Vancomycin-resistant enterococci colonise the antibiotic-treated intestine by occupying overlapping but distinct nutrient- and metabolite-defined intestinal niches

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ABSTRACT

Antibiotic treatment significantly disrupts the gut microbiome and promotes vancomycin-resistant enterococci (VRE) intestinal colonisation. These disruptions cause the intestine to act 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 found significant but incomplete suppression of VRE growth by individual short chain fatty acids that were decreased in antibiotic-treated faecal microbiomes. However, we showed that mixtures of short chain fatty acids provided complete or near complete suppression of VRE growth. We also showed that VRE could use most nutrients increased in antibiotic-treated faecal microbiomes as carbon or nitrogen sources to support their growth. *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 inhibitory microbial metabolites.

41 **INTRODUCTION**

42

43 Vancomycin-resistant enterococci (VRE) are multidrug-resistant pathogens and a major
44 cause of nosocomial infections worldwide[1,2]. These hospital-acquired pathogens pose a
45 major therapeutic challenge as they have intrinsic resistance to several commonly prescribed
46 antibiotics and have also acquired resistance to many available antibiotics[3].

47

48 *E. faecium* and *E. faecalis* are responsible for 75% of enterococcal infections and are
49 commonly associated with hospital outbreaks of invasive infections such as bloodstream
50 infections, urinary tract infections, and endocarditis[2,4,5]. *E. faecalis* was more virulent and more
51 prevalent in healthcare-associated infections than *E. faecium*, however, the prevalence of *E.*
52 *faecium* is increasing due to the rise of vancomycin-resistant and lactam-resistant *E. faecium*
53 strains[6]. *E. faecium* has higher levels of intrinsic and acquired resistance than *E. faecalis*,
54 where 80% of *E. faecium* isolates are vancomycin-resistant compared to 10% of *E. faecalis*
55 isolates[4]. Vancomycin-resistant *E. faecium* has gained attention as the World Health
56 Organisation classified it as a high priority pathogen due to its unfavourable rankings in several
57 criteria, including mortality, trends of resistance, transmissibility, preventability, treatability,
58 and pipelines for new medicines and diagnostics[7].

59

60 The intestine is a major reservoir for VRE that seed difficult-to-treat invasive infections[7,8]. VRE
61 can be acquired from other patients, hospital rooms, or healthcare workers in acute and long-
62 term healthcare facilities[9-11]. Patients exposed to broad-spectrum antibiotics (that significantly
63 disrupt the gut microbiome) are highly susceptible to intestinal colonisation and domination by
64 VRE[12-16]. Conversely, healthy individuals have intact colonisation resistance, where their gut
65 microbiomes protect them from VRE intestinal colonisation. To develop targeted and effective
66 therapies to restrict VRE intestinal colonisation we must first understand what drives VRE
67 colonisation in an antibiotic-disrupted gut microbiome.

68

69 Currently, we have an incomplete understanding of the mechanisms of colonisation resistance
70 that protect the host against VRE intestinal colonisation. However, a few studies have provided
71 some insights. Previous work demonstrated that antibiotic treatment downregulated the
72 expression of RegIIIγ, a C-type lectin that kills VRE in the small intestine[17]. Another study
73 demonstrated that a gut commensal strain of *Blautia producta* can produce a lantibiotic that
74 can reduce VRE growth[18]. Finally, another study demonstrated that lactulose treatment in
75 patients with severe liver disease increased *Bifidobacterium* growth, increased the production
76 of microbial metabolites, acidified the gut lumen, and reduced the incidence of systemic
77 infections and mortality[19]. However, we lack information on how broad-spectrum antibiotics
78 that promote VRE intestinal colonisation impact nutrient competition and inhibitory metabolite
79 production by the gut microbiome, and how this in turn impacts VRE growth.
80
81 Pathogens must have access to nutrients that they can utilise to support their growth and
82 colonise the intestine. In a healthy gut microbiome, commensal microbes compete intensely
83 for nutrients, resulting in nutrient depletion[20]. However, antibiotic-induced killing of gut
84 commensals reduces nutrient competition, making the nutrients available to support pathogen
85 growth. We have a very poor understanding of VRE nutrient utilisation in the antibiotic-treated
86 gut. A previous study demonstrated that vancomycin-treated mice had increased fructose in
87 their caecal contents compared to antibiotic-naïve mice, and vancomycin-resistant *E. faecium*
88 could use fructose as a carbon source[15]. However, this study used a targeted metabolomics
89 approach that likely missed changes in other nutrients that were increased with vancomycin
90 treatment. Moreover, this study only tested one antibiotic, whereas there are multiple broad-
91 spectrum antibiotics used clinically that can promote VRE intestinal colonisation[12-16]. It is
92 important to understand how other broad-spectrum antibiotics that promote VRE intestinal
93 colonisation affect the availability of nutrients that support VRE growth.
94
95 Microbiome therapeutics designed to restrict VRE growth based on nutrient utilisation profiles
96 will not be effective unless they deplete all available carbon and nitrogen sources that support

97 VRE growth. Otherwise, VRE can switch to using other available nutrients to grow. We
98 previously demonstrated that broad-spectrum antibiotics that promote the intestinal
99 colonisation of carbapenem-resistant *Enterobacteriaceae* (CRE) caused an increase in the
100 concentration of a wide range of nutrients[21]. As our previous study demonstrated that broad-
101 spectrum antibiotics had a severe impact on the gut microbiota, in this study we hypothesised
102 that broad-spectrum antibiotics that promote VRE intestinal colonisation would also increase
103 the concentration of a wide range of nutrients in the intestine, increasing the availability of
104 many nutrients that could support VRE growth.
105
106 It is also not clear how VRE occupy intestinal niches in antibiotic-treated gut microbiomes. A
107 previous study by Caballero and colleagues proposed that vancomycin-resistant *E. faecium*
108 and carbapenem-resistant *Klebsiella pneumoniae* occupied distinct but overlapping intestinal
109 niches, as they showed that growth of each strain was not impaired when they colonised the
110 mouse intestine simultaneously[22]. Shared bacterial niches have also been observed in
111 humans, as patients can be co-colonised with both CRE and VRE simultaneously[23]. However,
112 the mechanism of how VRE and CRE occupied the antibiotic-treated intestine was not shown.
113 Therefore, we hypothesised that VRE and CRE occupy distinct but overlapping nutrient-
114 defined intestinal niches, where VRE share the utilisation of some nutrients with CRE, but also
115 has its own distinct nutrient utilisation abilities.
116
117 In the healthy gut microbiome, nutrient utilisation results in the production of microbial
118 metabolites, some of which can inhibit pathogen growth[20]. Therefore, VRE must be able to
119 grow in the presence of microbial metabolites to successfully colonise the intestine. We
120 previously demonstrated that broad-spectrum antibiotics that promote CRE intestinal
121 colonisation decreased the concentration of a wide range of microbial metabolites in the gut
122 microbiome, and several of these metabolites highly inhibited CRE growth[21]. Another study
123 from our group demonstrated that antibiotic treatment promoted *Clostridioides difficile* growth
124 and decreased the concentration of valerate in the gut microbiome, and that valerate inhibited

125 *C. difficile* growth[24]. However, we do not understand how microbial metabolites may impact
126 VRE growth in the intestine. Therefore, we also wanted to determine whether microbial
127 metabolites that are found in healthy gut microbiomes and decreased with antibiotic treatment
128 could suppress VRE growth.
129
130 VRE are high-priority pathogens that are included in the "ESKAPE" group of pathogens, and
131 urgently require new treatments, including new therapies to prevent VRE intestinal
132 colonisation[25]. These therapies would remove a reservoir of VRE, thereby significantly
133 reducing the subsequent development of VRE invasive infections and patient-to-patient VRE
134 transmission[26]. Therefore, this study aimed to measure the nutrient and metabolite
135 landscapes encountered by VRE in a gut microbiome treated with multiple different antibiotics
136 that are known to promote VRE intestinal colonisation. Moreover, we wanted to define the
137 niche that VRE occupied in an antibiotic-treated gut microbiome, how distinct these niches
138 were between *E. faecium* and *E. faecalis*, and how different these niches were compared to
139 niches occupied by other multidrug-resistant pathogens. The results from this study will inform
140 the rational design of a new microbiome therapeutic that can occupy the same niche as VRE
141 to displace them from the intestine.
142
143 **RESULTS**
144
145 ***Antibiotics altered the nutrient- and metabolite-defined intestinal niches in a manner***
146 ***that supported VRE growth***
147
148 We performed *ex vivo* faecal culture experiments to determine how antibiotics that promote
149 VRE intestinal colonisation killed gut commensals, altered nutrient availability, and altered the
150 presence of microbial metabolites. In these experiments, faeces from healthy human donors
151 were cultured in a gut growth medium that was supplemented with water or an antibiotic known
152 to promote VRE intestinal colonisation: metronidazole (MTZ), clindamycin (CLI), vancomycin

153 (VAN), ceftriaxone (CRO), or piperacillin/tazobactam (TZP), at concentrations mimicking
154 those found in human faeces[27-32]. Samples were analysed by 16S rRNA gene sequencing to
155 measure changes in bacterial taxa and by ^{1}H-NMR spectroscopy to measure changes in
156 nutrients and metabolites.
157
158 Antibiotic-treated faecal culture samples were compared to water-treated faecal culture
159 samples. Principal component analysis (PCA) plots showed separation between antibiotic-
160 treated and water-treated faecal culture samples along the first principal component, which
161 corresponded to antibiotic treatment (**Fig. S1**). Faecal cultures treated with antibiotics that
162 promote VRE intestinal colonisation had significant decreases in many bacterial families, as
163 shown in **Fig. 1a**. *Ruminococcaceae* and *Bifidobacteriaceae* were decreased in all the
164 antibiotic-treated groups, while decreases in other bacterial families varied depending on the
165 antibiotic tested. We found that there was some heterogeneity in the responses of specific
166 bacterial families to antibiotic treatment in faecal cultures inoculated with faeces from different
167 donors (**Figs. S2 and S3**). Antibiotic-treated faecal cultures also had significant decreases in
168 taxa at the phylum, class, and order levels (**Fig. S4**).
169

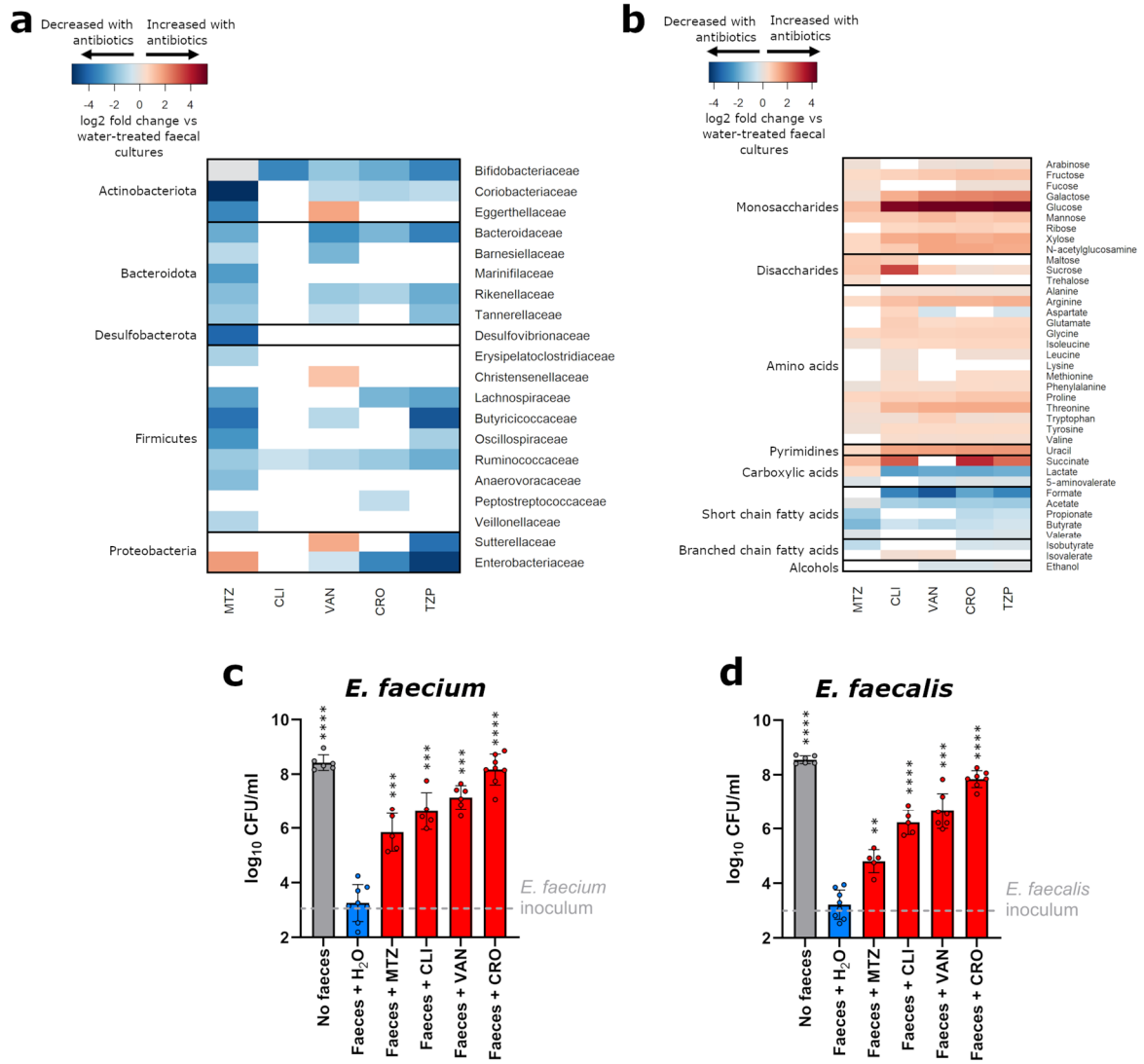


Figure 1: Antibiotic treatment significantly reduced gut commensals, increased nutrients, decreased microbial metabolites, and increased VRE growth. (a) Heatmap illustrating the log2 fold change (antibiotic-treated relative to water-treated) in bacterial taxa that were significantly decreased (shown 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 water-treated) 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* (NCTC 12202) and *E. faecalis* (NCTC 12201) 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 Dunnett'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.

