecalis* growth was measured in minimal
317 medium supplemented with each individual nutrient as a sole carbon source, under both
318 anaerobic and aerobic conditions.
319
320 Under anaerobic conditions *E. faecium* and *E. faecalis* growth was supported by
321 monosaccharides and disaccharides. *E. faecium* achieved high growth on fructose, galactose,
322 glucose, mannose, ribose, N-acetylglucosamine, maltose, and trehalose and moderate growth
323 on arabinose (**Figs. 7, S5**). *E. faecalis* achieved high growth on fructose, galactose, glucose,
324 mannose, ribose, N-acetylglucosamine, maltose, sucrose, and trehalose and low growth was
325 achieved on arabinose, xylose, alanine, and valine (**Figs. 7, S5, S6**). Negligible or no growth
326 was achieved on the other carbon sources tested (**Figs. S5, S6**).
327




**<u>Figure 7:</u> VRE growth was supported by individual monosaccharides and disaccharides as carbon sources.** AMiGA-predicted growth curves for vancomycin-resistant *E. faecium* and *E. faecalis* cultured in minimal medium supplemented with single carbon sources under anaerobic or aerobic conditions. The bold lines show the predicted mean of growth while the shaded bands show the predicted 95% credible intervals. n = 6 replicates in 2–3 independent experiments. OD = optical density.

328

329 Under aerobic conditions *E. faecium* and *E. faecalis* growth was also supported by
330 monosaccharides and disaccharides. *E. faecium* achieved high growth on arabinose, fructose,
331 glucose, mannose, ribose, N-acetylglucosamine, and maltose, moderate growth on galactose
332 and trehalose, and low growth on tryptophan (**Figs. 7, S5, S6**). *E. faecalis* achieved high
333 growth on fructose, galactose, glucose, mannose, ribose, N-acetylglucosamine, maltose,
334 sucrose, and trehalose (**Figs. 7, S5**). Negligible or no growth was achieved on the other
335 carbon sources (**Figs. S5, S6**).
336
337 Most amino acids were not used as carbon sources, however they could act as nitrogen
338 sources to support VRE growth. Therefore, next we tested the ability of nutrients that were
339 increased in antibiotic-treated faecal microbiota to act as nitrogen sources to support *E.*
340 *faecium* and *E. faecalis* growth under anaerobic and aerobic conditions. As *E. faecium* and *E.*
341 *faecalis* had undefined amino acid auxotrophies, nitrogen utilisation was tested in a leave-one-
342 out assay where growth in a mixture containing all nitrogen sources was compared to growth
343 in a mixture lacking one of the nitrogen sources.
344
345 Under anaerobic conditions *E. faecium* and *E. faecalis* growth was supported by specific
346 amino acids. *E. faecium* had a large decrease in growth when arginine, glutamate, glycine,
347 isoleucine, leucine, lysine, methionine, serine, threonine, or tryptophan were excluded, a
348 moderate decrease in growth when histidine and valine were excluded, and a small decrease
349 in growth when asparagine, cysteine, phenylalanine, and tyrosine were excluded (**Figs. 8, S7,**
350 **S8**). *E. faecalis* had a large decrease in growth when arginine, glutamate, glycine, isoleucine,
351 leucine, methionine, serine, tryptophan or valine were excluded, a moderate decrease in
352 growth when histidine was excluded, and a small decrease in growth when proline and
353 threonine were excluded (**Figs. 8, S7, S8**). Negligible or no difference in growth was observed
354 when the other nitrogen sources were excluded from the nitrogen mixture (**Figs. S7, S8**).
355