171 Antibiotic treatment also significantly altered the abundances of nutrients and metabolites that
172 would be encountered by VRE in an antibiotic treated faecal microbiome. Antibiotic treatment
173 resulted in a significant increase in a wide range of nutrients, including monosaccharides,
174 disaccharides, amino acids, uracil, and succinate (**Fig. 1b**). Monosaccharides increased with
175 antibiotics included arabinose, fructose, fucose, galactose, glucose, mannose, ribose, xylose,
176 and N-acetylglucosamine. Disaccharides increased with antibiotics included maltose, sucrose,
177 and trehalose. Amino acids increased with antibiotics included alanine, arginine, aspartate,
178 glutamate, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine,
179 tryptophan, tyrosine, and valine. Antibiotic treatment also resulted in a decrease in a wide
180 range of microbial metabolites, including short chain fatty acids (SCFAs; formate, acetate,
181 propionate, butyrate, and valerate), carboxylic acids (lactate and 5-aminovalerate),
182 isobutyrate, and ethanol (**Fig. 1b**). In contrast, isovalerate increased in CLI-treated and VAN-
183 treated faecal cultures. We found that there was less heterogeneity in the responses of
184 nutrients and metabolites to antibiotic treatment in faecal cultures inoculated with faeces from
185 different donors (**Fig. S5**).
186
187 We performed another set of faecal culture experiments to confirm whether vancomycin-
188 resistant *E. faecium* and *E. faecalis* were able to grow in these antibiotic-treated faecal
189 microbiomes that had increased nutrient concentrations and decreased metabolite
190 concentrations. First, MIC assays confirmed that the *E. faecium* and *E. faecalis* strains were
191 resistant to all the antibiotics tested (**Table S1**). *E. faecium* and *E. faecalis* were also grown in
192 pure cultures in the presence of the antibiotics at concentrations found in human faeces. Both
193 *E. faecium* and *E. faecalis* were able to grow axenically in faecal concentrations of MTZ, CRO,
194 CLI, and VAN, however, these strains were killed with faecal concentrations of TZP (**Fig. S6**).
195 Therefore, TZP was not tested further in the faecal culture experiments.
196
197 Faecal culture experiments were performed by spiking vancomycin-resistant *E. faecium* or *E.*
198 *faecalis* into antibiotic-treated faecal cultures as described above. We demonstrated that MTZ,

199 CLI, VAN, and CRO-treated faecal cultures promoted the growth of *E. faecium* and *E. faecalis*,
200 while water-treated faecal cultures restricted *E. faecium* and *E. faecalis* growth (**Figs. 1c and**
201 **1d**). We also found that there was a significant difference in VRE growth in some of the
202 antibiotic-treated faecal cultures compared to others (**Table S2**).
203
204 In summary, we demonstrated that antibiotic treatment killed gut commensals and promoted
205 VRE growth. This reduction in gut commensal abundance resulted in an increase in the
206 concentration of nutrients (including monosaccharides, disaccharides, amino acids, uracil, and
207 succinate) and a decrease in the concentration of microbial metabolites (including SCFAs,
208 lactate, 5-aminovalerate, isobutyrate, and ethanol).
209
210 ***Vancomycin treatment caused an increase in nutrients and a decrease in metabolites***
211 ***in mouse faeces and promoted vancomycin-resistant* E. faecium *growth***
212
213 We used a mouse model to demonstrate that antibiotic treatment altered nutrient availability
214 and metabolite production *in vivo*. Mice were treated with oral vancomycin as it has been
215 previously demonstrated to promote VRE intestinal colonisation in both humans and
216 mice[12,13,15,16]. Mice were colonised with *E. faecium* to confirm that vancomycin disrupts
217 colonisation resistance and creates an intestinal environment that promoted *E. faecium*
218 growth.
219
220 We measured changes in nutrient availability and metabolite production in the faeces of
221 vancomycin-treated mice compared to water-treated mice using ^{1}H-NMR spectroscopy.
222 Faeces from vancomycin-treated mice had an increase in the concentration of several
223 nutrients compared to water-treated mice, including arabinose, glucose, trehalose, arginine,
224 aspartate, glycine, phenylalanine, and tyrosine (**Fig. 2a**). Moreover, faeces from vancomycin-
225 treated mice had a decrease in the concentration of several metabolites compared to water-
226 treated mice, including propionate, butyrate, isobutyrate, lactate, and ethanol (**Fig. 2b**).

227 Fucose and alanine concentrations were decreased in the faeces of vancomycin-treated mice

228 compared to water-treated mice (**Fig. S7**).

229

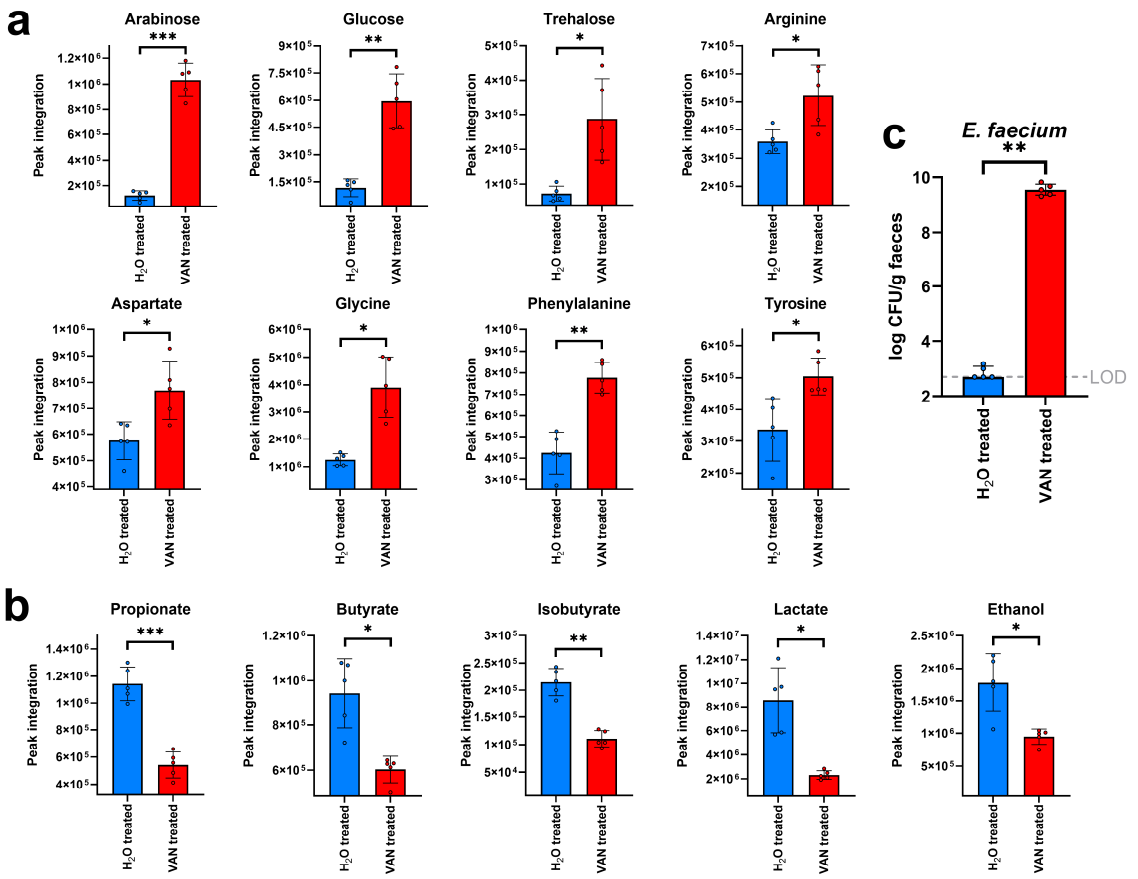


Figure 2: Nutrients were increased and metabolites were decreased in the faeces of vancomycin-treated mice. (a) Nutrients that were increased in the faeces of VAN-treated mice compared to H_2O-treated mice. **(b)** Metabolites that were decreased in the faeces of VAN-treated mice compared to H_2O-treated 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, data shown as mean ± SD. **(c)** Vancomycin-resistant *E. faecium* (NCTC 12202) 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 medians ± IQR. * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$. VAN = vancomycin, H_2O = water, LOD = limit of detection.

230

231 Vancomycin-treated and water-treated mice were gavaged with vancomycin-resistant *E.*

232 *faecium*, and *E. faecium* growth was quantified in mouse faecal samples (**Fig. 2c**). Mice

233 treated with vancomycin had high *E. faecium* counts in their faeces, while mice treated with

234 water had low *E. faecium* counts which were at or below the limit of detection.

235

236 In summary, the results from our mouse experiment were supportive of the results we
237 observed in our *ex vivo* human faecal culture experiments, where we showed that VAN-treated
238 faeces were enriched in nutrients (including sugars and amino acids) and depleted in microbial
239 metabolites (including SCFAs, isobutyrate, lactate, and ethanol). We also confirmed that
240 vancomycin-treated mice had disrupted colonisation resistance that promoted high levels of
241 vancomycin-resistant *E. faecium* intestinal colonisation.
242
243 ***Short chain fatty acids that were decreased in antibiotic-treated human faecal***
244 ***microbiomes significantly suppressed VRE growth***
245
246 We demonstrated that antibiotics that promote VRE intestinal colonisation reduced the
247 concentration of microbial metabolites in faecal microbiomes (**Figs. 1-2**). Previous work
248 demonstrated that microbial metabolites can inhibit the growth of pathogens in the intestine,
249 including CRE and *C. difficile*[21,24,33]. Therefore, we were interested in whether microbial
250 metabolites (that decreased in concentration with antibiotics) could also suppress VRE growth
251 at physiologically relevant concentrations. Previous studies also demonstrated that antibiotic
252 treatment increased the pH in the intestine[33,34]. Moreover, previous studies demonstrated that
253 short chain fatty acids were more inhibitory towards *Enterobacteriaceae* growth at more acidic
254 physiological pH values[21,33]. Therefore, we measured the effects of microbial metabolites at
255 physiological pH values found in the healthy large intestine (pH 6-7)[35,36].
256
257 First, we tested whether a mixture of 10 metabolites (containing formate, acetate, propionate,
258 butyrate, valerate, isobutyrate, isovalerate, lactate, 5-aminovalerate, ethanol) were able to
259 suppress *E. faecium* and *E. faecalis* growth. The mixture was tested at three concentrations
260 that represented the average, low, and high concentrations of these metabolites measured in
261 healthy human faeces[21]. The 10 metabolite mixture significantly suppressed *E. faecium* and
262 *E. faecalis* growth at the high metabolite concentration at pH 6 (**Fig. S8**).
263

Next, the growth of three *E. faecium* and three *E. faecalis* strains were measured in the presence of each individual metabolite. Individual metabolites were tested at concentrations that mimicked the average, low, and high concentrations of these metabolites measured in healthy human faeces at pH 6[21]. At average concentrations the growth of two *E. faecium* strains and one *E. faecalis* strain were significantly suppressed by acetate, and the growth of one *E. faecium* strain and three *E. faecalis* strains were significantly suppressed by propionate (**Fig. 3**). At high concentrations the growth of two *E. faecium* strains and two *E. faecalis* strains were significantly suppressed by acetate, the growth of three *E. faecium* strains and three *E. faecalis* strains were significantly suppressed by propionate, the growth of three *E. faecium* strains and two *E. faecalis* strains were significantly suppressed by butyrate, and the growth of three *E. faecium* strains and one *E. faecalis* strain were significantly suppressed by valerate (**Fig. 3**). Formate, isobutyrate, lactate, 5-aminovalerate, and ethanol did not suppress *E. faecium* or *E. faecalis* growth at these concentrations (**Figs. S9-S14**). However, the growth of one *E. faecalis* strain was significantly suppressed by isovalerate at the high concentration (**Fig. S12**).

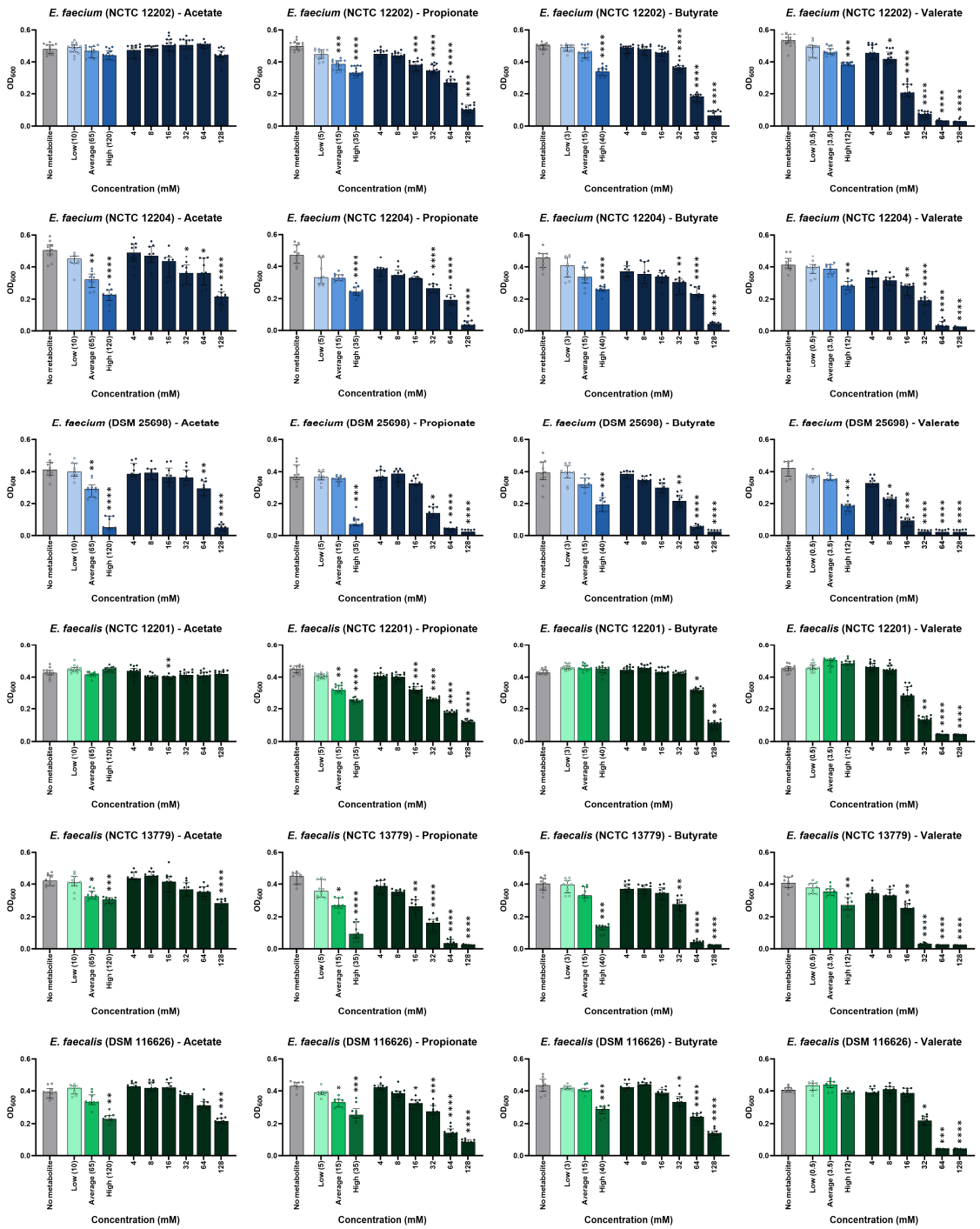


Figure 3: Individual SCFAs suppressed VRE growth at physiological concentrations. Vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* strains were grown in tryptic soy broth (pH 6) supplemented with an individual metabolite at low, average or high concentrations measured in healthy human faeces, concentrations spanning a defined range (4-128 mM), or unsupplemented (no metabolite control). Cultures were incubated under anaerobic conditions overnight. Growth of each isolate was measured with 10-14 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 medians ± IQR. Optical density at 600 nm, OD_{600}.