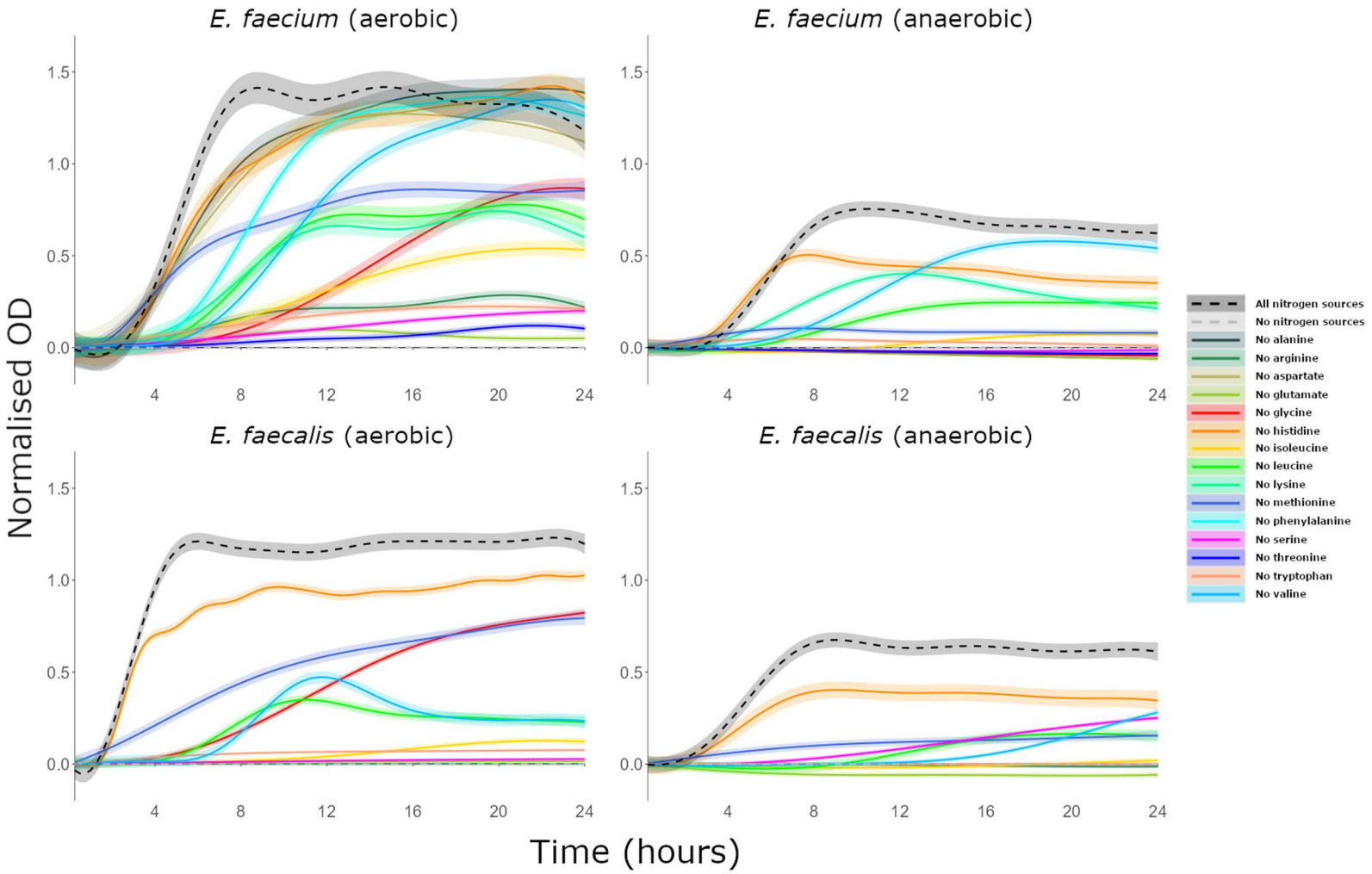


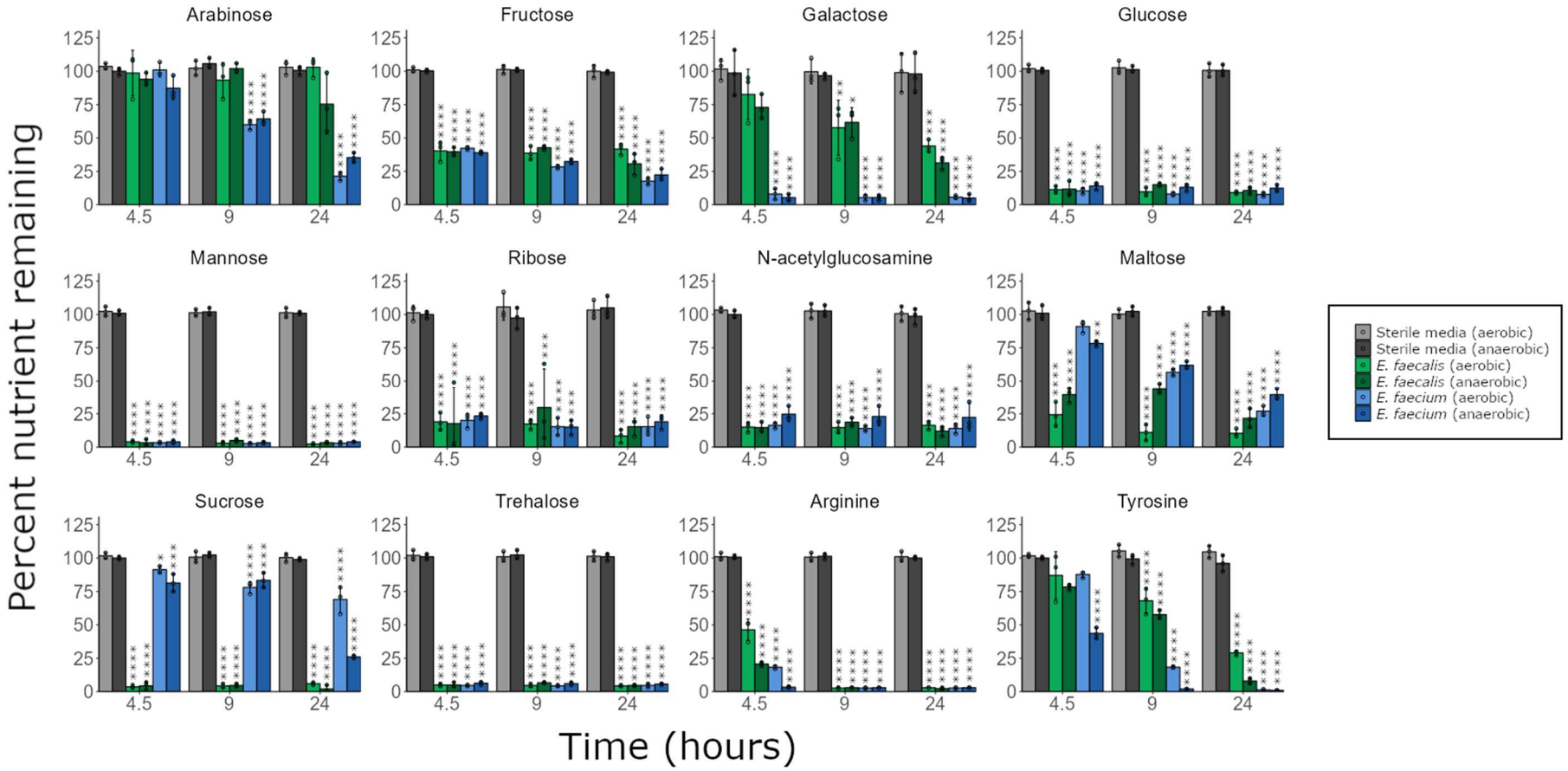

**Figure 8: Amino acids served as nitrogen sources to support VRE growth.** AMiGA-predicted growth curves for vancomycin-resistant *E. faecium* and *E. faecalis* cultured in minimal medium supplemented with a mixture of all nitrogen sources ("all nitrogen sources"), a leave-one-out mixture lacking a single nitrogen source, or lacking all nitrogen sources ("no nitrogen sources") under anaerobic or aerobic conditions. The bold lines show the predicted mean of growth while the shaded bands show the predicted 95% credible intervals. n = 6 replicates in 2 independent experiments. OD = optical density.

356

357 Under aerobic conditions *E. faecium* and *E. faecalis* growth was also supported by specific
358 amino acids. *E. faecium* had a large decrease in growth when arginine, glutamate, glycine,
359 isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, or valine
360 were excluded, a moderate decrease in growth when alanine, aspartate, or histidine were
361 excluded, and a small decrease in growth when cysteine was excluded (**Figs. 8, S7, S8**). *E.*
362 *faecalis* had a large decrease in growth when arginine, glutamate, glycine, isoleucine, leucine,
363 methionine, serine, tryptophan or valine were excluded, a moderate decrease in growth when
364 histidine was excluded, and a small decrease in growth when lysine, phenylalanine, threonine,
365 and tyrosine were excluded (**Figs. 8, S7, S8**). Negligible or no difference in growth was
366 observed when the other nitrogen sources were excluded from the nitrogen mixture (**Figs. S7,**
367 **S8**).
368
369 Overall, vancomycin-resistant *E. faecium* and *E. faecalis* grew on many nutrients that were
370 increased in antibiotic-treated faecal microbiomes. *E. faecium* and *E. faecalis* used
371 monosaccharides and disaccharides as carbon sources and used amino acids as nitrogen
372 sources. However, there were some differences in the utilisation of specific nutrients between
373 *E. faecium* and *E. faecalis*. Both strains generally used the same carbon sources under
374 anaerobic and aerobic conditions but grew to higher levels aerobically.
375
376 **VRE preferred to use specific nutrients over others in a mixture of nutrients that were**
377 **elevated in antibiotic-treated faecal microbiomes**
378
379 Previous work demonstrated that pathogens (such as *Escherichia coli*, *Klebsiella*
380 *pneumoniae*, and *Enterobacter hormaechei*) have a preference for some nutrients over others
381 when presented with a mixture of nutrients that they can use to support their growth[12,28].
382 Moreover, the presence of some nutrients can influence the utilisation of other nutrients[29,30].
383 This hierarchy of nutrient preferences may have implications for the designs of microbiome
384 therapeutics that are based on nutrient competition. However, nutrient preferences have not

been studied in vancomycin-resistant *E. faecium* and *E. faecalis*. Therefore, next we cultured VRE in a minimal medium supplemented with a mixture of nutrients that were increased in an antibiotic-treated intestine (as sole carbon and nitrogen sources) under both anaerobic and aerobic conditions and measured utilisation of these nutrients over time by $^{1}$H-NMR spectroscopy.

*E. faecium* and *E. faecalis* had some common and some distinct nutrient preferences, as demonstrated by how quickly they utilised each nutrient in the nutrient mixture (**Fig. 9**). Both *E. faecium* and *E. faecalis* highly utilised glucose, mannose, trehalose, ribose, N-acetylglucosamine, fructose, and arginine by 4.5 hours. However, *E. faecium* highly utilised galactose by 4.5 hours, while *E. faecalis* highly utilised galactose by 24 hours. Conversely, *E. faecalis* highly utilised maltose and sucrose by 4.5 hours, while *E. faecium* highly/moderately utilised maltose and sucrose by 24 hours. *E. faecium* highly used arabinose by 24 hours, while *E. faecalis* did not use arabinose. Finally, *E. faecium* highly used tyrosine by 9 hours, while *E. faecalis* highly utilised tyrosine by 24 hours. *E. faecalis* also had low utilisation of some amino acids under anaerobic conditions by 24 hours (**Fig. S9**).




**<u>Figure 9:</u> VRE preferred specific nutrients when grown in nutrient mixtures found in antibiotic-treated faecal microbiomes.** Percentage of nutrients remaining in vancomycin-resistant *E. faecium* and *E. faecalis* cultures grown in a minimal medium supplemented with a mixture of nutrients, under aerobic or anaerobic conditions. $^{1}$H-NMR spectroscopy peak integrations were used to measure nutrient concentrations. Comparisons were made using a two-way mixed ANOVA followed by pairwise comparisons with Bonferroni correction. n = 3 replicates per group, with data shown as mean ± SD. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.

401

402

403 In summary, both vancomycin-resistant *E. faecium* and *E. faecalis* showed a preference for
404 some nutrients over others when cultured with a mixture of nutrients that were elevated in
405 antibiotic-treated faecal microbiomes. Moreover, nutrient utilisation was generally comparable
406 for these strains when cultured under both anaerobic and aerobic conditions.

407

408 **VRE occupied overlapping but distinct intestinal niches with each other and with**
409 **carbapenem-resistant *Enterobacteriaceae***

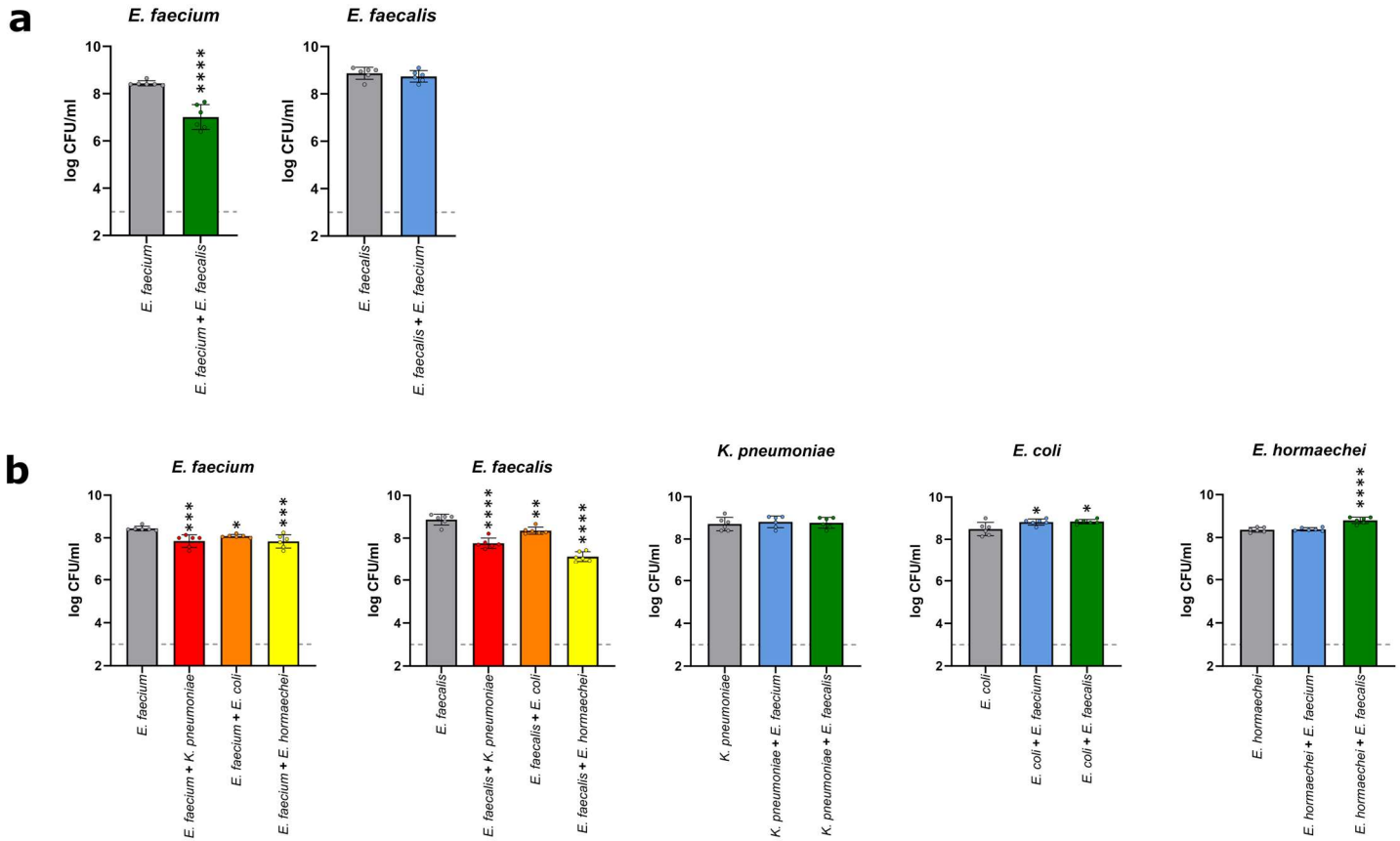
410

411 Our data demonstrated that both *E. faecium* and *E. faecalis* consumed a similar, but not
412 identical range of nutrients found in antibiotic-treated faecal microbiota, and that each strain
413 had similar but not identical preferences for these nutrients. Therefore, we wanted to
414 determine whether *E. faecium* and *E. faecalis* occupied distinct nutrient-defined intestinal
415 niches. If these strains occupy distinct intestinal niches, this may mean that different
416 approaches are required to design microbiome-based therapeutics to displace them from the
417 intestine.