281 Next, we compared the effects of each individual metabolite at the same concentrations
282 ranging from 4-128 mM, which could be considered for future therapeutic drug designs. For
283 *E. faecium* strains the minimum concentration that caused a significant suppression of growth
284 was 32-64 mM for acetate (1 strain suppressed at concentrations >128 mM), 16-32 mM for
285 propionate, 32 mM for butyrate, and 8-16 mM for valerate (**Fig. 3**). For *E. faecalis* the minimum
286 concentration that caused a significant suppression of growth was 128 mM for acetate (1 strain
287 suppressed at concentrations >128 mM)**,** 16 mM for propionate, 32-64 mM for butyrate, and
288 16-32 mM for valerate (**Fig. 3**). The effects of formate, isobutyrate, isovalerate, lactate, 5-
289 aminovalerate, and ethanol on *E. faecium* and *E. faecalis* growth at 4-128 mM are shown in
290 **Figs. S9-S14.** The half maximal inhibitory concentrations (IC_{50}) that provide growth
291 suppression against *E. faecium* and *E. faecalis* are shown in **Table S3**.
292
293 We showed that acetate, propionate, butyrate, and valerate were individually capable of
294 suppressing VRE growth at high concentrations found in human faeces. Therefore, next we
295 tested whether a mixture of propionate, butyrate, and valerate ("PBV" mixture) or a mixture of
296 acetate, propionate, butyrate, and valerate ("APBV" mixture) were able to more highly
297 suppress VRE growth. At pH 6 we showed that *E. faecium* growth was fully suppressed by
298 the APBV mixture and nearly fully suppressed by the PBV mixture, while *E. faecalis* growth
299 was fully suppressed by both the APBV mixture and PBV mixture (**Fig. 4**). At pH 6.5 both the
300 APBV and PBV mixtures significantly suppressed *E. faecium* and *E. faecalis* growth (**Fig. 4**).
301 At pH 7 the APBV mixture significantly suppressed the growth of one *E. faecium* strain and
302 three *E. faecalis* strains, while the PBV mixture significantly suppressed the growth of two *E.*
303 *faecium* strains and two *E. faecalis* strains (**Fig. 4**).
304

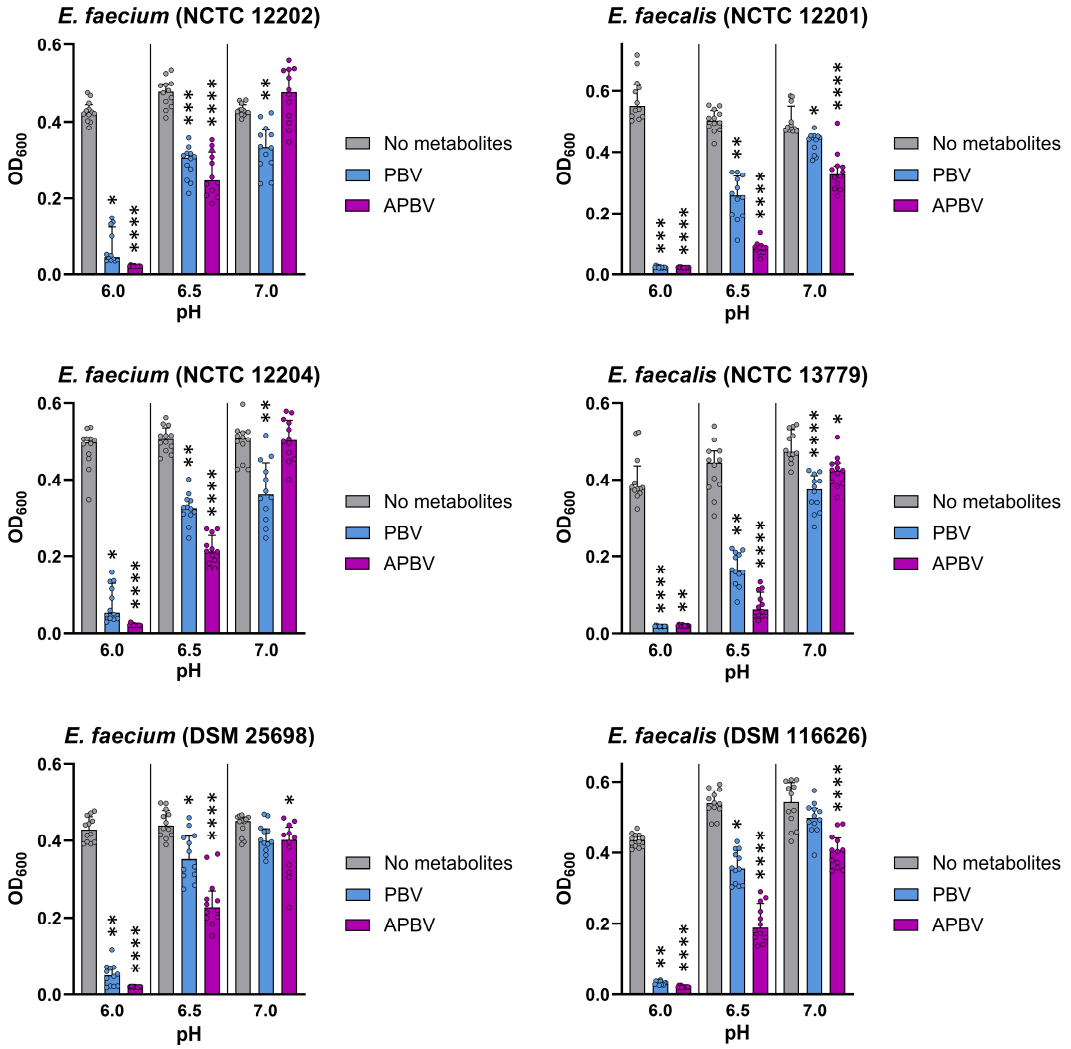
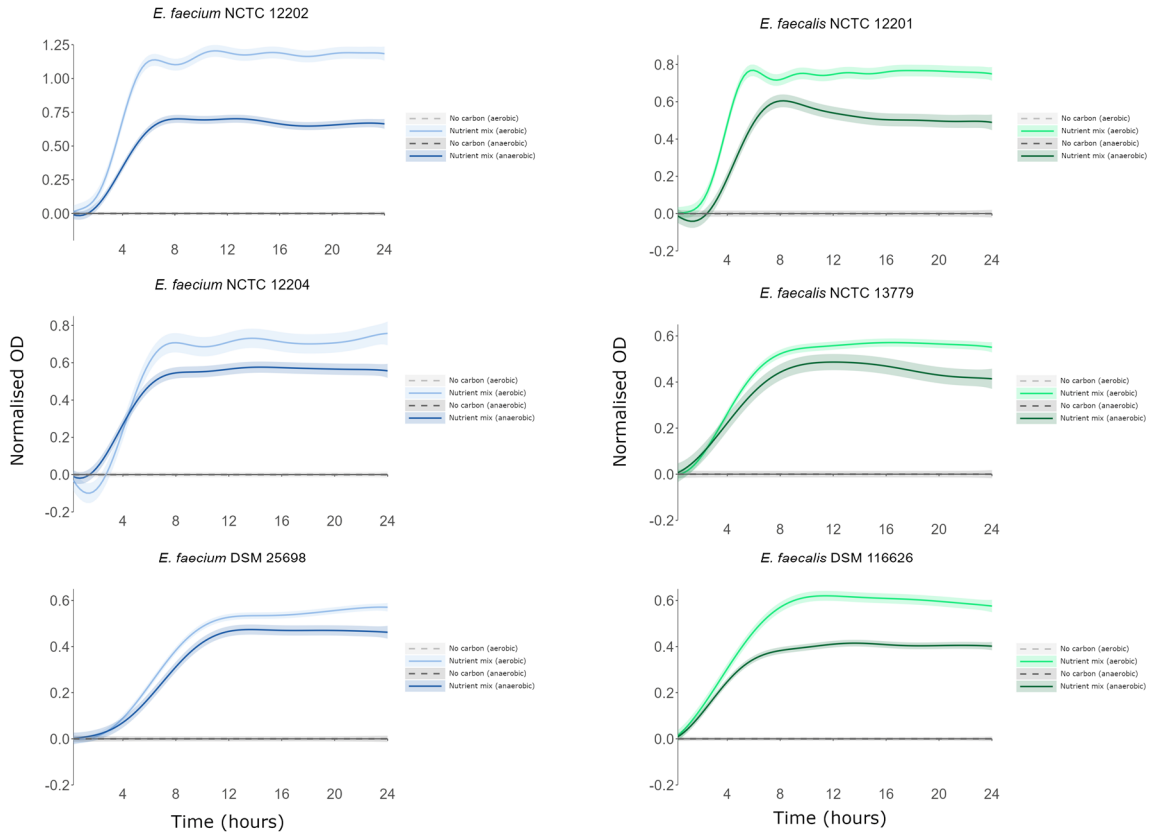


Figure 4: VRE growth was highly suppressed by mixtures of SCFAs at high concentrations. Vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* strains were grown in tryptic soy broth supplemented with a mixture of propionate, butyrate, and valerate ("PBV") or a mixture of acetate, propionate, butyrate, and valerate ("APBV") at concentrations mimicking the high concentrations measured in human faeces, or unsupplemented ("No metabolites" control). Broth was adjusted to pH 6, 6.5, or 7 to mimic the pH of the healthy large intestine, and cultures were incubated under anaerobic conditions overnight. Data shown as medians ± IQR, with 12 replicates from 3 independent experiments. Kruskal-Wallis with Dunn's multiple comparison test comparing the no metabolites control to each 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.

Overall, we observed significant but incomplete suppression of vancomycin-resistant *E. faecium* and *E. faecalis* growth by acetate, propionate, butyrate, and valerate (at high concentrations) when tested individually at pH 6. However, the APBV and PBV mixtures provided complete or near complete suppression of *E. faecium* and *E. faecalis* growth at pH 6.

312 ***Nutrients enriched in antibiotic-treated human faecal microbiomes supported VRE***
313 ***growth***
314
315 We demonstrated that antibiotics that promote VRE intestinal colonisation increased nutrient
316 availability in faecal microbiomes (**Figs. 1-2**). Therefore, next we measured whether the VRE
317 isolates were able to grow in a mixture of nutrients that were enriched with antibiotic treatment.
318 Growth was measured in the presence and absence of oxygen as previous studies
319 demonstrated that antibiotic treatment increased oxygen availability in the intestine, which
320 could impact nutrient utilisation by pathogens[21,37]. Therefore, we wanted to determine whether
321 the presence of oxygen also impacted nutrient utilisation by VRE.
322
323 Vancomycin-resistant *E. faecium* and *E. faecalis* strains were cultured in a minimal growth
324 medium supplemented with a mixture of nutrients that were increased in antibiotic-treated
325 faecal microbiomes. VRE growth was supported by this mixture of nutrients under both
326 anaerobic and aerobic conditions (**Fig. 5**). Growth was higher for *E. faecium* and *E. faecalis*
327 under aerobic conditions compared to anaerobic conditions.
328



<u>Figure 5</u>: 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 vancomycin-resistant *E. faecalis* strains 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 = 6 replicates in two independent experiments. OD = optical density.

Next, we measured whether a healthy faecal microbiota was able to deplete nutrients (that we demonstrated were increased in antibiotic-treated faecal microbiomes) to restrict VRE growth. We demonstrated that VRE growth was suppressed in spent faecal supernatants and spent VRE supernatants supplemented with water, compared to growth in a minimal medium supplemented with the nutrient mixture (**Fig. 6**). We also demonstrated that glucose supplementation of spent faecal supernatants and spent VRE supernatants caused an increase in growth that was comparable to growth achieved in the nutrient-supplemented minimal medium. These results support the hypothesis that nutrient depletion contributes to the restriction of VRE growth in a healthy gut microbiome.