418

419 To test this, *E. faecium* and *E. faecalis* were co-cultured in a minimal medium supplemented
420 with a mixture of nutrients that were elevated in antibiotic-treated faecal microbiota. We
421 demonstrated that both *E. faecium* and *E faecalis* achieved high growth when co-cultured
422 together (**Fig. 10a**). However, *E. faecium* growth had low level inhibition in the co-culture.
423 These results indicate that there is partial, but not complete overlap of the nutrient-defined
424 niches, and both strains can simultaneously grow together on nutrients that are enriched in
425 antibiotic-treated faecal microbiomes.

426




**<u>Figure 10:</u> VRE and CRE achieve high growth in co-culture, but VRE growth is slightly inhibited. (a)** *E. faecium* and *E. faecalis* were co-cultured in a minimal medium supplemented with a mixture of nutrients that are enriched in antibiotic-treated faecal microbiomes. **(b)** *E. faecium* or *E. faecalis* were co-cultured with *K. pneumoniae*, *E. coli*, or *E. hormaechei* in a minimal medium supplemented with a mixture of nutrients that are enriched in antibiotic-treated faecal microbiomes. Each strain was inoculated into the minimal media at a concentration of $10^3$ CFU/ml (represented as the grey dashed line) and the cultures were incubated for 24 hours under anaerobic conditions. Samples were plated on Brilliance VRE plates to quantify *E. faecium* or *E. faecalis* growth and on Brilliance CRE to quantify *K. pneumoniae*, *E. coli*, or *E. hormaechei* growth. Growth of each isolate was measured with 6 replicates in two independent experiments. Comparison of *E. faecium* and *E. faecalis* monoculture growth to co-culture growth was made using an unpaired t-test (two-sided). Comparison of VRE strain and CRE strain monoculture growth to co-culture growth was made using ANOVA with Dunn's multiple comparison test. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$. Data are presented as mean values ± SD.

427

428 Both VRE and CRE intestinal colonisation are promoted by broad spectrum antibiotics, which
429 we demonstrated enriched for a similar range of nutrients. Therefore, we aimed to determine
430 whether VRE and CRE occupied distinct nutrient-defined intestinal niches which permitted for
431 simultaneous growth of VRE and CRE. Again, if VRE and CRE occupy distinct intestinal
432 niches this may have implications for the design of new microbiome-based therapeutics to
433 decolonise these pathogens.
434
435 Co-culture experiments were carried out as described above, except that in this experiment
436 one VRE strain was co-cultured with one CRE strain. Although *E. faecium* and *E. faecalis*
437 growth were slightly inhibited in the co-culture with each CRE strain, we showed that both
438 VRE and CRE strains achieved high growth when co-cultured together (**Fig. 10b**). These
439 results suggested that there is partial, but not complete overlap of the nutrient-defined niches
440 between VRE and CRE, allowing both types of pathogens to simultaneously grow together to
441 high levels on nutrients that are elevated in antibiotic-treated faecal microbiomes.
442
443 **DISCUSSION**
444
445 We used both *ex vivo* and *in vivo* models to demonstrate that five antibiotics that promoted
446 VRE intestinal colonisation disrupted colonisation resistance, leading to increased availability
447 of a wide range of nutrients and decreased production of a wide range of microbial
448 metabolites. We demonstrated that VRE were inhibited by specific short and branched chain
449 fatty acids that were decreased in antibiotic-treated faecal microbiota. However, these
450 metabolites were not sufficient to fully inhibit VRE growth at physiologically relevant
451 concentrations found in healthy human faeces, suggesting that another mechanism of
452 colonisation resistance was important for restricting VRE growth. We also demonstrated that
453 VRE could use most monosaccharides and disaccharides that were increased in antibiotic-
454 treated gut microbiomes as carbon sources, and many amino acids that were enriched in in
455 antibiotic-treated gut microbiomes as nitrogen sources. When cultured with a mixture of

456 nutrients that were found in antibiotic-treated faecal microbiomes, *E. faecium* and *E. faecalis*
457 preferentially utilised specific nutrients over others, where the nutrient preferences for each
458 strain was the same for some nutrients and different for others. Finally, we also showed that
459 vancomycin-resistant *E. faecium* and *E. faecalis* occupied overlapping but distinct intestinal
460 niches in an antibiotic-treated gut microbiome that permitted high growth with each other and
461 with carbapenem-resistant *Enterobacteriaceae* as co-cultures.
462
463 Antibiotics are a major risk factor for VRE intestinal colonisation, including vancomycin, third
464 generation cephalosporins, and antibiotics with potent activity against anaerobic bacteria[31,32].
465 In this study we measured the effects of several antibiotics (VAN, CRO, CLI, MTZ, and TZP)
466 on the human faecal microbiome, where these antibiotics are frequently used clinically and
467 have been shown to promote VRE intestinal colonisation in humans and mice. In agreement
468 with previous studies[7-11], we demonstrated that VAN, CRO, CLI, and MTZ promoted the
469 growth of vancomycin-resistant *E. faecium* and *E. faecalis*. Previous studies demonstrated
470 that prior exposure to TZP was associated with VRE intestinal colonisation[8,9]. However, TZP
471 has anti-enterococcal activity and is secreted in human bile at high concentrations[9]. In this
472 study, we demonstrated that *E. faecium* and *E. faecalis* were killed in the presence of TZP at
473 concentrations found in human faeces. However, TZP may promote VRE intestinal
474 colonisation *in vivo* if the patient is exposed to VRE shortly after finishing a course of TZP
475 (when TZP concentrations have decreased, but before the gut microbiota has recovered). A
476 previous study demonstrated that mice exposed to VRE following the cessation of TZP
477 treatment had high levels of VRE intestinal colonisation[9]. In this study we demonstrated that
478 TZP-treated human faecal cultures had a similar change in nutrients and metabolites as CRO-
479 treated faecal cultures. We concluded that antibiotics could promote pathogen intestinal
480 colonisation by disrupting the gut microbiome, and colonisation could occur during a course
481 of antibiotics if VRE is resistant to the antibiotic, or shortly after stopping a course of antibiotics
482 (prior to recovery of the gut microbiome) if VRE was susceptible to the antibiotic. This

483 emphasises the need to study VRE growth promotion in an antibiotic-treated faecal microbiota
484 over the course of antibiotic treatment and following cessation of antibiotics.
485
486 We demonstrated that antibiotics that promote VRE intestinal colonisation caused a significant
487 decrease in the abundance of a wide range of bacterial families, including *Ruminococcaceae*
488 and *Bifidobacteriaceae* (which were significantly decreased with all antibiotics tested).
489 However, there were many other bacterial families that were changed with the different
490 antibiotics, and it is difficult to understand the impact of changes to these bacterial families
491 without first understanding the functions that these bacteria perform in the gut microbiome.
492 This finding is further complicated by functional redundancy between gut commensals, where
493 different bacterial taxa possess similar genes that perform similar functions[33]. Therefore, we
494 measured changes in nutrients and metabolites directly, in order to understand the nutrient
495 and metabolite landscape that VRE are exposed to during antibiotic treatment.
496
497 We showed that antibiotics that promote VRE intestinal colonisation killed gut commensals
498 that produce a wide range of microbial metabolites. These metabolites have the potential to
499 inhibit pathogen growth, and therefore we tested a mixture of these metabolites in a growth
500 inhibition assay. Growth inhibition was only found at the high concentration measured in
501 healthy human faeces at pH 6, with a 40% reduction in *E. faecium* $OD_{600}$ and a 55% reduction
502 in *E. faecalis* $OD_{600}$. By contrast, in a previous study we demonstrated that CRE were more
503 highly inhibited by a mixture of metabolites found in healthy faeces[12]. At average
504 concentrations measured in healthy faeces at pH 6, $OD_{600}$ was reduced by 85% for *E. coli*,
505 82% for *K. pneumoniae*, and 79% for *E. hormaechei*. Therefore, while the metabolites were
506 less inhibitory for VRE as compared to other multidrug-resistant pathogens like CRE, the
507 metabolites may still be beneficial for restricting VRE growth if they reach the gut in adequate
508 concentrations at a lower pH.
509

510 Next, we investigated the ability of each metabolite to individually inhibit VRE at physiologically
511 relevant concentrations. We saw moderate inhibition of *E. faecium* by the average and high
512 propionate concentrations, the high butyrate concentration, and the high valerate
513 concentration. We also only saw moderate inhibition of *E. faecalis* by the average and high
514 propionate concentration, and the high isovalerate concentration. It may be that the additive
515 effects of multiple inhibitory metabolites administered simultaneously could result in higher
516 VRE growth inhibition than each of the metabolites administered individually.
517
518 Comparing the $IC_{50}$ values of the individual metabolites showed that some metabolites were
519 more inhibitory than others at the same concentrations. Valerate had the lowest $IC_{50}$ value for
520 both *E. faecium* (14 mM) and *E. faecalis* (19 mM). For *E. faecium* and *E. faecalis*, full inhibition
521 with valerate was achieved between 32-64 mM. Although full VRE inhibition is only achieved
522 at high concentrations above the maximum valerate concentration measured in healthy
523 human faeces, it does approach the maximum concentrations of other short chain fatty acids
524 such as acetate, propionate, or butyrate[12]. Therefore, valerate may hold some therapeutic
525 potential if it is well tolerated at higher concentrations. Valerate has previously been
526 demonstrated to be inhibitory against other pathogens including CRE and *C. difficile*[12,13]. Like
527 butyrate, valerate has also been demonstrated to promote intestinal barrier function which
528 may help to prevent dissemination of VRE from the intestine[34].
529
530 Another mechanism of colonisation resistance that may be involved in restricting VRE growth
531 is nutrient competition. We demonstrated that killing of gut commensals with antibiotics that
532 promote VRE intestinal colonisation resulted in an increase in a broad range of nutrients that
533 contained both carbon and nitrogen sources (including monosaccharides, disaccharides,
534 amino acids, uracil, and succinate). Our group also recently demonstrated that antibiotics that
535 promote CRE intestinal colonisation had a similar increase in the concentration of a wide range
536 of nutrients and decrease in the concentration of microbial metabolites in human and mouse
537 faecal microbiota[12]. This observation is consistent with the literature, which showed that VRE

538 and CRE intestinal colonisation are both promoted by broad-spectrum antibiotics – for
539 example, both CRO and TZP promote the intestinal colonisation of VRE and CRE[8-10,35].
540 Moreover, VAN, MTZ, CRO, CLI, and TZP have been demonstrated to cause blooms in
541 *Enterobacteriaceae* growth in the intestine[11,12,36-38]. Therefore, broad-spectrum antibiotics that
542 promote VRE and CRE intestinal colonisation resulted in an increase in the availability of
543 similar nutrients, from which we conclude that this may be a broader phenomenon that may
544 apply to other intestinal pathogens that have their growth promoted in the intestine following
545 antibiotic treatment.
546
547 Microbes with similar or "overlapping" nutrient utilisation abilities will compete for similar
548 intestinal niches[39]. In this study we demonstrated that *E. faecium* and *E. faecalis* occupy
549 distinct but overlapping intestinal niches. *E. faecium* and *E. faecalis* can both use
550 monosaccharides (fructose, galactose, glucose, mannose, ribose, N-acetylglucosamine) and
551 disaccharides (maltose, trehalose) as carbon sources, and can use amino acids (arginine,
552 glutamate, glycine, histidine, isoleucine, leucine, methionine, serine, tryptophan, valine) as
553 nitrogen sources. However, there were some differences in the utilisation of specific nutrients
554 between *E. faecium* and *E. faecalis*. For example, *E. faecium* showed higher growth on
555 arabinose, lysine, and threonine, while *E. faecalis* showed higher growth on sucrose.
556 Therefore, if we can identify gut commensal strains that can deplete these nutrients, we can
557 develop novel microbiome therapeutics to re-establish colonisation resistance and restrict
558 VRE intestinal growth. However, we demonstrated that both *E. faecium* and *E. faecalis* can
559 utilise a wide range of nutrients, and this flexibility in nutrient utilisation suggests that
560 microbiome therapeutics targeting VRE must contain several gut commensals that together
561 can deplete all the nutrients that VRE can use to grow.
562
563 A previous study demonstrated that antibiotics increase oxygen availability in the intestine[40].
564 Therefore, we also investigated nutrient utilisation by VRE under both anaerobic and aerobic
565 conditions. We found that *E. faecium* and *E. faecalis* generally used the same nutrients under

566 both aerobic and anaerobic conditions, but growth was typically higher under aerobic
567 conditions. This scenario is in contrast to CRE, where CRE were able to use more nutrients
568 under aerobic conditions compared to anaerobic conditions[12]. However, elevated oxygen
569 levels present in the intestine during antibiotic treatment could give VRE a competitive
570 advantage in the intestine, as their overall growth is higher in the presence of oxygen.
571 Furthermore, the presence of oxygen would also be detrimental to the growth of strictly
572 anaerobic gut pathogens that may provide colonisation resistance against VRE. New
573 microbiome therapeutics should aim to deplete oxygen to further restrict VRE growth. For
574 example, oxygen could be depleted by increasing butyrate, a metabolite that is consumed by
575 intestinal epithelial cells in a process that consumes oxygen, or by including facultative
576 anaerobes that can consume oxygen in the design of new microbiome therapeutics[41].
577
578 Previous studies demonstrated that the presence of some nutrients may influence the
579 utilisation of others by bacteria, including stimulating or repressing the utilisation of specific
580 nutrients[12,29,30,42,43]. We demonstrated that *E. faecium* and *E. faecalis* showed a preference for
581 some nutrients over others when cultured with a mixture of nutrients that were elevated in
582 antibiotic-treated faecal microbiomes. Some nutrients followed expected nutrient utilisation
583 patterns - nutrients that were highly used in the individual nutrient utilisation assays were
584 quickly depleted when *E. faecium* and *E. faecalis* were cultured in a mixture of nutrients (e.g.
585 glucose and arginine). However, other nutrients that were not used when tested individually
586 were used when VRE were cultured in a mixture of nutrients. For example, we showed that *E.*
587 *faecium* was not able to use sucrose as a sole carbon source but was able to use sucrose
588 when grown in a mixture of nutrients. In contrast, some nutrients that were used in the
589 individual nutrient utilisation assays were not used by VRE when cultured in the mixture of
590 nutrients. For example, we showed that *E. faecium* highly used threonine when tested in the
591 nitrogen leave-one-out assay, but *E. faecium* did not use threonine when grown in a mixture
592 of nutrients. Therefore, nutrient utilisation and preferences in mixtures of nutrients are complex
593 and it is not always straightforward to translate the utilisation of nutrients as single carbon or

594 nitrogen sources to their utilisation in mixtures that VRE would encounter in the intestine *in*
595 *vivo*. Nutrient utilisation may also be context dependent, meaning that VRE nutrient utilisation
596 and preferences may change under different environmental conditions. For example, future
597 studies should investigate VRE nutrient utilisation and preferences in the context of different
598 host diets, or in different host microbiomes that vary in terms of composition and antibiotic-
599 resistance profiles. Therefore, the best approach to restrict VRE growth may be to deplete all
600 nutrients that are elevated with antibiotic treatment, so there are no alternative nutrient sources
601 for VRE to utilise.
602
603 We demonstrated that antibiotics that promote VRE and CRE intestinal colonisation enriched
604 for a similar broad range of nutrients, and depleted a similar broad range of microbial
605 metabolites[12]. However, it was not clear whether VRE and CRE occupied distinct intestinal
606 niches, which may impact the design of microbiome therapeutics to restrict the growth of these
607 pathogens. A previous study demonstrated that vancomycin-resistant *E. faecium* and
608 carbapenem-resistant *Klebsiella pneumoniae* occupied distinct but overlapping intestinal
609 niches, however, the mechanism of how these pathogens occupied their intestinal niches were
610 not defined[15]. We hypothesised that VRE growth was promoted in an antibiotic-treated faecal
611 microbiome because VRE were able to occupy a distinct nutrient-defined intestinal niche from
612 CRE. Compared to our previous study of CRE nutrient utilisation[12], we demonstrated that VRE
613 and CRE had an overlap in the utilisation of many but not all nutrients. For example, *E. faecium*
614 and *K. pneumoniae* can both use monosaccharides (arabinose, fructose, galactose, glucose,
615 mannose, ribose, N-acetylglucosamine) and disaccharides (maltose, sucrose, and trehalose)
616 as carbon sources, and can use amino acids (alanine, arginine, aspartate, glutamate, glycine,
617 isoleucine, leucine, lysine, and methionine) as nitrogen sources. However, *E. faecium* can use
618 tryptophan and valine as carbon and nitrogen sources and can use phenylalanine and tyrosine
619 as nitrogen sources (nutrients not used by *K. pneumoniae*). Conversely, *K. pneumoniae* can
620 use xylose as a carbon source, proline as a carbon and nitrogen source, and uracil as a
621 nitrogen source (nutrients not used by *E. faecium*). Moreover, VRE and CRE also had different

622 preferences for nutrients that they could use. For example, although both *E. faecium* and *K.*
623 *pneumoniae* can use aspartate, arginine, lysine, and ribose, *E. faecium* prefers to use arginine
624 and ribose while *K. pneumoniae* prefers to use aspartate and lysine. It is also possible that
625 CRE and VRE may be able to share nutrients to simultaneously grow together. For example,
626 each species may only need one of the many carbon sources that are available in the intestine
627 following antibiotic treatment to support its growth. Therefore, although similar nutrient- and
628 metabolite-defined landscapes are generated in the intestine with broad-spectrum antibiotics
629 that promote VRE and CRE intestinal colonisation, the niches that these pathogens occupy in
630 the intestine are different. This highlights that VRE have different strategies for utilising
631 nutrients to colonise and bloom in an antibiotic-treated gut microbiome compared to other
632 multidrug-resistant pathogens, which may have implications for the design of new microbiome
633 therapeutics to decolonise pathogens from the intestine.
634
635 To design microbiome therapeutics that deplete nutrients found in antibiotic-treated intestines
636 we also need to understand the nutrient utilisation abilities and preferences of gut
637 commensals. We need more information about the gut commensals that are sensitive or
638 resistant to antibiotics that promote VRE intestinal colonisation, as well as the impact of host
639 diet on VRE and gut commensal intestinal colonisation. Gut commensals that are killed by
640 antibiotics that promote VRE intestinal colonisation may be important for restoring colonisation
641 resistance. Moreover, understanding the nutrient utilisation abilities and preferences of
642 antibiotic-resistant gut commensals that persist or bloom in the intestine during antibiotic
643 treatment is also important. These antibiotic-resistant gut commensals would impact the
644 nutrient landscape of the intestine and could influence engraftment of gut commensals in
645 microbiome therapeutics.
646
647 In summary, we demonstrated that VRE take advantage of the nutrient-enriched and
648 metabolite-depleted intestinal niches that are generated during antibiotic treatment to colonise
649 the intestine. We demonstrated that several antibiotics that promote VRE intestinal