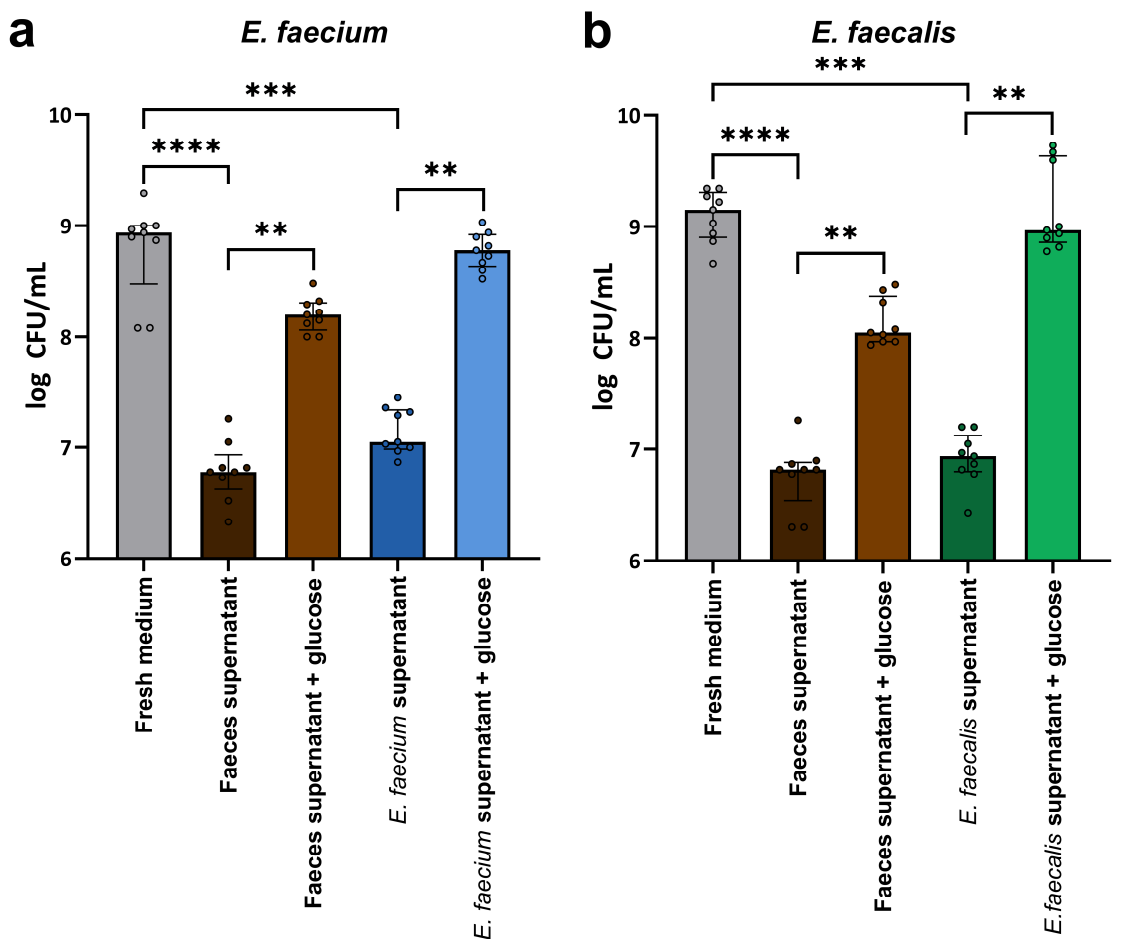


Figure 6: Faecal culture supernatants suppressed VRE growth through nutrient depletion. Minimal medium was supplemented with a mixture of nutrients that were increased with antibiotics and inoculated with either fresh healthy human faeces (n = 3 donors for 9 replicates from 3 independent experiments), a single VRE strain (*E. faecium* NCTC 12202 or *E. faecalis* NCTC 12201), 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 medians ± IQR.

340

341 ***Many nutrients enriched in antibiotic-treated faecal microbiomes acted as sole carbon***

342 ***or nitrogen sources to support VRE growth***

343

344 We measured VRE growth on individual carbon and nitrogen sources that were enriched in

345 antibiotic-treated faecal microbiomes to determine which specific nutrients were capable of

346 supporting VRE growth. This information could help inform the design of new microbiome

347 therapeutics composed of gut commensals that can outcompete the VRE strains for use of
348 these specific nutrients. We also wanted to determine whether vancomycin-resistant *E.*
349 *faecium* and *E. faecalis* used different nutrients, as this may indicate whether these strains
350 could share the same intestinal niches.
351
352 First, the growth of three vancomycin-resistant *E. faecium* strains and three vancomycin-
353 resistant *E. faecalis* strains were measured in minimal medium supplemented with each
354 individual nutrient as a sole carbon source, under both anaerobic and aerobic conditions.
355
356 Under anaerobic conditions *E. faecium* and *E. faecalis* growth was supported by
357 monosaccharides and disaccharides. High or moderate growth was achieved for three *E.*
358 *faecium* strains on arabinose, fructose, galactose, glucose, mannose, ribose, N-
359 acetylglucosamine, maltose, and trehalose, and high growth was achieved for two *E. faecium*
360 strains on sucrose (**Figs. 7, S15**). High or moderate growth was achieved for three *E. faecalis*
361 strains on fructose, glucose, mannose, ribose, N-acetylglucosamine, maltose, and trehalose,
362 and high or moderate growth was achieved for two *E. faecalis* strains on galactose and
363 sucrose (**Figs. 7, S16**). Low growth or negligible growth was achieved on the other carbon
364 sources tested (**Figs. S15- S18**).
365

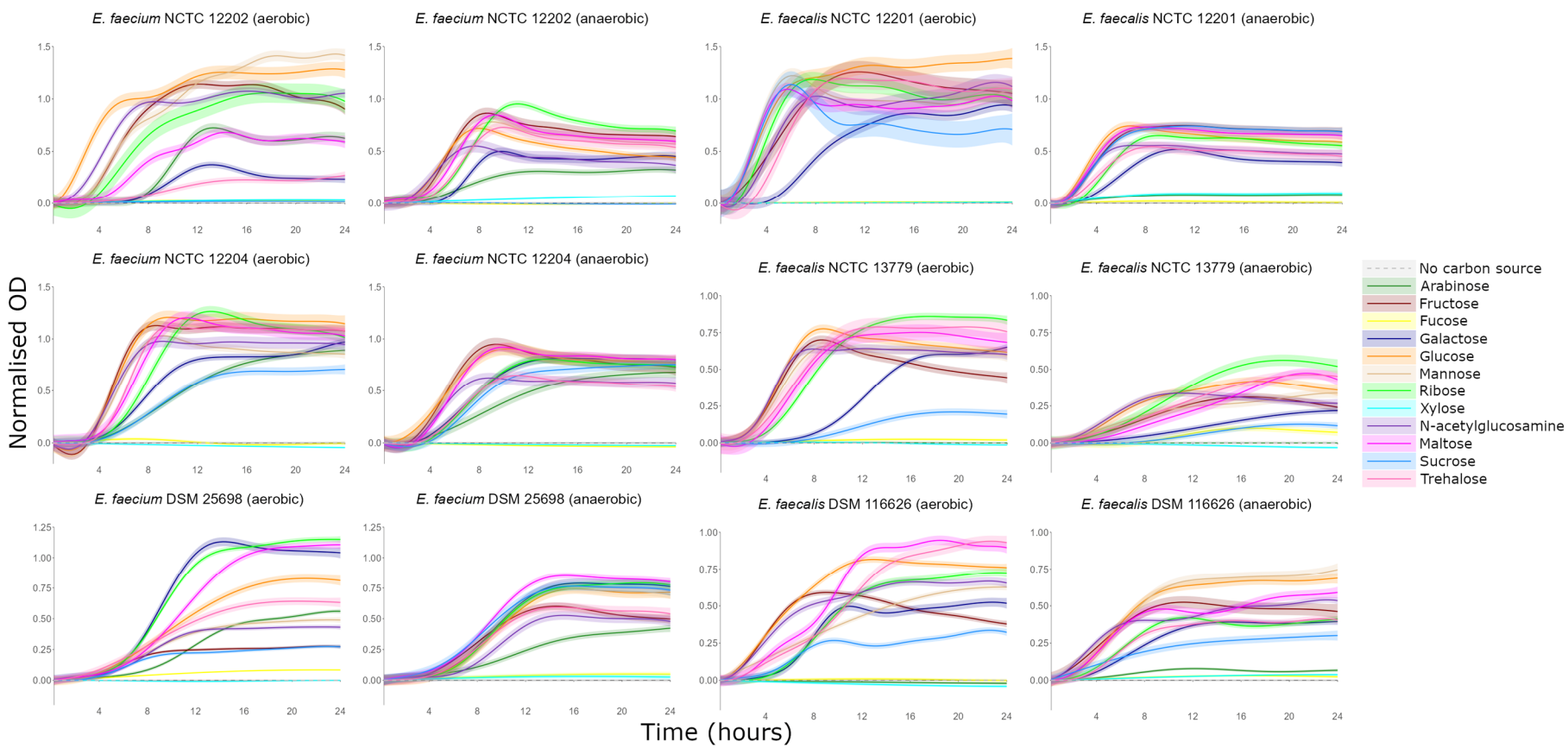


Figure 7: VRE growth was supported by individual monosaccharides and disaccharides as carbon sources. AMiGA-predicted growth curves for vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* strains cultured in minimal medium supplemented with single carbon sources under anaerobic or 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.

366

367 Under aerobic conditions *E. faecium* and *E. faecalis* growth was also supported by
368 monosaccharides and disaccharides. High or moderate growth was achieved for three *E.*
369 *faecium* strains on arabinose, fructose, galactose, glucose, mannose, ribose, N-
370 acetylglucosamine, maltose, and trehalose, and high or moderate growth was achieved for
371 two *E. faecium* strains on sucrose (**Figs. 7, S15**). High or moderate growth was achieved for
372 three *E. faecalis* strains on fructose, galactose, glucose, mannose, ribose, N-
373 acetylglucosamine, maltose, and trehalose, and high or moderate growth was achieved for
374 two *E. faecalis* strains on sucrose (**Figs. 7, S16**). Low growth or negligible growth was
375 achieved on the other carbon sources tested (**Figs. S15-S18**).
376
377 Most amino acids were not used as carbon sources; however, they could act as nitrogen
378 sources to support VRE growth. Therefore, next we tested the ability of nutrients that were
379 increased in antibiotic-treated faecal microbiota to act as nitrogen sources to support the
380 growth of three vancomycin-resistant *E. faecium* strains and three vancomycin-resistant *E.*
381 *faecalis* strains under anaerobic and aerobic conditions. As *E. faecium* and *E. faecalis* had
382 undefined amino acid auxotrophies, nitrogen utilisation was tested in a leave-one-out assay
383 where growth in a mixture containing all nitrogen sources was compared to growth in a mixture
384 lacking one of the nitrogen sources.
385
386 Under anaerobic conditions *E. faecium* and *E. faecalis* growth was supported by amino acids.
387 There was a large or moderate decrease in growth for three *E. faecium* strains when arginine,
388 glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, serine, threonine,
389 tryptophan, or valine were excluded, a large or moderate decrease in growth for two *E.*
390 *faecium* strains when phenylalanine was excluded, and a moderate decrease in growth for
391 one *E. faecium* strain when cysteine or tyrosine were excluded (**Figs. 8, S19**). There was a
392 large or moderate decrease in growth for three *E. faecalis* strains when arginine, glutamate,
393 histidine, isoleucine, leucine, methionine, serine, tryptophan, or valine were excluded, a large
394 or moderate decrease in growth for two *E. faecalis* strains when glycine or phenylalanine were

395 excluded, and a moderate decrease in growth for one *E. faecalis* strain when asparagine or
396 lysine were excluded (**Figs. 8, S20**). There was a small decrease in growth, negligible
397 decrease in growth, or an increase in growth when the other nitrogen sources were excluded
398 (**Figs. 8, S19, S20**).
399

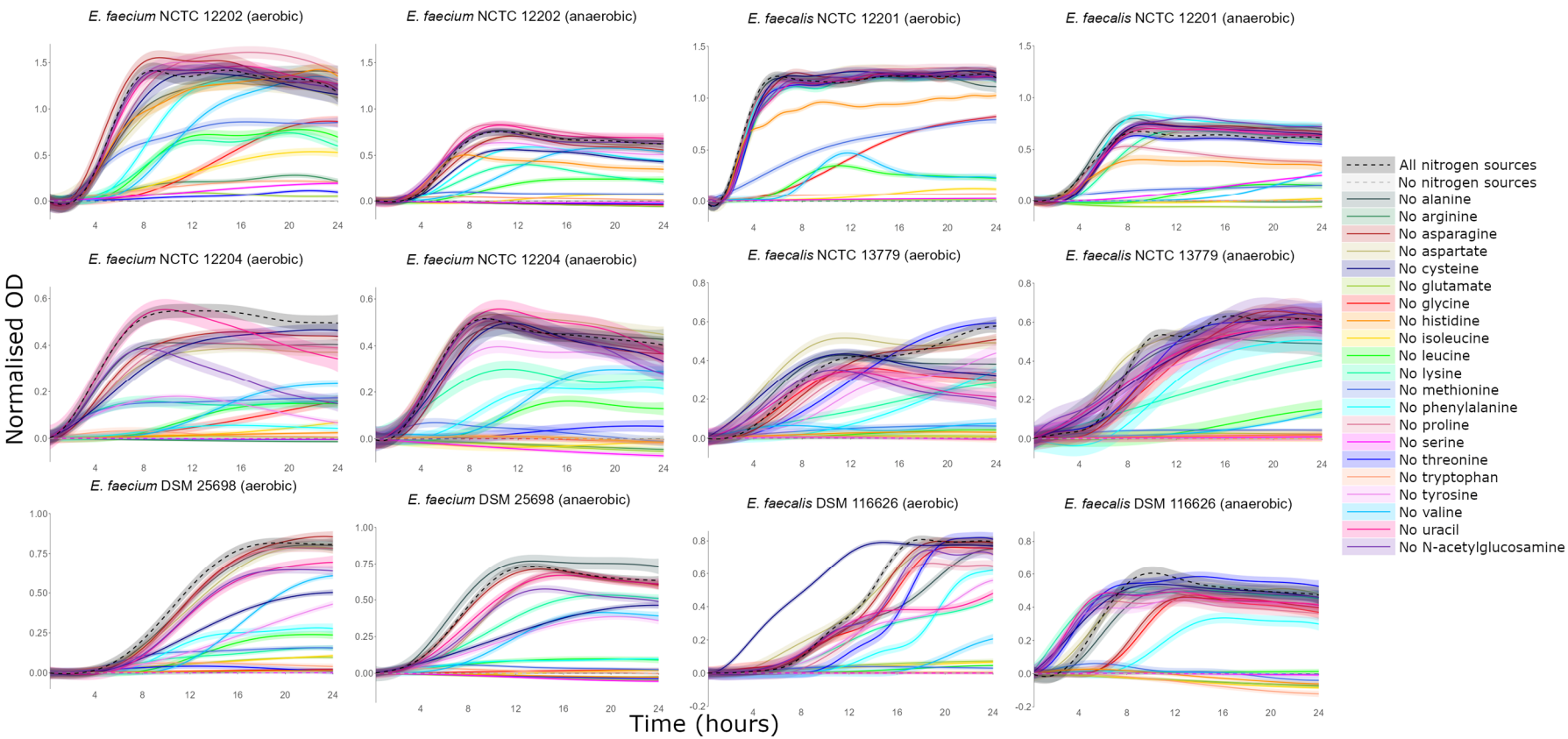


Figure 8: Amino acids served as nitrogen sources to support VRE growth. AMiGA-predicted growth curves for vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* strains cultured in minimal medium supplemented with a mixture of all nitrogen sources ("all nitrogen sources"), a leave-one-out 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 2-3 independent experiments. OD = optical density.