650 colonisation changed the abundance of many nutrients and metabolites. Although microbial
651 metabolites showed some growth inhibition at physiologically relevant concentrations, this
652 inhibition was incomplete. Healthy gut microbiomes depleted nutrients though nutrient
653 competition, however in antibiotic-treated faecal microbiomes killing of gut commensals
654 enriched for nutrients. VRE can use most of these nutrients as carbon or nitrogen sources,
655 where *E. faecium* and *E. faecalis* have some similar and some different preferences for these
656 nutrients. Finally, we showed that *E. faecium* and *E. faecalis* occupy overlapping but distinct
657 intestinal niches that permit high simultaneous growth with each other and with CRE.
658 Therefore, designing a microbiome therapeutic consisting of gut commensals that deplete
659 these nutrients could restore colonisation resistance and restrict or prevent VRE intestinal
660 colonisation. Removing an intestinal reservoir for VRE would lead to a reduction in the
661 development of invasive VRE infections. Our study also suggested that designing a
662 microbiome therapeutic consisting of a mixture of gut commensals that can deplete the whole
663 range of nutrients that are elevated with broad-spectrum antibiotics could be used to restrict
664 both VRE and CRE growth.
665
666 **METHODS**
667
668 ***Bacterial isolates:***
669
670 Vancomycin-resistant *E. faecalis* (NCTC 12201) and vancomycin-resistant *E. faecium* (NCTC
671 12202) were purchased from Public Health England (now the UK Health Security Agency) in
672 February 2021. These strains were isolated from infected human patients in the Dulwich Public
673 Health Laboratory[44]. Both strains have *vanA*-type glycopeptide resistance. VRE isolates with
674 the *vanA*-type resistance gene were chosen as this gene confers a high level of vancomycin
675 resistance and it is one of the most frequently detected vancomycin-resistance genes in
676 enterococci[45,46].
677

678 Carbapenem-resistant *E. coli* ST617 (NDM-5), *K. pneumoniae* ST1026 (NDM-1) and *E.*
679 *hormaechei* ST278 (NDM-1) were isolated and characterised in a previously published study
680 by Yip and colleagues[12]. These carbapenem-resistant *Enterobacteriaceae* were isolated from
681 rectal swabs from patients in the Imperial College Healthcare NHS Trust (London, UK), with
682 ethical approval from the London - Queen Square Research Ethics Committee, 19/LO/0112.
683
684 ***Minimum inhibitory concentrations (MICs):***
685
686 VAN, MTZ, CRO, TZP and CLI MICs were measured for *E. faecium* and *E. faecalis* following
687 standard protocols[47]. EUCAST guidelines provided clinical breakpoints for *Enterococcus* spp.
688 for VAN (≤4 mg/L sensitive, >4 mg/L resistant)[48]. According to EUCAST guidance,
689 *Enterococcus* spp. can be reported as resistant to MTZ, CRO, TZP, and CLI without further
690 testing.
691
692 ***Growth of VRE in faecal concentrations of antibiotics:***
693
694 Distal gut growth medium was supplemented with water or with one of the following antibiotics
695 at concentrations found in human faeces: 1000 µg/mL VAN, 25 µg/mL MTZ, 152 µg/mL CRO,
696 139 µg/mL TZP, and 383 µg/mL CLI[19-24,49]. *E. faecium* or *E. faecalis* were inoculated into the
697 growth medium at a concentration of $10^3$ CFU/ml and cultures were incubated at 37°C
698 anaerobically for 24 hours. VRE growth was quantified by plating on Columbia agar and
699 incubating the plates at 37°C for 24 hours.
700
701 ***Human ethical approval:***
702
703 Ethical approval was received by the South Central - Oxford C Research Ethics Committee
704 (16/SC/0021 and 20/SC/0389) to collect fresh faecal samples from healthy human donors.
705 Faecal donors were aged between 18-65 years old. There is no biological reason to favour

706 one sex over the other, and therefore both male and female donors were recruited, with
707 approximately equal numbers of male and female donors included in each experiment. Faecal
708 donors did not receive antibiotic treatment in at least 6 months prior to donation. We obtained
709 informed consent from each participant.
710
711 ***Human faecal culture experiments:***
712
713 *Ex vivo* faecal culture experiments were performed using a previously published protocol[12].
714 These experiments were performed in a standardised distal gut growth medium under
715 anaerobic conditions[49]. The faecal slurry was inoculated into the distal gut growth medium at
716 a final concentration of 2% (w/v). For each donor, fresh faeces were used to seed all
717 experimental conditions, including each of the antibiotic-treated faecal cultures and the
718 antibiotic-naïve faecal culture. Each treatment was performed in triplicate for each donor.
719
720 The first set of faecal culture experiments measured the impact of five broad-spectrum
721 antibiotics (known to promote VRE intestinal colonisation) on the faecal microbiome from 12
722 healthy human donors. Donors were aged 29.5±9.5 years old and consisted of 7 female
723 donors and 5 male donors. The distal gut growth medium was supplemented with water
724 (antibiotic-naïve control) or with one of the following antibiotics at concentrations measured in
725 human faeces: 1000 µg/mL VAN, 25 µg/mL MTZ, 152 µg/mL CRO, 139 µg/mL TZP, and 383
726 µg/mL CLI[19-24]. Faecal cultures were incubated at 37°C anaerobically for 24 hours. Samples
727 were collected for 16S rRNA gene sequencing, 16S rRNA gene qPCR, and $^{1}$H-NMR
728 spectroscopy.
729
730 The second set of faecal culture experiments were performed to measure VRE growth in
731 antibiotic-treated and antibiotic-naïve faecal microbiomes. Faecal samples were collected
732 from 5-8 healthy human donors (5 females, 3 males) aged 26.8±4.3 years old. The distal gut
733 growth medium was supplemented with water, MTZ (25 µg/mL), CLI (383 µg/mL), VAN (1000

734 µg/mL), or CRO (152 µg/mL) at concentrations measured in human faeces. TZP was excluded
735 from this experiment as the VRE isolates used in this study were killed by TZP at faecal
736 concentrations. Faecal cultures were incubated at 37°C anaerobically for 24 hours. The next
737 day, *E. faecium* or *E. faecalis* were inoculated into the faecal cultures at a concentration of $10^3$
738 CFU/mL and cultures were incubated at 37°C anaerobically for an additional 24 hours.
739 Cultures were plated on Brilliance VRE agar (Thermo Fisher Scientific, UK) to measure VRE
740 growth. VRE growth was also measured in distal gut growth medium not supplemented with
741 faeces or antibiotics after incubation at 37°C anaerobically for 24 hours, followed by plating
742 on Brilliance VRE agar.
743
744 ***16S rRNA gene sequencing and 16S rRNA gene qPCR:***
745
746 The DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germany) was used to extract DNA from
747 faecal culture samples (DNA extracted from 250 µL per sample). The extraction protocol
748 followed the manufacturer's instructions, with the addition of 3x 1 minute bead beating cycles
749 at speed 8 (with a 1-minute rest between each cycle) using a FastPrep 24 bead beater (MP
750 Biomedicals, USA). The extracted DNA was stored at -80°C until it was ready to be analysed.
751
752 Sample libraries were prepared for 16S rRNA gene sequencing following Illumina's 16S
753 metagenomic sequencing library preparation protocol and as previously described by our
754 group[12,50]. The V1-V2 region was amplified using a forward primer mix (a 4:1:1:1 ratio of 28F-
755 YM, 28F-Borrellia, 28F-Chloroflex, and 28F-Bifdo primers) and the reverse primer 388R[12,50].
756 The index PCR reactions were cleaned up and normalised using the SequalPrep
757 Normalisation Plate Kit (Life Technologies, UK). Sample libraries were quantified using the
758 NEBNext Library Quant Kit for Illumina (New England Biolabs, UK). Sequencing was
759 performed on an Illumina MiSeq platform (Illumina Inc., UK) with paired-end 300 bp chemistry
760 using the MiSeq Reagent Kit V3 (Illumina). The resulting data was imported into R where it
761 was processed using the standard DADA2 pipeline (version 1.18.0)[51]. Version 138.1 of the

762 SILVA bacterial database was used for sequence variant classification (https://www.arb-
763 silva.de/).
764
765 Bacterial biomass was quantified using 16S rRNA gene qPCR, following a previously
766 published protocol[13]. PCR reactions were prepared in 20 µL volumes, containing 1x Platinum
767 Supermix with ROX, 225 nmol/L BactQUANT probe, 1.8 µmol/L BactQUANT forward primer,
768 1.8 µmol/L BactQUANT reverse primer, 5 µL DNA, and PCR grade water. A 10-fold dilution
769 series of *E. coli* DNA was used to generate the standard curve. All samples, standards, and
770 controls were analysed in triplicate. Amplification and real-time fluorescence detection was
771 performed on the Applied Biosystems StepOnePlus Real-Time PCR System (Applied
772 Biosystems, Waltham, Massachusetts). The PCR cycle conditions for qPCR were as follows:
773 50 °C for 3 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60°C for 1 min.
774
775 The 16S rRNA gene sequencing data was converted to absolute abundances using the 16S
776 rRNA gene qPCR data using the following formula, as previously described[13]:
777
778 $$\text{Absolute abundance of taxa} =$$
779 $$\text{relative abundance of taxa} \times \left(\frac{\text{16S rRNA gene copy number in sample}}{\text{highest 16S rRNA gene copy number in sample set}}\right)$$
780
781 ***$^1$H-NMR spectroscopy:***
782
783 $^1$H-NMR spectroscopy was performed on human faecal culture samples, VRE culture
784 samples, and mouse faecal samples following a previously published protocol[12,13].
785
786 Human faecal culture samples and VRE culture samples were prepared as follows.
787 Supernatants were prepared by centrifuging samples at 17,000 × g for 10 min at 4°C (the pellet
788 was discarded), and supernatants were frozen down until ready to be analysed. Then,