400

401 Under aerobic conditions *E. faecium* and *E. faecalis* growth was also supported by amino
402 acids, as well as uracil and N-acetylglucosamine. There was a large decrease in growth for
403 three *E. faecium* strains when arginine, glutamate, glycine, isoleucine, leucine, lysine,
404 methionine, phenylalanine, serine, threonine, tryptophan, or valine were excluded, a large or
405 moderate decrease in growth for two *E. faecium* strains when aspartate, histidine, or tyrosine
406 were excluded, and a moderate decrease in growth for one *E. faecium* strain when cysteine
407 or N-acetylglucosamine were excluded (**Figs. 8, S19**). There was a large or moderate
408 decrease in growth for three *E. faecalis* strains when arginine, glutamate, histidine, isoleucine,
409 leucine, methionine, serine, tryptophan, or valine were excluded, a large or moderate
410 decrease in growth for two *E. faecalis* strains when lysine, phenylalanine, or uracil were
411 excluded, and a large or moderate decrease in growth for one *E. faecalis* strain when alanine,
412 glycine, threonine, tyrosine, or N-acetylglucosamine were excluded (**Figs. 8, S20**). There was
413 a small decrease in growth, negligible decrease in growth, or an increase in growth when the
414 other nitrogen sources were excluded (**Figs. 8, S19, S20**).
415
416 Overall, vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* strains grew
417 on many nutrients that were increased in antibiotic-treated faecal microbiomes. *E. faecium*
418 and *E. faecalis* used monosaccharides and disaccharides as carbon sources and used amino
419 acids as nitrogen sources. However, there were some differences in the utilisation of specific
420 nutrients between *E. faecium* and *E. faecalis*, and some differences between different strains
421 of the same species.
422
423 ***VRE preferred to use specific nutrients over others in a mixture of nutrients that were***
424 ***elevated in antibiotic-treated faecal microbiomes***
425
426 Previous work demonstrated that pathogens (such as *Escherichia coli*, *Klebsiella*
427 *pneumoniae*, and *Enterobacter hormaechei*) have a preference for some nutrients over others
428 when presented with a mixture of nutrients that they can use to support their growth[21,38].

429 Moreover, the presence of some nutrients can influence the utilisation of other nutrients[39,40].
430 This hierarchy of nutrient preferences may have implications for the designs of microbiome
431 therapeutics that are based on nutrient competition. However, nutrient preferences have not
432 been studied in vancomycin-resistant *E. faecium* and *E. faecalis*. Therefore, next we cultured
433 a vancomycin-resistant *E. faecium* strain and a vancomycin-resistant *E. faecalis* strain in a
434 minimal medium supplemented with a mixture of nutrients that were increased in an antibiotic-
435 treated intestine (as sole carbon and nitrogen sources) under both anaerobic and aerobic
436 conditions and measured utilisation of these nutrients over time by ^{1}H-NMR spectroscopy.
437
438 The *E. faecium* and *E. faecalis* strains had some common and some distinct nutrient
439 preferences, as demonstrated by how quickly they utilised each nutrient in the nutrient mixture
440 (**Fig. 9**). Both *E. faecium* and *E. faecalis* highly utilised glucose, mannose, trehalose, ribose,
441 N-acetylglucosamine, fructose, and arginine by 4.5 hours. However, *E. faecium* highly utilised
442 galactose by 4.5 hours, while *E. faecalis* highly utilised galactose by 24 hours. Conversely, *E.*
443 *faecalis* highly utilised maltose and sucrose by 4.5 hours, while *E. faecium* highly/moderately
444 utilised maltose and sucrose by 24 hours. *E. faecium* highly used arabinose by 24 hours, while
445 *E. faecalis* did not use arabinose. Finally, *E. faecium* highly used tyrosine by 9 hours, while *E.*
446 *faecalis* highly utilised tyrosine by 24 hours. *E. faecalis* also had low utilisation of some amino
447 acids under anaerobic conditions by 24 hours (**Fig. S21**).

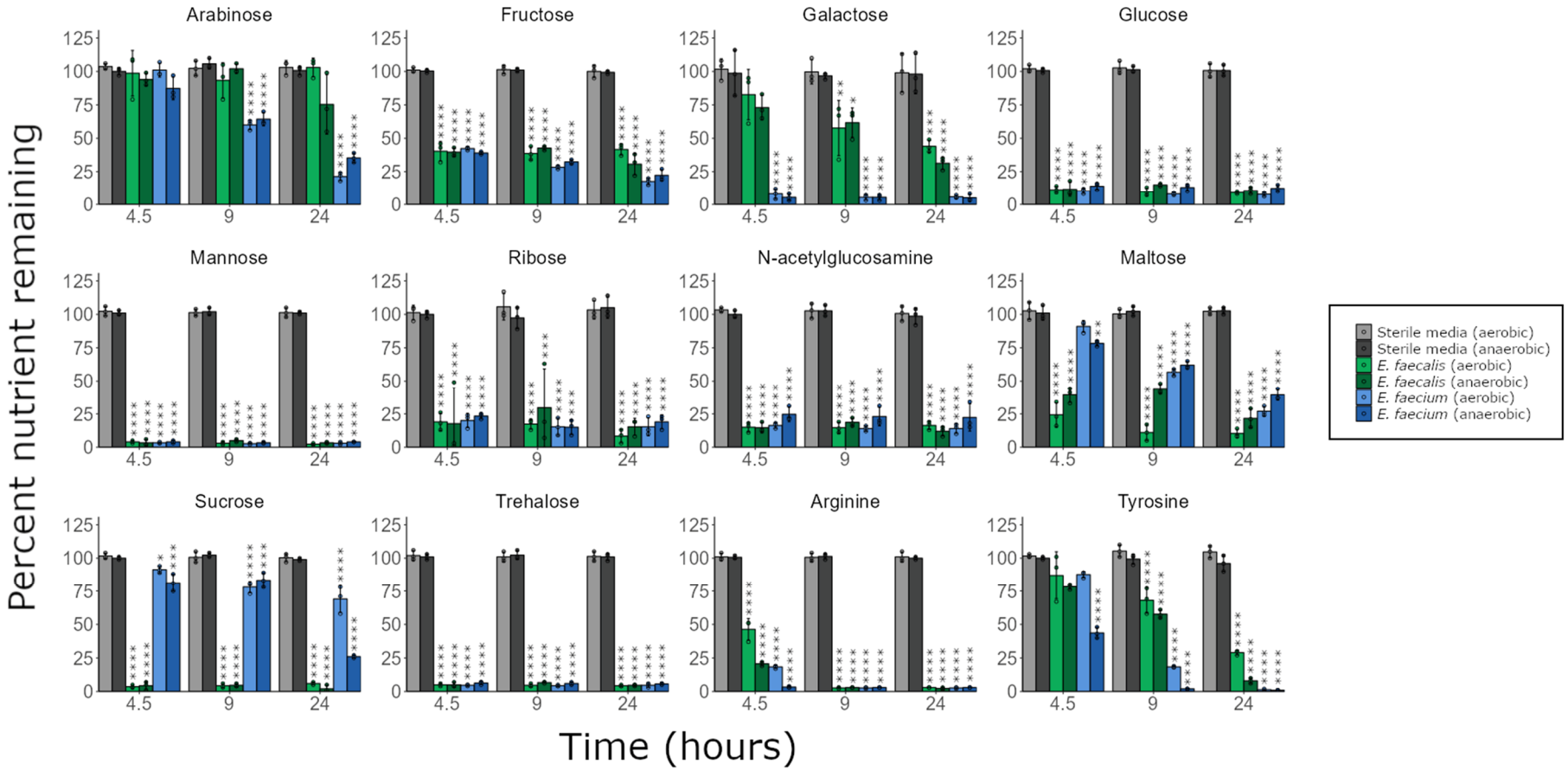


Figure 9: VRE preferred specific nutrients when grown in nutrient mixtures found in antibiotic-treated faecal microbiomes. Percentage of nutrients remaining in vancomycin-resistant *E. faecium* (NCTC 12202) and *E. faecalis* (NCTC 12201) cultures grown in a minimal medium supplemented with a mixture 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.

448

449 In summary, both *E. faecium* and *E. faecalis* strains showed a preference for some nutrients
450 over others when cultured with a mixture of nutrients that were elevated in antibiotic-treated
451 faecal microbiomes. Moreover, nutrient utilisation was generally comparable for these strains
452 when cultured under both anaerobic and aerobic conditions.
453
454 ***VRE occupied overlapping but distinct intestinal niches with each other and with***
455 ***carbapenem-resistant* Enterobacteriaceae**
456
457 Our data demonstrated that *E. faecium* and *E. faecalis* strains consumed a similar, but not
458 identical range of nutrients found in antibiotic-treated faecal microbiomes. Moreover, our data
459 demonstrated that *E. faecium* and *E. faecalis* strains had similar but not identical preferences
460 for these nutrients. Therefore, we wanted to determine whether *E. faecium* and *E. faecalis*
461 occupied distinct nutrient-defined intestinal niches. If these strains occupy distinct intestinal
462 niches, this may mean that different approaches are required to design microbiome-based
463 therapeutics to displace them from the intestine.
464
465 To test this, *E. faecium* and *E. faecalis* strains were co-cultured in a minimal medium
466 supplemented with a mixture of nutrients that were elevated in antibiotic-treated faecal
467 microbiota. We demonstrated that both *E. faecium* and *E faecalis* achieved high growth when
468 co-cultured together (**Fig. 10a**). However, for *E. faecium* there was a small but significant
469 suppression of growth in the co-culture compared to growth in monoculture. These results
470 indicate that there is partial, but not complete overlap of the nutrient-defined niches, and both
471 strains can simultaneously grow together on nutrients that are enriched in antibiotic-treated
472 faecal microbiomes.
473

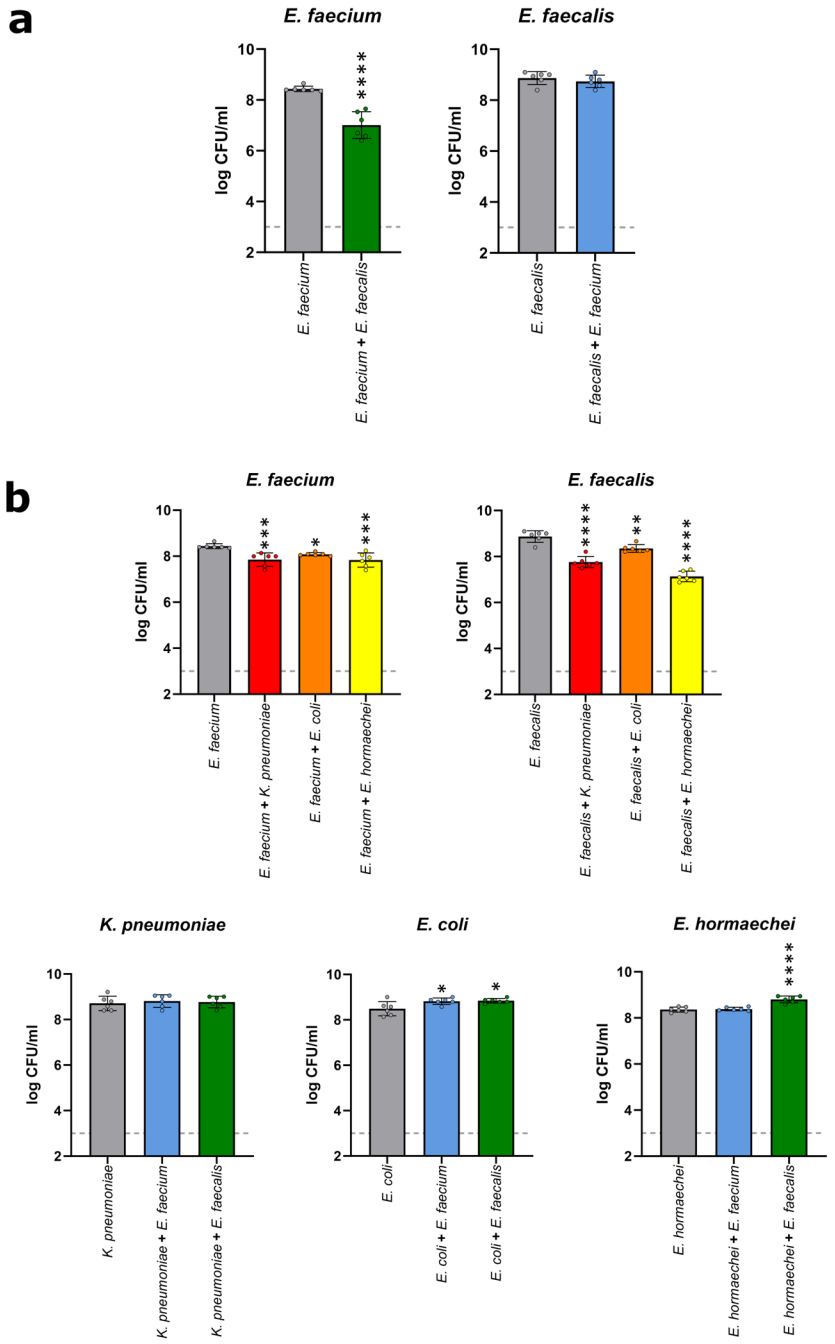


Figure 10: VRE and CRE achieved high growth in co-culture, but VRE growth was slightly suppressed. (a) *E. faecium* (NCTC 12202) and *E. faecalis* (NCTC 12201) were co-cultured in a minimal medium supplemented with a mixture of nutrients that were enriched in antibiotic-treated faecal microbiomes. **(b)** *E. faecium* (NCTC 12202) or *E. faecalis* (NCTC 12201) were co-cultured with *K. pneumoniae*, *E. coli*, or *E. hormaechei* in a minimal medium supplemented with a mixture of nutrients that were 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 Dunnett'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.

474

475 Both VRE and CRE intestinal colonisation are promoted by broad spectrum antibiotics, which
476 we demonstrated enriched for a similar range of nutrients. Therefore, we aimed to determine
477 whether VRE and CRE occupied distinct nutrient-defined intestinal niches which permitted for
478 simultaneous growth of VRE and CRE. Again, if VRE and CRE occupy distinct intestinal
479 niches this may have implications for the design of new microbiome-based therapeutics to
480 decolonise these pathogens.

481

482 Co-culture experiments were carried out as described above, except that in this experiment
483 one VRE strain was co-cultured with one CRE strain. Although *E. faecium* and *E. faecalis*
484 growth were slightly suppressed in the co-culture with each CRE strain, we showed that both
485 VRE and CRE strains achieved high growth when co-cultured together (**Fig. 10b**). These
486 results suggested that there is partial, but not complete overlap of the nutrient-defined niches
487 between VRE and CRE, allowing both types of pathogens to simultaneously grow together to
488 high levels on nutrients that are elevated in antibiotic-treated faecal microbiomes.

489

490 **DISCUSSION**

491

492 We used both *ex vivo* and *in vivo* models to demonstrate that five antibiotics that promoted
493 VRE intestinal colonisation disrupted colonisation resistance, leading to increased availability
494 of a wide range of nutrients and decreased production of a wide range of microbial
495 metabolites. We found significant but incomplete suppression of VRE growth by individual
496 SCFAs (that were decreased in antibiotic-treated faecal microbiomes). However, we found
497 that mixtures of SCFAs provided complete or near complete suppression of VRE growth. We
498 also demonstrated that VRE could use most monosaccharides and disaccharides that were
499 increased in antibiotic-treated gut microbiomes as carbon sources, and most amino acids that
500 were enriched in antibiotic-treated gut microbiomes as nitrogen sources. When cultured with
501 a mixture of nutrients that were found in antibiotic-treated faecal microbiomes, *E. faecium* and

502 *E. faecalis* strains preferentially utilised specific nutrients over others, where the nutrient
503 preferences for each strain were the same for some nutrients and different for others. Finally,
504 we also showed that vancomycin-resistant *E. faecium* and *E. faecalis* occupied overlapping
505 but distinct intestinal niches in an antibiotic-treated gut microbiome that permitted high growth
506 with each other and with carbapenem-resistant *Enterobacteriaceae* as co-cultures.
507
508 Antibiotics are a major risk factor for VRE intestinal colonisation, including vancomycin, third
509 generation cephalosporins, and antibiotics with potent activity against anaerobic bacteria[41,42].
510 In this study we measured the effects of several antibiotics (VAN, CRO, CLI, MTZ, and TZP)
511 on the human faecal microbiome, where these antibiotics are frequently used clinically and
512 have been shown to promote VRE intestinal colonisation in humans and mice. In agreement
513 with previous studies[12-16], we demonstrated that VAN, CRO, CLI, and MTZ promoted the
514 growth of vancomycin-resistant *E. faecium* and *E. faecalis*.
515
516 Previous studies demonstrated that prior exposure to TZP was associated with VRE intestinal
517 colonisation[13,14]. However, TZP has anti-enterococcal activity and is secreted in human bile at
518 high concentrations[14]. In this study, we demonstrated that *E. faecium* and *E. faecalis* were
519 killed in the presence of TZP at concentrations found in human faeces. However, TZP may
520 promote VRE intestinal colonisation *in vivo* if the patient is exposed to VRE shortly after
521 finishing a course of TZP (when TZP concentrations have decreased, but before the gut
522 microbiota has recovered). A previous study demonstrated that mice exposed to VRE
523 following the cessation of TZP treatment had high levels of VRE intestinal colonisation[14]. In
524 this study we demonstrated that TZP-treated human faecal cultures had a similar change in
525 nutrients and metabolites as CRO-treated faecal cultures. We concluded that antibiotics could
526 promote pathogen intestinal colonisation by disrupting the gut microbiome, and colonisation
527 could occur during a course of antibiotics if VRE is resistant to the antibiotic, or shortly after
528 stopping a course of antibiotics (prior to recovery of the gut microbiome) if VRE was
529 susceptible to the antibiotic. This emphasises the need to study VRE growth promotion in an

530 antibiotic-treated faecal microbiota over the course of antibiotic treatment and following
531 cessation of antibiotics.
532
533 We demonstrated that antibiotics that promote VRE intestinal colonisation caused a significant
534 decrease in the abundance of a wide range of bacterial families, including *Ruminococcaceae*
535 and *Bifidobacteriaceae* (which were significantly decreased with all antibiotics tested).
536 Moreover, we saw some heterogeneity in the responses of specific bacterial families to
537 antibiotic treatment in faecal cultures seeded with faeces from different donors. This
538 heterogeneity may be due to differences in the presence of different antibiotic-resistance
539 genes in bacterial species in the gut microbiotas from different donors. Finally, because certain
540 bacterial families were found in some donors but not others, changes in these families did not
541 reach statistical significance, even though they appeared to change in faecal cultures from
542 donors that possessed these bacterial families.
543
544 It is difficult to understand the impact of antibiotic treatment on the abundance of specific gut
545 commensals without first understanding the functions that these bacteria perform in the gut
546 microbiome. Therefore, we measured changes in nutrients and metabolites directly to define
547 the nutrient and metabolite landscape that VRE encounter during antibiotic treatment. We
548 found that antibiotic treatment enriched for a wide range of nutrients and depleted a wide
549 range of microbial metabolites, and that changes in nutrients and metabolites were less
550 heterogeneous between different donors than changes in bacterial families. This may be due
551 to shared functional redundancy between gut commensals, where different bacterial taxa
552 possess genes that perform similar functions[43].
553
554 We showed that the antibiotics which promoted VRE intestinal colonisation killed gut
555 commensals that produced a wide range of microbial metabolites. These metabolites had the
556 potential to restrict pathogen growth, and therefore we tested these metabolites in growth
557 assays both individually and as mixtures. When tested individually we showed that acetate,

558 propionate, butyrate, and valerate significantly suppressed *E. faecium* and *E. faecalis* growth
559 at high concentrations found in healthy human faeces at pH 6 (acetate and propionate also
560 significantly suppressed the growth of some *E. faecium* and *E. faecalis* strains at average
561 concentrations). However, there were some differences in the susceptibility of VRE strains to
562 each metabolite. At high concentrations the growth of all *E. faecium* strains were suppressed
563 by propionate, butyrate, and valerate, while the growth of two *E. faecium* strains were
564 suppressed by acetate. At high concentrations the growth of all *E. faecalis* strains were
565 suppressed by propionate, the growth of two *E. faecalis* strains were suppressed by acetate
566 and butyrate, and the growth of one *E. faecalis* strain was suppressed by valerate. In a
567 previous study we demonstrated that the growth of CRE strains were also suppressed by
568 acetate, propionate, butyrate, and valerate at average and high concentrations measured in
569 healthy human faeces, both when tested individually and when tested as a mixture[21].
570 Therefore, development of a mixture of SCFAs as a new therapeutic could be beneficial to
571 restrict the growth of both VRE and CRE in the intestine.
572
573 Although individually acetate, propionate, butyrate, and valerate significantly suppressed *E.*
574 *faecium* and *E. faecalis* growth at high concentrations, growth was not fully suppressed.
575 Therefore, we were also interested in whether a mixture of these metabolites showed
576 synergistic or additive effects, where the metabolite mixture provided higher growth
577 suppression than each metabolite provided individually. We showed that the APBV and PBV
578 mixtures provided complete or near complete suppression of *E. faecium* and *E. faecalis* growth
579 at pH 6. The additive effects of these metabolites suggests that they act through a similar
580 mechanism. A previous study by Sorbara and colleagues demonstrated that acetate,
581 propionate, and butyrate inhibited antibiotic-resistant *Enterobacteriaceae* growth through
582 intracellular acidification[33]. Although the inhibitory mechanism of valerate has not been
583 demonstrated yet, it has also been shown to inhibit *C. difficile* and CRE growth[21,24]. Both
584 butyrate and valerate have also been demonstrated to promote intestinal barrier function
585 which may help to prevent dissemination of VRE from the intestine[44]. Further dose testing is

586 required to determine the optimal concentration of each metabolite within this inhibitory
587 metabolite mixture. However, this metabolite mixture shows potential for the use to restrict
588 VRE growth when administered to a patient with VRE intestinal colonisation or when
589 administered to a patient as a prophylactic treatment (e.g. during antibiotic treatment when
590 the patient is susceptible to VRE intestinal colonisation).
591
592 We investigated the ability of each metabolite to individually suppress VRE growth at
593 physiologically relevant concentrations, where we tested each metabolite at their low,
594 average, and high concentrations measured in healthy human faeces. However, metabolite
595 concentrations vary along the length of the gastrointestinal tract. SCFA and branched chain
596 fatty acid concentrations are high in the caecum and ascending colon and progressively
597 decrease in the transverse and descending colon, with a small increase in the
598 sigmoid/rectum[35]. Therefore, microbial metabolites may provide greater suppression of VRE
599 growth in different regions of the intestine.
600
601 Another mechanism of colonisation resistance involved in restricting VRE growth is nutrient
602 competition. We demonstrated that killing gut commensals with antibiotics that promote VRE
603 intestinal colonisation resulted in an increase in a broad range of nutrients that contained both
604 carbon and nitrogen sources (including monosaccharides, disaccharides, amino acids, uracil,
605 and succinate). Our group recently demonstrated that antibiotics that promote CRE intestinal
606 colonisation had a similar increase in the concentration of a wide range of nutrients and
607 decrease in the concentration of microbial metabolites in human and mouse faecal
608 microbiomes[21]. This observation is consistent with the literature, which showed that VRE and
609 CRE intestinal colonisation are both promoted by broad-spectrum antibiotics – for example,
610 both CRO and TZP promote the intestinal colonisation of VRE and CRE[13-15,45]. Therefore,
611 broad-spectrum antibiotics that promote VRE and CRE intestinal colonisation resulted in an
612 increase in the availability of similar nutrients, and we conclude that this may be a broader

613 phenomenon that may apply to other intestinal pathogens that have their growth promoted in
614 the intestine following antibiotic treatment.
615
616 Microbes with similar or "overlapping" nutrient utilisation abilities will compete for similar
617 intestinal niches[46]. In this study we demonstrated that *E. faecium* and *E. faecalis* occupy
618 distinct but overlapping intestinal niches. *E. faecium* and *E. faecalis* can both use
619 monosaccharides (fructose, galactose, glucose, mannose, ribose, N-acetylglucosamine) and
620 disaccharides (maltose, sucrose, trehalose) as carbon sources, and can use amino acids
621 (arginine, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine,
622 serine, tryptophan, valine) as nitrogen sources under anaerobic and aerobic conditions.
623 However, there were some differences in the utilisation of specific nutrients between *E.*
624 *faecium* and *E. faecalis*. For example, *E. faecium* showed higher growth on arabinose,
625 cysteine, threonine, and tyrosine under both anaerobic and aerobic conditions, and showed
626 higher growth on aspartate under aerobic conditions. *E. faecalis* showed higher growth on
627 asparagine under anaerobic conditions and higher growth on alanine and uracil under aerobic
628 conditions. Therefore, identification of gut commensal strains that can deplete these nutrients
629 will allow us to develop novel microbiome therapeutics that can deplete these nutrients to re-
630 establish colonisation resistance and restrict VRE intestinal growth. However, we
631 demonstrated that both *E. faecium* and *E. faecalis* can utilise a wide range of nutrients, and
632 this flexibility in nutrient utilisation suggests that microbiome therapeutics targeting VRE must
633 contain several gut commensals that together can deplete all the nutrients that VRE can use
634 to grow.
635
636 A previous study by Lebreton and colleagues found that the hospital-associated *E. faecium*
637 lineage lost pathways related to complex carbohydrate utilisation (found in the community-
638 associated *E. faecium* lineage), which were replaced by pathways on mobile elements that
639 were related to the utilisation of amino sugars[47]. This could be advantageous for hospital-
640 associated *E. faecium* strains in an antibiotic-treated intestine, which we demonstrated had

641 increased concentrations of sugars (including increased concentrations of N-
642 acetylglucosamine) that VRE could utilise.
643
644 Previous studies demonstrated that the presence of some nutrients may influence the
645 utilisation of others by bacteria, including stimulating or repressing the utilisation of specific
646 nutrients[21,39,40,48,49]. We demonstrated that *E. faecium* and *E. faecalis* strains showed a
647 preference for some nutrients over others when cultured with a mixture of nutrients that were
648 elevated in antibiotic-treated faecal microbiomes. Some nutrients followed expected nutrient
649 utilisation patterns - nutrients that were highly used in the individual nutrient utilisation assays
650 were quickly depleted when *E. faecium* and *E. faecalis* were cultured in a mixture of nutrients
651 (e.g. glucose and arginine). However, other nutrients that were not used when tested
652 individually were used when VRE were cultured in a mixture of nutrients. For example, we
653 showed that *E. faecium* (NCTC 12202) was not able to use sucrose as a sole carbon source
654 but was able to use sucrose when grown in a mixture of nutrients. In contrast, some nutrients
655 that were used in the individual nutrient utilisation assays were not used by VRE when cultured
656 in the mixture of nutrients. For example, we showed that *E. faecium* (NCTC 12202) highly
657 used threonine when tested in the nitrogen leave-one-out assay, but did not use threonine
658 when grown in a mixture of nutrients. Therefore, nutrient utilisation and preferences in mixtures
659 of nutrients are complex and it is not always straightforward to translate the utilisation of
660 nutrients as single carbon or nitrogen sources to their utilisation in mixtures that VRE would
661 encounter in the intestine *in vivo*. Nutrient utilisation may also be context dependent, meaning
662 that VRE nutrient utilisation and preferences may change under different environmental
663 conditions. For example, future studies should investigate VRE nutrient utilisation and
664 preferences in the context of different host diets, or in different host microbiomes that vary in
665 terms of composition and antibiotic-resistance profiles. Therefore, the best approach to restrict
666 VRE growth may be to deplete all nutrients that are elevated due to antibiotic treatment, so
667 there are no alternative nutrient sources for VRE to utilise.
668

669 We demonstrated that antibiotics that promote VRE and CRE intestinal colonisation enriched
670 for a similar broad range of nutrients, and depleted a similar broad range of microbial
671 metabolites[21]. However, it was not clear whether VRE and CRE occupied distinct intestinal
672 niches, which may impact the design of microbiome therapeutics to restrict the growth of these
673 pathogens. A previous study demonstrated that vancomycin-resistant *E. faecium* and
674 carbapenem-resistant *K. pneumoniae* occupied distinct but overlapping intestinal niches,
675 however, the mechanism of how these pathogens occupied their intestinal niches was not
676 defined[22]. We hypothesised that VRE growth was promoted in an antibiotic-treated faecal
677 microbiome because VRE were able to occupy a distinct nutrient-defined intestinal niche from
678 CRE. Compared to our previous study of CRE nutrient utilisation[21], we demonstrated that VRE
679 and CRE had an overlap in the utilisation of many, but not all nutrients. Under anaerobic
680 conditions both *E. faecium* and *K. pneumoniae* could use arabinose, fructose, galactose,
681 glucose, mannose, N-acetylglucosamine, maltose, sucrose, and trehalose as carbon sources
682 and could use arginine as a nitrogen source. However, *K. pneumoniae* could also use xylose
683 as a carbon source and could use aspartate as a nitrogen source. *E. faecium* could also use
684 ribose as a carbon source and could use glutamate, glycine, isoleucine, leucine, lysine,
685 methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine as nitrogen sources.
686 Under aerobic conditions both *E. faecium* and *K. pneumoniae* could use arabinose, fructose,
687 galactose, glucose, mannose, ribose, N-acetylglucosamine, maltose, sucrose, and trehalose
688 as carbon sources and could use arginine, aspartate, glutamate, and uracil as nitrogen
689 sources. However, *K. pneumoniae* could also use xylose, alanine, proline, and glutamate as
690 carbon sources, and could grow more highly on alanine as a nitrogen source. *E. faecium* could
691 also use tryptophan and leucine as carbon sources, could grow more highly on glycine,
692 isoleucine, leucine, lysine, and methionine as nitrogen sources, and could also use
693 phenylalanine, threonine, tryptophan, tyrosine, and valine as nitrogen sources. These
694 differences in nutrient utilisation between *E. faecium* and *K. pneumoniae* support the
695 observations from our co-culture experiments where both strains were able to grow to high
696 levels in the presence of these nutrients.