789 supernatants were randomised and defrosted at room temperature. Next, supernatants were
790 centrifuged for 10 min at 17,000 × g and 4 °C (the pellet was discarded). The NMR buffer
791 contained the following per L: 28.85 g $Na_2HPO_4$, 5.25 g $NaH_2PO_4$, 1 mM 3-
792 (trimethylsilyl)propionic-2,2,3,3-$d_4$ acid sodium salt (TSP), 3 mM $NaN_3$, deuterium oxide to 1 L,
793 pH 7.4. For each sample, 400 µl of sample supernatant was mixed with 250 µl NMR buffer.
794
795 Mouse faecal samples were prepared as follows. Mouse faecal samples were randomised
796 and defrosted at room temperature. Mouse faecal supernatants were prepared by
797 homogenising 20 mg of mouse faeces in 560 µl of high-performance liquid chromatography-
798 grade water by vortexing for 10 minutes at 3000 rpm, followed by centrifuging samples at
799 17,000 × g for 10 min at 4°C (the pellet was discarded). The NMR buffer contained the
800 following per 100 mL: 20.4 g $KH_2PO_4$, 5.8 mM TSP, 2 mM $NaN_3$, deuterium oxide to 100 mL,
801 pH 7.4. For each sample, 540 µl of supernatant was mixed with 60 µl NMR buffer.
802
803 For each sample 600 µl of sample supernatant mixed with NMR buffer was loaded into 5mm
804 NMR tubes and loaded into the SampleJet system. $^1$H-NMR spectra were acquired at 300 K
805 using a Bruker AVANCE III 800 MHz NMR spectrometer (Bruker Bio-Spin, Rheinstetten,
806 Germany) fitted with a 5mm CPTCI 1H-13C/15N D Z-gradient cryoprobe. 1D spectra were
807 acquired using a standard NOESYGPPR1D pulse sequence (RD-90°-t1-90°-tm-90°-ACQ). A
808 4 s recycle delay, 100 ms mixing time, and ~10 µs 90° pulse length were used. For the human
809 faecal culture samples and VRE culture samples 32 scans were recorded. For the mouse
810 faecal samples 128 scans were recorded.
811
812 NMR spectra pre-processing was performed in Topspin (v3.2.6), with an exponential window
813 function (line broadened by 0.3 Hz), zero-order auto phasing (command apk0), baseline
814 correction (command abs), and referencing TSP (command sref). Further processing was
815 performed by importing NMR spectra into MATLAB r2019b (The Mathworks, USA) using a
816 custom script, as previously described[12]. Following alignment of the spectra the water peak

817 was removed by cutting region 4.6 - 5.0 ppm and the TSP peak was removed by cutting region
818 -0.2 - 0.2 ppm. Chenomx NMR Suite v9.02 (Chenomx, Canada) was used for peak
819 identification and representative peaks were selected and integrated. To define the limit of
820 detection, a signal-to-noise ratio cut-off of 3:1 was used[52]. The minimal signal-to-noise ratio
821 for each dataset was as follows: 8.3:1 for the human faecal culture samples, 8.5:1 for the VRE
822 culture samples, 3.2:1 for the mouse faecal samples.
823
824 ***Mouse model of intestinal colonisation with vancomycin-resistant* E. faecium*:***
825
826 The Imperial College London Animal Welfare and Ethical Review Body (PF93C158E) provided
827 ethical approval for the mouse experiments. The mouse experiment was performed under the
828 authority of the UK Home Office, as described in the Animals (Scientific Procedures) Act 1986.
829 We adhered to standards outlined in the Animal Research: Reporting of In Vivo Experiments
830 (ARRIVE) guidelines.
831
832 Female wild-type C57BL/6 mice (8-10 weeks old) were purchased from Envigo (Huntingdon,
833 UK). Mice were housed 5 per cage in individually ventilated cages with bedding (Aspen chip
834 2 bedding, NEPCO, Warrensburg, New York). Mice were maintained at 20-22°C and 45-65%
835 humidity, with 12-hour light and dark cycles. Mice were given autoclaved food (RM1, Special
836 Diet Services, Essex, UK) and water (provided ad libitum). Prior to the start of the experiment
837 mice were acclimatised for 1 week.
838
839 The mouse experiment was designed to measure the impact of VAN treatment on nutrient
840 availability, metabolite production, and vancomycin-resistant *E. faecium* intestinal growth.
841 Mice were orally gavaged with VAN or water once daily for 7 days, and faecal samples were
842 collected for $^{1}$H-NMR spectroscopy 24 hours after the last dose of VAN was administered.
843 VAN dosing was based on previously published study, where mice were orally gavaged with
844 water or with VAN at a dose of 50 mg/kg/day[53]. Both groups of mice were orally gavaged with

845 $10^6$ CFU per 200 µl of vancomycin-resistant *E. faecium* 4 days after the last dose of VAN, and
846 4 days later faecal samples were collected from mice to plate on Brilliance VRE agar to
847 quantify *E. faecium* growth.
848
849 ***Mixed metabolite inhibition assay:***
850
851 Microbial metabolites that were decreased with antibiotic treatment were tested together as a
852 mixture in an inhibition assay to see if they could inhibit VRE growth. The metabolite
853 concentrations found in healthy faeces were obtained from a previously published study, and
854 included the low, average, and high concentrations measured in healthy faeces[12]. We tested
855 a mixture of formate, acetate, propionate, butyrate, valerate, isobutyrate, isovalerate, lactate,
856 5-aminovalerate, and ethanol, where metabolites mimicked their concentrations measured in
857 healthy human faeces. For the low mixture all metabolites were tested at the low
858 concentrations measured in faeces, for the average mixture all metabolites were tested at the
859 average concentrations measured in faeces, and for the high mixture all metabolites were
860 tested at the high concentrations measured in faeces. Tryptic soy broth was supplemented
861 with the metabolite mixture and the pH of the supplemented broth was adjusted to 6.0, 6.5, or
862 7.0 to mimic the pH of the healthy intestine[26,54]. *E. faecium* or *E. faecalis* were inoculated into
863 the broth at a concentration of $10^3$ CFU/ml, cultures were incubated at 37°C anaerobically
864 overnight, and $OD_{600}$ measurements were taken the next day.
865
866 ***Individual metabolite inhibition assay:***
867
868 Microbial metabolites were individually tested to see if they could inhibit VRE growth. Each
869 metabolite was tested at concentrations mimicking the low, average, and high concentrations
870 measured in healthy human faeces[12]. We tested the following individual metabolites: formate,
871 acetate, propionate, butyrate, valerate, isobutyrate, isovalerate, lactate, 5-aminovalerate, and
872 ethanol. We also tested each individual metabolite as 2-fold dilutions between 0.016-512 mM

873 to calculate the half-maximal inhibitory concentration ($IC_{50}$). Tryptic soy broth was
874 supplemented with each metabolite and the pH of the supplemented broth was adjusted to
875 6.0 (based on the results from the mixed metabolite inhibition assay). *E. faecium* or *E. faecalis*
876 were inoculated into the broth at a concentration of $10^3$ CFU/ml, cultures were incubated at
877 37°C anaerobically overnight, and $OD_{600}$ measurements were taken the next day.
878
879 ***Growth of VRE in faecal culture supernatants and VRE supernatant:***
880
881 Basal minimal medium (see Supplementary Data 1) was supplemented with a mixture of
882 nutrients that were enriched in antibiotic-treated faecal microbiomes at a final concentration of
883 0.015% (w/v): L-alanine, L-arginine, L-aspartate, L-glutamate, L-glycine, L-isoleucine, L-
884 leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-
885 tyrosine, L-valine, L-arabinose, D-fructose, L-fucose, D-galactose, D-glucose, D-mannose, D-
886 ribose, D-xylose, N-acetylglucosamine, D-maltose, sucrose, D-trehalose, uracil and succinate.
887 The supplemented basal minimal medium was inoculated with fresh healthy human faeces (2
888 female donors and 1 male donor, average age 29.3±4.0 years old), a single VRE strain (*E.*
889 *faecium* or *E. faecalis*), or left uninoculated. To prepare the faecal inoculum, fresh faeces were
890 prepared as a 10% (w/v) faecal slurry in 0.9% (w/v) saline and homogenised in a stomacher
891 at 250 rpm for 1 minute. The faecal slurry was diluted 1 in 10 into degassed supplemented
892 basal minimal medium to give a final concentration of 1%. *E. faecium* and *E. faecalis* were
893 inoculated into degassed supplemented basal minimal medium at a concentration of $10^7$
894 CFU/ml. Cultures were incubated at 37°C anaerobically for 24 hours. The next day, the spent
895 supernatants were adjusted to pH 7, filter sterilised, and inoculated with a single VRE strain
896 at $10^6$ CFU/ml. As a control, spent supernatants were also supplemented with 0.5% (w/v)
897 glucose to confirm nutrient depletion. Cultures were incubated at 37°C anaerobically for 24
898 hours, then cultures were plated on fastidious anaerobe agar with 5% (v/v) defibrinated horse
899 blood to quantify VRE growth.
900

901 ***Carbon and nitrogen utilisation assays:***

902

903 Nutrients increased in antibiotic-treated faecal cultures were tested for their potential to serve
904 as individual carbon and/or nitrogen sources for *E. faecium* and *E. faecalis*. We designed an
905 *Enterococcus* minimal medium to test the individual nutrients, which did not allow any
906 background growth without a supplemented carbon and nitrogen source. The minimal medium
907 consisted of the following: potassium phosphate monobasic (4 g/L), potassium phosphate
908 dibasic (14 g/L), sodium citrate (1 g/L), magnesium sulphate (0.2 g/L), ammonium sulphate (2
909 g/L), adenosine (0.04 g/L), thymine (0.04 g/L), Kao and Michayluk vitamin solution (Merck,
910 Cat. No. K3129; 10 mL/L). If the study was performed under anaerobic conditions, fumarate
911 (5.2 g/L) was added to increase growth of VRE strains. The minimal medium was sterilised
912 with a 0.22 µm filter and used immediately for aerobic incubations or degassed overnight if
913 used for anaerobic incubations.

914

915 For the carbon assays the minimal medium was supplemented with the RPMI-1640 amino
916 acid solution (Merck, Cat. No. R7131; 50 mL/L) as nitrogen sources, and each individual
917 nutrient was supplemented in at a concentration of 0.5% (w/v) to test their utilisation as a
918 carbon source.