697

698 VRE and CRE also had different preferences for nutrients that they could use. For example,
699 although both *E. faecium* and *K. pneumoniae* can use aspartate, arginine, lysine, and ribose,
700 *E. faecium* prefers to use arginine and ribose while *K. pneumoniae* prefers to use aspartate
701 and lysine. It is also possible that CRE and VRE may be able to share nutrients to
702 simultaneously grow together. For example, each species may only need one of the many
703 carbon sources that are available in the intestine following antibiotic treatment to support its
704 growth. Therefore, although similar nutrient- and metabolite-defined landscapes are
705 generated in the intestine with broad-spectrum antibiotics that promote VRE and CRE
706 intestinal colonisation, the niches that these pathogens occupy in the intestine are different.
707 This highlights that VRE have different strategies for utilising nutrients to colonise an antibiotic-
708 treated gut microbiome compared to other multidrug-resistant pathogens, which may have
709 implications for the design of new microbiome therapeutics to decolonise pathogens from the
710 intestine.

711

712 The results from this study can be used to inform the rational design of microbiome
713 therapeutics that can re-establish colonisation resistance to restrict VRE intestinal growth. A
714 novel microbiome therapeutic could be composed of a cocktail of live gut commensals that
715 can together deplete nutrients that were enriched with antibiotic treatment and restore the
716 production of microbial metabolites that were decreased with antibiotic treatment. Another
717 option could be a mixture of microbial metabolites that can restrict VRE growth, such as the
718 APBV or PBV mixtures tested in this study. Finally, these two new therapeutics could also be
719 combined, such that we could administer the cocktail of live gut commensals and the mixture
720 of metabolites simultaneously, or we could administer the mixture of metabolites while the
721 patient is on antibiotic treatment and then administer the cocktail of live gut commensals after
722 antibiotic treatment is completed.

723

More research is required before we can design effective microbiome therapeutics to restrict VRE growth. Gut commensals that are killed by antibiotics that promote VRE intestinal colonisation may be important candidates to reintroduce into the intestine to restore colonisation resistance. We need more information on the nutrient utilisation abilities and metabolite production abilities of these gut commensals (both individually and together as mixtures) to determine how they would grow in a nutrient-enriched and metabolite-depleted intestinal environment following antibiotic treatment. We also need more information about the impact of host diet on VRE and gut commensal intestinal colonisation.

There are some limitations in this study that highlight additional areas that require further research. Faecal samples are more commonly used than small intestine samples to investigate VRE intestinal colonisation in both humans and mice, and as such this study investigated VRE intestinal colonisation in the context of the faecal microbiome, which is more reflective of the large intestine microbiome than other regions of the intestine[12-16,50,51]. VRE colonises the antibiotic-treated large intestine to higher concentrations, however VRE can also colonise the antibiotic-treated small intestine[17]. It would also be important to investigate nutrient availability and microbial metabolite production in other regions of the intestine that VRE can colonise (such as the small intestine) to design microbiome therapeutics that can restrict VRE growth at all intestinal regions. Moreover, we have only tested *vanA* VRE strains in this study as *vanA* type resistance is the most dominant genotype worldwide, *vanA* shows higher levels of overall antibiotic-resistance compared to *vanB*, and *vanA* strains were the most commercially available stains to test[52-54]. However, future studies should investigate whether there is a difference in nutrient utilisation and metabolite inhibition of other non-*vanA* type resistant VRE.

In this study we used a mouse model to confirm that our *ex vivo* results held up in an *in vivo* intestinal environment. However, like all experimental models, mice have their limitations. For example, humans and mice have some differences in the bacterial species that compose their

752 gut microbiome, and humans and mice consume different diets[55]. However, it is not possible
753 to perform pathogen intestinal colonisation experiments in humans due to ethical limitations,
754 and as such mice are often used in these studies[14-16,21,33,56]. Humans and mice with healthy
755 gut microbiomes have intact colonisation resistance, where both humans and mice are
756 resistant to VRE intestinal colonisation. Moreover, like humans, mice treated with broad
757 spectrum antibiotics become highly susceptible to VRE intestinal colonisation, where VRE
758 intestinal growth reaches high densities. In both our *ex vivo* human faecal culture experiments
759 and our *in vivo* mouse experiments we showed that antibiotic treatment caused an enrichment
760 of nutrients (such as sugars and amino acids) and a depletion of metabolites (such as SCFAs,
761 isobutyrate, lactate, and ethanol). Therefore, despite differences in the gut microbiome
762 composition and diets between humans and mice, our *in vivo* mouse results were supportive
763 of our *ex vivo* human faecal culture results.
764
765 In summary, we demonstrated that VRE take advantage of the nutrient-enriched and
766 metabolite-depleted intestinal niches that are generated during antibiotic treatment to colonise
767 the intestine. We demonstrated that several antibiotics that promote VRE intestinal
768 colonisation changed the abundance of many nutrients and metabolites. We showed that
769 individually acetate, propionate, butyrate, and valerate partially suppressed VRE growth, but
770 a mixture of these metabolites provided complete or near complete suppression of VRE
771 growth. Healthy gut microbiomes depleted nutrients through nutrient competition, however in
772 antibiotic-treated faecal microbiomes killing of gut commensals enriched for nutrients. VRE
773 can use most of these nutrients as carbon or nitrogen sources, where *E. faecium* and *E.*
774 *faecalis* have some similar and some different preferences for these nutrients. Finally, we
775 showed that *E. faecium* and *E. faecalis* occupied overlapping but distinct intestinal niches that
776 permit high simultaneous growth with each other and with CRE. Therefore, designing a
777 microbiome therapeutic consisting of gut commensals that deplete these nutrients could
778 restore colonisation resistance and restrict or prevent VRE intestinal colonisation. Removing
779 an intestinal reservoir for VRE would lead to a reduction in the development of invasive VRE

780 infections. Our study also suggested that designing a microbiome therapeutic consisting of a
781 mixture of gut commensals that can deplete nutrients that were enriched with antibiotic
782 treatment and restore the production of microbial metabolites that were depleted with antibiotic
783 treatment could be used to restrict both VRE and CRE growth.
784
785 **METHODS**
786
787 ***Materials:***
788
789 **Table S4** lists the reagents and oligonucleotides used in this study.
790
791 ***Bacterial isolates:***
792
793 Vancomycin-resistant *E. faecium* (NCTC 12202 and NCTC 12204) and vancomycin-resistant
794 *E. faecalis* (NCTC 12201 and NCTC 13779) were purchased from the UK Health Security
795 Agency. Vancomycin-resistant *E. faecium* (DSM 25698) and vancomycin-resistant *E. faecalis*
796 (DSM 116626) were purchased from the Leibniz Institute DSMZ. All strains have *vanA*-type
797 glycopeptide resistance. VRE isolates with the *vanA*-type resistance gene were chosen as
798 this gene confers a high level of vancomycin resistance and it is one of the most frequently
799 detected vancomycin-resistance genes in enterococci[53,57].
800
801 Carbapenem-resistant *E. coli* ST617 (NDM-5), *K. pneumoniae* ST1026 (NDM-1) and *E.*
802 *hormaechei* ST278 (NDM-1) were isolated and characterised in a previously published study
803 by Yip and colleagues[21]. These carbapenem-resistant *Enterobacteriaceae* were isolated from
804 rectal swabs from patients in the Imperial College Healthcare NHS Trust (London, UK), with
805 ethical approval from the London - Queen Square Research Ethics Committee, 19/LO/0112.
806
807 ***Minimum inhibitory concentrations (MICs):***

808

809 VAN, MTZ, CRO, TZP and CLI MICs were measured for *E. faecium* (NCTC 12202) and *E.*
810 *faecalis* (NCTC 12201) following standard protocols[58]. Briefly, MIC assays were carried out
811 using Mueller-Hinton broth in 96-well plates, where the broth was supplemented with no
812 antibiotics or with 2-fold dilutions of each antibiotic ranging from 0.125-128 mg/L. Each VRE
813 strain was inoculated into the supplemented broth at $5x10^5$ CFU/mL and plates were incubated
814 aerobically at 37°C for 18 hours. Growth was assessed by taking OD_{600} measurements.
815 EUCAST guidelines provided clinical breakpoints for *Enterococcus* spp. for VAN (≤4 mg/L
816 sensitive, >4 mg/L resistant)[59]. According to EUCAST guidance, *Enterococcus* spp. can be
817 reported as resistant to MTZ, CRO, TZP, and CLI without further testing.

818

819 ***Growth of VRE in faecal concentrations of antibiotics:***

820

821 VRE growth was measured in gut growth medium supplemented with antibiotics at
822 concentrations found in human faeces. The gut growth medium was composed of the
823 following: unmodified starch (5 g/L), casein (3 g/L), inulin (1 g/L), sodium chloride (0.1 g/L),
824 peptone water (2 g/L), yeast extract (2 g/L), sodium bicarbonate (2 g/L), pectin (2 g/L), xylan
825 (2 g/L), arabinogalactan (2 g/L), calcium chloride (0.01 g/L), porcine gastric mucin type II (4
826 g/L), potassium phosphate monobasic (0.04 g/L), potassium phosphate dibasic (0.04 g/L),
827 magnesium sulphate heptahydrate (0.01 g/L), hemin (0.005 g/L), menadione (0.001 g/L), bile
828 salts (0.5 g/L), and L-cysteine hydrochloride (0.5 g/L)[60]. Gut growth medium was
829 supplemented with water or with one of the following antibiotics at concentrations found in
830 human faeces: 1000 µg/mL VAN, 25 µg/mL MTZ, 152 µg/mL CRO, 139 µg/mL TZP, and 383
831 µg/mL CLI[27-32,60]. *E. faecium* (NCTC 12202) or *E. faecalis* (NCTC 12201) were inoculated into
832 the growth medium at a concentration of 10^3 CFU/ml and cultures were incubated at 37°C
833 anaerobically for 24 hours. VRE growth was quantified by plating on Columbia agar and
834 incubating the plates at 37°C for 24 hours.

835

836 ***Human ethical approval:***

837

838 Ethical approval was received by the South Central - Oxford C Research Ethics Committee
839 (16/SC/0021 and 20/SC/0389) to collect fresh faecal samples from healthy human donors.
840 Faecal donors were aged between 18-65 years old, did not have a personal or family history
841 of gastrointestinal disease, and did not have any chronic health issues. There is no biological
842 reason to favour one sex over the other, and therefore both male and female donors were
843 recruited, with approximately equal numbers of male and female donors included in each
844 experiment. Faecal donors did not receive antibiotic treatment for at least 6 months prior to
845 donation. Ten donors consumed an omnivore diet, one donor consumed a vegetarian diet,
846 and one donor consumed a mostly vegetarian diet. None of the donors used regular
847 medication or consumed prebiotics, probiotics, or postbiotics. We obtained informed consent
848 from each participant.

849

850 ***Human faecal culture experiments:***

851

852 *Ex vivo* faecal culture experiments were performed using a previously published protocol[21].
853 These experiments were performed in the standardised gut growth medium under anaerobic
854 conditions, where the anaerobic gas mixture contained 10% CO_2, 10% H_2, 80% N_2[60]. Faecal
855 slurries were prepared by homogenising fresh faeces in degassed 0.9% saline. Each faecal
856 slurry was inoculated into the gut growth medium at a final concentration of 2% (w/v). For each
857 donor, fresh faeces were used to seed all experimental conditions, including each of the
858 antibiotic-treated faecal cultures and the water-treated faecal culture. Each treatment was
859 performed in triplicate for each donor.