919

920 For the nitrogen assays the minimal medium was supplemented with 0.5% (w/v) glucose as a
921 carbon source. As *E. faecium* and *E. faecalis* had undefined amino acid auxotrophies, nitrogen
922 utilisation was assessed by comparing growth of the nitrogen mixture lacking one of the
923 nitrogen sources to growth on the complete nitrogen mixture (leave-one-out experimental
924 design). The complete “all nitrogen” mixture contained the following: L-alanine (2 g/L), L-
925 arginine (2.5 g/L), L-asparagine (0.71 g/L), L-aspartate (0.25 g/L), L-cysteine (0.625 g/L), L-
926 glutamate (0.25 g/L), L-glycine (0.125 g/L), L-histidine (0.1875 g/L), L-isoleucine (0.625 g/L),
927 L-leucine (0.625 g/L), L-lysine HCl (0.5 g/L), L-methionine (0.1875 g/L), L-phenylalanine
928 (0.1875 g/L), L-proline (0.25 g/L), L-serine (0.375 g/L), L-threonine (0.25 g/L), L-tryptophan

929 (0.0625 g/L), L-tyrosine (0.145 g/L), L-valine (0.25 g/L), N-acetylglucosamine (2 g/L), and
930 uracil (0.5 g/L). Each leave-one-out mixture excluded one of the nitrogen sources found in the
931 all nitrogen mixture.
932
933 For both carbon and nitrogen assays, VRE strains were grown on Reasoner's 2A (R2A) agar
934 overnight and inoculated into supplemented minimal medium at a starting $OD_{600}$ of 0.06.
935 Cultures were incubated either anaerobically or aerobically in a plate reader which measured
936 $OD_{600}$ every 15 minutes for 24 hours.
937
938 ***Mixed nutrient preference assays:***
939
940 Nutrient preference was assessed for *E. faecium* and *E. faecalis* by inoculating each strain in
941 a minimal medium supplemented with a mixture of nutrients that were increased in antibiotic-
942 treated faecal cultures. Decreases in the concentrations of each nutrient were measured over
943 time. The *Enterococcus* minimal medium was supplemented with a mix of the following
944 nutrients at 0.015% (w/v) for each nutrient: L-alanine, L-arginine, L-aspartate, L-glutamate, L-
945 glycine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-
946 threonine, L-tryptophan, L-tyrosine, L-valine, L-arabinose, D-fructose, L-fucose, D-galactose,
947 D-glucose, D-mannose, D-ribose, D-xylose, N-acetylglucosamine, D-maltose, sucrose, D-
948 trehalose, uracil and succinate.
949
950 VRE strains were grown on Reasoner's 2A (R2A) agar overnight and inoculated into the
951 supplemented minimal medium at a starting $OD_{600}$ of 0.06. Cultures were incubated either
952 anaerobically or aerobically in a plate reader which measured $OD_{600}$ every 15 minutes for 24
953 hours. Samples were collected for $^{1}$H-NMR spectroscopy at 0, 4.5, 9, and 24 hours. Nutrient
954 utilisation was expressed as a percentage of the peak integration value for each nutrient at 0
955 hours.
956

957 ***Analysis of Microbial Growth Assays (AMiGA):***

958

959 The growth curve data generated for *E. faecium* and *E. faecalis* was analysed using the
960 AMiGA software (available at https://github.com/firasmidani/amiga) in Python (v3.6.5) [55]. The
961 carbon source utilisation growth curves and mixed nutrient growth curves were normalised to
962 the no carbon control using the subtraction method. The nitrogen utilisation growth curves
963 were normalised to the no nitrogen control using the subtraction method. Differential growth
964 was compared between the substrate and the no carbon control for the carbon assays, or to
965 the all nitrogen control for the nitrogen assays by performing Gaussian Process regression in
966 AMiGA. The functional difference and its credible interval were also calculated between the
967 substrate and no carbon control for the carbon assay, or to the all nitrogen control for the
968 nitrogen assays.

969

970 ***VRE and CRE co-culture assays:***

971

972 VRE and CRE strains were co-cultured in a basal minimal medium supplemented with a
973 mixture of nutrients that are enriched in antibiotic-treated faecal microbiomes. The
974 composition of the basal minimal medium and the steps to prepare it are shown in
975 Supplementary Data 1. The minimal medium was supplemented with a mixture of nutrients
976 that were enriched in antibiotic-treated faecal microbiomes at a final concentration of 0.015%
977 (w/v): L-alanine, L-arginine, L-aspartate, L-glutamate, L-glycine, L-isoleucine, L-leucine, L-
978 lysine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-tyrosine, L-
979 valine, L-arabinose, D-fructose, L-fucose, D-galactose, D-glucose, D-mannose, D-ribose, D-
980 xylose, N-acetylglucosamine, D-maltose, sucrose, D-trehalose, uracil and succinate.

981

982 The following co-cultures were tested and compared to their growth as monocultures: *E.*
983 *faecium* + *E. faecalis*, *E. faecium* + *K. pneumoniae*, *E. faecium* + *E. coli*, *E. faecium* + *E.*
984 *hormaechei*, *E. faecalis* + *K. pneumoniae*, *E. faecalis* + *E. coli*, *E. faecalis* + *E. hormaechei*.

985 Each strain was inoculated into the minimal medium at a concentration of $10^3$ CFU/ml and the
986 cultures were incubated for 24 hours under anaerobic conditions. Pathogen growth was
987 quantified by plating samples on Brilliance VRE plates to quantify *E. faecium* or *E. faecalis*
988 growth and on Brilliance CRE to quantify *K. pneumoniae*, *E. coli*, or *E. hormaechei* growth.
989 Growth of each isolate was measured with 6 replicates from two independent experiments.
990
991 ***Statistical analysis:***
992
993 GraphPad Prism 10.1.0 (La Jolla, California) or R (v4.2.3) were used to perform the statistical
994 tests. P values or q values less than 0.05 were considered to be significant. For the *ex vivo*
995 human faecal culture experiment, the 16S rRNA gene sequencing data (log transformed
996 absolute abundances) and the $^1$H-NMR spectroscopy data (peak integrations) were analysed
997 using the Wilcoxon signed rank test (two-sided) with Benjamini Hochberg false discovery rate
998 (FDR) correction using the DA.wil function within in the DAtest R package version 2.7.18. For
999 the log transformed *E. faecium* and *E. faecalis* plate counts, antibiotic-treated faecal culture
1000 counts were compared to water-treated faecal culture counts using a mixed effects model with
1001 Dunn's multiple comparison, and no faeces counts were compared to water-treated faecal
1002 culture counts using an unpaired t-test (two-sided). For the experiment measuring *E. faecium*
1003 and *E. faecalis* growth in concentrations of antibiotics measured in human faeces, antibiotic-
1004 or water-treated samples were compared to the inoculum using a one-way ANOVA with
1005 Dunn's multiple comparison test. For the mouse experiment, the $^1$H-NMR spectroscopy data
1006 (peak integrations) were analysed using an unpaired t-test (two-sided) with Benjamini
1007 Hochberg FDR using the t_test function within the rstatix R package version 0.7.2. The log
1008 transformed *E. faecium* plate counts were compared using Mann–Whitney U test (two-sided).
1009 For the mixed metabolite inhibition assay, a Kruskal-Wallis with Dunn's multiple comparison
1010 test compared the no metabolite control to each concentration of the metabolite mixture at the
1011 same pH. For the individual metabolite inhibition assay, a Kruskal-Wallis test with Dunn's
1012 multiple comparison test compared the no metabolite control to each concentration of the

1013 individual metabolite. $IC_{50}$ values were calculated for each individual metabolite in Graphpad
1014 Prism. The mixed nutrient growth curves, carbon assay growth curves, and nitrogen assay
1015 growth curves were analysed using the AMiGA software in Python (v3.6.5) as described
1016 above. For the supernatant growth tests, comparisons between growth in the spent
1017 supernatants and fresh medium used a Kruskal-Wallis with Dunn's multiple comparison test,
1018 while comparison of unsupplemented vs glucose-supplemented supernatants used a
1019 Wilcoxon signed rank test. For the nutrient preference experiment comparisons were made
1020 between the *E. faecium* or *E. faecalis* cultures and the sterile media controls over time using
1021 a two-way mixed ANOVA followed by pairwise comparisons with Bonferroni correction. For
1022 the *E. faecium* and *E. faecalis* co-culture experiment, log transformed monoculture plate
1023 counts were compared to log transformed co-culture plate counts using an unpaired t-test
1024 (two-sided). For the VRE strain and CRE strain co-culture experiment, log transformed
1025 monoculture plate counts were compared to log transformed co-culture plate counts using an
1026 ANOVA with Dunn's multiple comparison test.
1027
1028 **DATA AVAILABILITY**
1029
1030 We will provide source data with the final published version of this paper. The 16S rRNA gene
1031 sequencing, 16S rRNA gene qPCR, and $^{1}$H-NMR spectroscopy datasets will be deposited into
1032 the Figshare repository upon publication. The 16S rRNA gene sequencing raw reads will also
1033 be deposited into the ENA upon publication. The SILVA bacterial database version 138.1 can
1034 be found at https://www.arb-silva.de/. The compound database for the Chenomx NMR Suite
1035 v9.02 can be found at https://www.chenomx.com/.
1036

1037 **CODE AVAILABILITY**

1038

1039 MATLAB r2019b was used to import and process $^{1}$H-NMR spectra using a custom script which
1040 will be available in the Figshare repository upon publication, using code found in the Zenodo
1041 repository at https://doi.org/10.5281/zenodo.3077413.

1042

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1193

1194 **AUTHOR CONTRIBUTIONS**

1195

1196 OGK—formal analysis: equal; investigation: lead; methodology: equal; visualisation: equal;
1197 writing – review & editing: supporting. AYGY— formal analysis: supporting; investigation:
1198 supporting; writing – review & editing: supporting. VH—formal analysis: supporting;
1199 investigation: supporting; writing – review & editing: supporting. JMB—investigation:
1200 supporting. JRM—resources: supporting; writing – review & editing: supporting. BHM—
1201 resources: supporting; writing – review & editing: supporting. TBC—investigation: supporting;

1202 methodology: supporting; writing – review & editing: supporting. JAKM—conceptualisation:
1203 lead; data curation: lead; formal analysis: equal; funding acquisition: lead; methodology: equal;
1204 project administration: lead; resources: lead; supervision: lead; visualisation: equal; writing –
1205 original draft: lead; writing – review & editing: lead.
1206

1207 **COMPETING INTERESTS**

1208 JAKM, AYGY and OGK filed a patent application (patent application number 2217266.2)
1209 related to this work. BHM received consultancy fees from Finch Therapeutics Group and
1210 Ferring Pharmaceuticals outside the submitted work. JRM received consultancy fees from
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1212 other authors declare no competing interests.
1213

1214 **FIGURE LEGENDS**

1215

1216 **Figure 1: Antibiotic treatment significantly reduced gut commensals, increased**
1217 **nutrients, decreased microbial metabolites, and increased VRE growth. (a)** Heatmap
1218 illustrating the log2 fold change (antibiotic-treated relative to antibiotic-naïve) in bacterial taxa
1219 that were significantly decreased (shown in blue) or increased (shown in red) in faecal cultures
1220 following treatment with antibiotics that promote VRE intestinal colonisation. Taxa that were
1221 not significantly changed with antibiotic treatment were not plotted (shown in white). n = 12
1222 healthy human faecal donors, Wilcoxon signed rank test (two-sided) of log-transformed
1223 abundances with Benjamini Hochberg false discovery rate (FDR) correction, $p < 0.05$. **(b)**
1224 Heatmap illustrating the log2 fold change (antibiotic-treated relative to antibiotic-naïve) in
1225 nutrients and metabolites that were significantly decreased (shown in blue) or increased
1226 (shown in red) in faecal cultures following treatment with antibiotics that promote VRE
1227 intestinal colonisation. Nutrients and metabolites that were not significantly changed with
1228 antibiotic treatment were not plotted (shown in white). n = 12 healthy human faecal donors,
1229 Wilcoxon signed rank test (two-sided) with Benjamini Hochberg FDR correction, $p < 0.05$. **(c-**

1230 **d)** Antibiotic treatment significantly promoted the growth of vancomycin-resistant *E. faecium*
1231 and *E. faecalis* in faecal microbiomes. *E. faecium* or *E. faecalis* were spiked into faecal
1232 cultures at $10^3$ CFU/ml (horizontal dashed line). n = 5-8 healthy human faecal donors, with
1233 data shown as mean ± SD. Antibiotic-treated faecal culture counts were compared to water-
1234 treated faecal culture counts using a mixed effects model of log transformed CFU/ml with
1235 Dunn's multiple comparison. No faeces counts were compared to water-treated faecal culture
1236 counts using an unpaired t-test (two-sided) of log transformed CFU/ml. ** = P ≤ 0.01,
1237 *** = P ≤ 0.001, **** = P ≤ 0.0001. MTZ = metronidazole, CLI = clindamycin, VAN =
1238 vancomycin, CRO = ceftriaxone, TZP = piperacillin/tazobactam.
1239
1240 **<u>Figure 2:</u> Nutrients were increased and metabolites were decreased in the faeces of**
1241 **vancomycin-treated mice. (a)** Nutrients that were increased in the faeces of VAN-treated
1242 mice compared to $H_2O$-treated mice. **(b)** Metabolites that were decreased in the faeces of
1243 VAN-treated mice compared to $H_2O$-treated mice. Comparison of nutrients and metabolites in
1244 the VAN-treated faeces to the $H_2O$-treated faeces used an unpaired t-test (two-sided) with
1245 Benjamini Hochberg FDR. **(c)** Vancomycin-resistant *E. faecium* counts were high in the faeces
1246 from vancomycin-treated mice and nearly undetectable in the faeces of water-treated mice.
1247 Comparisons of the *E. faecium* plate counts were made using a Mann–Whitney U test (two-
1248 sided) of log10-transformed counts. n = 5 mice per group from one independent experiment,
1249 with data shown as mean ± SD. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001. VAN = vancomycin,
1250 $H_2O$ = water, LOD = limit of detection.
1251
1252 **<u>Figure 3:</u> VRE growth was inhibited at pH 6.0 by a mixture of microbial metabolites at**
1253 **high concentrations.** Vancomycin-resistant *E. faecium* and *E. faecalis* were grown in tryptic
1254 soy broth supplemented with a metabolite mixture mimicking the low, average, or high
1255 concentrations measured in human faeces, or unsupplemented ("No metabolite" control).
1256 Cultures were incubated at pH 6.0, 6.5, and 7.0 to mimic the pH of the healthy large intestine.
1257 Data shown as mean ± SD, with 24-36 replicates from 3 independent experiments. Kruskal-

1258 Wallis with Dunn's multiple comparison test comparing the No metabolite control to each
1259 concentration of the metabolite mixture at the same pH. * $P \leq 0.05$, ** = $P \leq 0.01$,
1260 *** = $P \leq 0.001$, **** = $P \leq 0.0001$. $OD_{600}$ = optical density at 600 nm.
1261
1262 **<u>Figure 4:</u> Some individual microbial metabolites moderately inhibited VRE growth.** (**a**)
1263 Vancomycin-resistant *E. faecium* and (**b**) vancomycin-resistant *E. faecalis* were grown in
1264 tryptic soy broth (pH 6) supplemented with an individual metabolite at low, average or high
1265 concentrations measured in healthy human faeces, concentrations spanning a defined range
1266 (0.016-512 mM), or unsupplemented (no metabolite control). Growth of each isolate was
1267 measured with 14 replicates in two independent experiments. Kruskal-Wallis test with Dunn's
1268 multiple comparison test (each metabolite was compared to the no metabolite control).
1269 * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$. Data are presented as mean
1270 values ± SD. Optical density at 600 nm, $OD_{600}$.
1271
1272 **<u>Figure 5:</u> VRE used nutrients enriched in antibiotic-treated faecal microbiome as sole**
1273 **carbon and nitrogen sources.** AMiGA-predicted growth curves for vancomycin-resistant *E.*
1274 *faecium* and *E. faecalis* cultured in minimal medium supplemented with a mixture of nutrients
1275 under anaerobic or aerobic conditions. The bold lines show the predicted mean of growth while
1276 the shaded bands show the predicted 95% credible intervals. N = 24 replicates in 3
1277 independent experiments for each strain under each condition, OD = optical density.
1278
1279 **<u>Figure 6:</u> Faecal culture supernatants suppressed VRE growth through nutrient**
1280 **depletion.** Minimal medium was supplemented with a mixture of nutrients that were increased
1281 with antibiotics and inoculated with either fresh healthy human faeces (n = 3 donors for 9
1282 replicates from 3 independent experiments), a single VRE strain (*E. faecium* in panel **a** or *E.*
1283 *faecalis* in panel **b**), or left uninoculated (n = 9 replicates from 3 independent experiments).
1284 Spent supernatants were filter sterilised and then inoculated with a single VRE strain. As a
1285 control, spent supernatants were also supplemented with 0.5% glucose to confirm nutrient

1286 depletion. VRE growth was measured after overnight incubation using plate counts.
1287 Comparisons between growth in the spent supernatants and fresh medium were assessed
1288 using a Kruskal-Wallis with Dunn's multiple comparison test. Comparison of unsupplemented
1289 vs glucose-supplemented supernatants was assessed through a Wilcoxon signed rank test.
1290 ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001. Data are presented as mean values ± SD.
1291
1292 **Figure 7: VRE growth was supported by individual monosaccharides and disaccharides**
1293 **as carbon sources.** AMiGA-predicted growth curves for vancomycin-resistant *E. faecium* and
1294 *E. faecalis* cultured in minimal medium supplemented with single carbon sources under
1295 anaerobic or aerobic conditions. The bold lines show the predicted mean of growth while the
1296 shaded bands show the predicted 95% credible intervals. n = 6 replicates in 2–3 independent
1297 experiments. OD = optical density.
1298
1299 **Figure 8: Amino acids served as nitrogen sources to support VRE growth.** AMiGA-
1300 predicted growth curves for vancomycin-resistant *E. faecium* and *E. faecalis* cultured in
1301 minimal medium supplemented with a mixture of all nitrogen sources ("all nitrogen sources"),
1302 a leave-one-out mixture lacking a single nitrogen source, or lacking all nitrogen sources ("no
1303 nitrogen sources") under anaerobic or aerobic conditions. The bold lines show the predicted
1304 mean of growth while the shaded bands show the predicted 95% credible intervals. n = 6
1305 replicates in 2 independent experiments. OD = optical density.
1306
1307 **Figure 9: VRE preferred specific nutrients when grown in nutrient mixtures found in**
1308 **antibiotic-treated faecal microbiomes.** Percentage of nutrients remaining in vancomycin-
1309 resistant *E. faecium* and *E. faecalis* cultures grown in a minimal medium supplemented with a
1310 mixture of nutrients, under aerobic or anaerobic conditions. $^{1}$H-NMR spectroscopy peak
1311 integrations were used to measure nutrient concentrations. Comparisons were made using a
1312 two-way mixed ANOVA followed by pairwise comparisons with Bonferroni correction. n = 3

1313 replicates per group, with data shown as mean ± SD. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001,
1314 **** = P ≤ 0.0001.
1315
1316 **<u>Figure 10:</u> VRE and CRE achieve high growth in co-culture, but VRE growth is slightly**
1317 **inhibited. (a)** *E. faecium* and *E. faecalis* were co-cultured in a minimal medium supplemented
1318 with a mixture of nutrients that are enriched in antibiotic-treated faecal microbiomes. **(b)** *E.*
1319 *faecium* or *E. faecalis* were co-cultured with *K. pneumoniae*, *E. coli*, or *E. hormaechei* in a
1320 minimal medium supplemented with a mixture of nutrients that are enriched in antibiotic-
1321 treated faecal microbiomes. Each strain was inoculated into the minimal media at a
1322 concentration of $10^3$ CFU/ml (represented as the grey dashed line) and the cultures were
1323 incubated for 24 hours under anaerobic conditions. Samples were plated on Brilliance VRE
1324 plates to quantify *E. faecium* or *E. faecalis* growth and on Brilliance CRE to quantify *K.*
1325 *pneumoniae*, *E. coli*, or *E. hormaechei* growth. Growth of each isolate was measured with 6
1326 replicates in two independent experiments. Comparison of *E. faecium* and *E. faecalis*
1327 monoculture growth to co-culture growth was made using an unpaired t-test (two-sided).
1328 Comparison of VRE strain and CRE strain monoculture growth to co-culture growth was made
1329 using ANOVA with Dunn's multiple comparison test. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001,
1330 **** = P ≤ 0.0001. Data are presented as mean values ± SD.