860

861 The first set of faecal culture experiments measured the impact of five broad-spectrum
862 antibiotics (known to promote VRE intestinal colonisation) on the faecal microbiome from 12
863 healthy human donors. Donors were aged 29.5±9.5 years old and consisted of 7 female

864 donors and 5 male donors. The gut growth medium was supplemented with water (vehicle
865 control) or with one of the following antibiotics at concentrations measured in human faeces:
866 1000 µg/mL VAN, 25 µg/mL MTZ, 152 µg/mL CRO, 139 µg/mL TZP, and 383 µg/mL CLI[27-32].
867 Faecal cultures were incubated at 37°C anaerobically for 24 hours. Samples were collected
868 for 16S rRNA gene sequencing, 16S rRNA gene qPCR, and ^{1}H-NMR spectroscopy. Antibiotic-
869 treated faecal culture samples were compared to water-treated faecal culture samples.
870
871 The second set of faecal culture experiments were performed to measure VRE growth in
872 antibiotic-treated and water-treated faecal microbiomes. Faecal samples were collected from
873 5-8 healthy human donors (5 females, 3 males) aged 26.8±4.3 years old. The gut growth
874 medium was supplemented with water, MTZ (25 µg/mL), CLI (383 µg/mL), VAN (1000 µg/mL),
875 or CRO (152 µg/mL) at concentrations measured in human faeces. TZP was excluded from
876 this experiment as the VRE isolates used in this study were killed by TZP at faecal
877 concentrations. Faecal cultures were incubated at 37°C anaerobically for 24 hours. The next
878 day, *E. faecium* (NCTC 12202) or *E. faecalis* (NCTC 12201) were inoculated into the faecal
879 cultures at a concentration of 10^3 CFU/mL and cultures were incubated at 37°C anaerobically
880 for an additional 24 hours. Cultures were plated on Brilliance VRE agar (Thermo Fisher
881 Scientific, UK) to measure VRE growth. VRE growth was also measured in gut growth medium
882 not supplemented with faeces or antibiotics after incubation at 37°C anaerobically for 24 hours,
883 followed by plating on Brilliance VRE agar.
884
885 ***16S rRNA gene sequencing and 16S rRNA gene qPCR:***
886
887 The DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germany) was used to extract DNA from
888 faecal culture samples (DNA extracted from 250 µL per sample). The extraction protocol
889 followed the manufacturer's instructions, with the addition of 3x 1 minute bead beating cycles
890 at speed 8 (with a 1-minute rest between each cycle) using a FastPrep 24 bead beater (MP
891 Biomedicals, USA). The extracted DNA was stored at -80°C until it was ready to be analysed.

892

893 Sample libraries were prepared for 16S rRNA gene sequencing following Illumina's 16S
894 metagenomic sequencing library preparation protocol and as previously described by our
895 group[21,61]. The V1-V2 region was amplified using a forward primer mix (a 4:1:1:1 ratio of 28F-
896 YM, 28F-Borrellia, 28F-Chloroflex, and 28F-Bifdo primers) and the reverse primer 388R[21,61].
897 The index PCR reactions were cleaned up and normalised using the SequalPrep
898 Normalisation Plate Kit (Life Technologies, UK). Sample libraries were quantified using the
899 NEBNext Library Quant Kit for Illumina (New England Biolabs, UK). Sequencing was
900 performed on an Illumina MiSeq platform (Illumina Inc., UK) with paired-end 300 bp chemistry
901 using the MiSeq Reagent Kit V3 (Illumina). The resulting data was imported into R where it
902 was processed using the standard DADA2 pipeline (version 1.18.0)[62]. Version 138.1 of the
903 SILVA bacterial database was used for sequence variant classification (https://www.arb-
904 silva.de/).

905

906 Bacterial biomass was quantified using 16S rRNA gene qPCR, following a previously
907 published protocol[24]. PCR reactions were prepared in 20 µL volumes, containing 1x Platinum
908 Supermix with ROX, 225 nmol/L BactQUANT probe, 1.8 µmol/L BactQUANT forward primer,
909 1.8 µmol/L BactQUANT reverse primer, 5 µL DNA, and PCR grade water. A 10-fold dilution
910 series of *E. coli* DNA was used to generate the standard curve. All samples, standards, and
911 controls were analysed in triplicate. Amplification and real-time fluorescence detection was
912 performed on the Applied Biosystems StepOnePlus Real-Time PCR System (Applied
913 Biosystems, Waltham, Massachusetts). The PCR cycle conditions for qPCR were as follows:
914 50°C for 3 min for UNG treatment, 95°C for 10 min for *Taq* polymerase activation, and 40
915 cycles of 95°C for 15 s for denaturation and 60°C for 1 min for annealing and extension.

916

917 The 16S rRNA gene sequencing data was converted to absolute abundances using the 16S
918 rRNA gene qPCR data using the following formula, as previously described[24]:

919

920 Absolute abundance of taxa =

921 $$\text{relative abundance of taxa} \times \left(\frac{\text{16S rRNA gene copy number in sample}}{\text{highest 16S rRNA gene copy number in sample set}}\right)$$

922

923 ***^{1}H-NMR spectroscopy:***

924

925 ^{1}H-NMR spectroscopy was performed on human faecal culture samples, VRE culture
926 samples, and mouse faecal samples to measure absolute quantification of metabolites,
927 following previously published protocols[21,24].

928

929 Human faecal culture samples and VRE culture samples were prepared as follows.
930 Supernatants were prepared by centrifuging samples at 17,000 × g for 10 min at 4°C (the pellet
931 was discarded), and supernatants were frozen down until ready to be analysed. Then,
932 supernatants were randomised and defrosted at room temperature. Next, supernatants were
933 centrifuged for 10 min at 17,000 × g and 4 °C (the pellet was discarded). The NMR buffer
934 contained the following per L: 28.85 g Na_2HPO_4, 5.25 g NaH_2PO_4, 1 mM 3-
935 (trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP), 3 mM NaN_3, deuterium oxide to 1 L,
936 pH 7.4. For each sample, 400 µl of sample supernatant was mixed with 250 µl NMR buffer.

937

938 Mouse faecal samples were prepared as follows. Mouse faecal samples were randomised
939 and defrosted at room temperature. Mouse faecal supernatants were prepared by
940 homogenising 20 mg of mouse faeces in 560 µl of high-performance liquid chromatography-
941 grade water by vortexing for 10 minutes at 3000 rpm, followed by centrifuging samples at
942 17,000 × g for 10 min at 4°C (the pellet was discarded). The NMR buffer contained the
943 following per 100 mL: 20.4 g KH_2PO_4, 5.8 mM TSP, 2 mM NaN_3, deuterium oxide to 100 mL,
944 pH 7.4. For each sample, 540 µl of supernatant was mixed with 60 µl NMR buffer.

945

946 For each sample 600 µl of sample supernatant mixed with NMR buffer was loaded into 5mm
947 NMR tubes and loaded into the SampleJet system. ^{1}H-NMR spectra were acquired at 300 K
948 using a Bruker AVANCE III 800 MHz NMR spectrometer (Bruker Bio-Spin, Rheinstetten,
949 Germany) fitted with a 5mm CPTCI 1H-13C/15N D Z-gradient cryoprobe. 1D spectra were
950 acquired using a standard NOESYGPPR1D pulse sequence (RD-90°-t1-90°-tm-90°-ACQ). A
951 4 s recycle delay, 100 ms mixing time, and ~10 µs 90° pulse length were used. For the human
952 faecal culture samples and VRE culture samples 32 scans were recorded. For the mouse
953 faecal samples 128 scans were recorded.
954
955 NMR spectra pre-processing was performed in Topspin (v3.2.6), with an exponential window
956 function (line broadened by 0.3 Hz), zero-order auto phasing (command apk0), baseline
957 correction (command abs), and referencing TSP (command sref). Further processing was
958 performed by importing NMR spectra into MATLAB r2019b (The Mathworks, USA) using a
959 custom script, as previously described[21]. Following alignment of the spectra the water peak
960 was removed by cutting region 4.6 - 5.0 ppm and the TSP peak was removed by cutting region
961 -0.2 - 0.2 ppm. Chenomx NMR Suite v9.02 (Chenomx, Canada) was used for peak
962 identification and representative peaks were selected and integrated. To define the limit of
963 detection, a signal-to-noise ratio cut-off of 3:1 was used[63]. The minimal signal-to-noise ratio
964 for each dataset was as follows: 8.3:1 for the human faecal culture samples, 8.5:1 for the VRE
965 culture samples, 3.2:1 for the mouse faecal samples.
966
967 ***Mouse model of intestinal colonisation with vancomycin-resistant* E. faecium*:***
968
969 The Imperial College London Animal Welfare and Ethical Review Body (PF93C158E) provided
970 ethical approval for the mouse experiments. The mouse experiment was performed under the
971 authority of the UK Home Office, as described in the Animals (Scientific Procedures) Act 1986.
972 We adhered to standards outlined in the Animal Research: Reporting of In Vivo Experiments
973 (ARRIVE) guidelines.

974

975 Female wild-type C57BL/6 mice (8-10 weeks old) were purchased from Envigo (Huntingdon,
976 UK). Mice were housed 5 per cage in individually ventilated cages with bedding (Aspen chip
977 2 bedding, NEPCO, Warrensburg, New York). Mice were maintained at 20-22°C and 45-65%
978 humidity, with 12-hour light and dark cycles. Mice were given autoclaved food (RM1, Special
979 Diet Services, Essex, UK) and water (provided ad libitum). Prior to the start of the experiment
980 mice were acclimatised for 1 week.

981

982 The mouse experiment was designed to measure the impact of VAN treatment on nutrient
983 availability, metabolite production, and vancomycin-resistant *E. faecium* intestinal growth.
984 Mice were orally gavaged with VAN or water once daily for 7 days, and faecal samples were
985 collected for ^{1}H-NMR spectroscopy 24 hours after the last dose of VAN was administered.
986 VAN dosing was based on previously published study, where mice were orally gavaged with
987 water or with VAN at a dose of 50 mg/kg/day[64]. Both groups of mice were orally gavaged with
988 10^{6} CFU per 200 µl of vancomycin-resistant *E. faecium* (NCTC 12202) 4 days after the last
989 dose of VAN, and 4 days later faecal samples were collected from mice to plate on Brilliance
990 VRE agar to quantify *E. faecium* growth.

991

992 ***Mixed metabolite inhibition assay:***

993

994 Microbial metabolites that were decreased with antibiotic treatment were tested together as a
995 mixture in an inhibition assay to see if they could suppress VRE growth. The metabolite
996 concentrations found in healthy faeces were obtained from a previously published study, and
997 included the low, average, and high concentrations measured in healthy faeces (see **Table**
998 **S5**)[21].

999

1000 In the first experiment we tested a mixture of formate, acetate, propionate, butyrate, valerate,
1001 isobutyrate, isovalerate, lactate, 5-aminovalerate, and ethanol, where metabolites mimicked

1002 their concentrations measured in healthy human faeces. For the low mixture all metabolites
1003 were tested at the low concentrations measured in faeces, for the average mixture all
1004 metabolites were tested at the average concentrations measured in faeces, and for the high
1005 mixture all metabolites were tested at the high concentrations measured in faeces. Tryptic soy
1006 broth was supplemented with the metabolite mixture and the pH of the supplemented broth
1007 was adjusted to 6, 6.5, or 7 to mimic the pH of the healthy intestine[36,65]. *E. faecium* (NCTC
1008 12202) or *E. faecalis* (NCTC 12201) were inoculated into the broth at a concentration of 10^3
1009 CFU/ml, cultures were incubated at 37°C anaerobically overnight, and OD_{600} measurements
1010 were taken the next day.
1011
1012 In the second experiment we tested a mixture of acetate, propionate, butyrate, and valerate
1013 ("APBV mixture"), or a mixture of propionate, butyrate, and valerate ("PBV mixture"), where
1014 metabolite concentrations mimicked high concentrations measured in healthy human faeces.
1015 Tryptic soy broth was supplemented with the metabolite mixture or unsupplemented ("no
1016 metabolite" control) and the pH of the broth was adjusted to 6, 6.5, or 7. Vancomycin-resistant
1017 *E. faecium* strains or *E. faecalis* strains were inoculated into the broth at a concentration of
1018 10^3 CFU/ml, cultures were incubated at 37°C anaerobically overnight, and OD_{600}
1019 measurements were taken the next day.
1020
1021 ***Individual metabolite inhibition assay:***
1022
1023 Microbial metabolites were individually tested to determine whether they could individually
1024 suppress VRE growth. Each metabolite was tested at concentrations mimicking the low,
1025 average, and high concentrations measured in healthy human faeces[21]. We tested the
1026 following individual metabolites: formate, acetate, propionate, butyrate, valerate, isobutyrate,
1027 isovalerate, lactate, 5-aminovalerate, and ethanol. We also tested each individual metabolite
1028 as 2-fold dilutions between 4-512 mM to calculate the half-maximal inhibitory concentrations
1029 (IC_{50}). Tryptic soy broth was supplemented with each metabolite and the pH of the

1030 supplemented broth was adjusted to 6. Vancomycin-resistant *E. faecium* strains or *E. faecalis*
1031 strains were inoculated into the broth at a concentration of 10^3 CFU/ml, cultures were
1032 incubated at 37°C anaerobically overnight, and OD_{600} measurements were taken the next day.
1033
1034 ***Growth of VRE in faecal culture supernatants and VRE supernatant:***
1035
1036 Basal minimal medium (see Supplementary Data 1) was supplemented with a mixture of
1037 nutrients that were enriched in antibiotic-treated faecal microbiomes at a final concentration of
1038 0.015% (w/v): L-alanine, L-arginine, L-aspartate, L-glutamate, L-glycine, L-isoleucine, L-
1039 leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-
1040 tyrosine, L-valine, L-arabinose, D-fructose, L-fucose, D-galactose, D-glucose, D-mannose, D-
1041 ribose, D-xylose, N-acetylglucosamine, D-maltose, sucrose, D-trehalose, uracil and succinate.
1042 The supplemented basal minimal medium was inoculated with fresh healthy human faeces (2
1043 female donors and 1 male donor, average age 29.3±4.0 years old), a single VRE strain (*E.*
1044 *faecium* NCTC 12202 or *E. faecalis* NCTC 12201), or left uninoculated. To prepare the faecal
1045 inoculum, fresh faeces were prepared as a 10% (w/v) faecal slurry in 0.9% (w/v) saline and
1046 homogenised in a stomacher at 250 rpm for 1 minute. The faecal slurry was diluted 1 in 10
1047 into degassed supplemented basal minimal medium to give a final concentration of 1%. *E.*
1048 *faecium* (NCTC 12202) and *E. faecalis* (NCTC 12201) were inoculated into degassed
1049 supplemented basal minimal medium at a concentration of 10^7 CFU/ml. Cultures were
1050 incubated at 37°C anaerobically for 24 hours. The next day, the spent supernatants were
1051 adjusted to pH 7, filter sterilised, and inoculated with a single VRE strain at 10^6 CFU/ml. As a
1052 control, spent supernatants were also supplemented with 0.5% (w/v) glucose to confirm
1053 nutrient depletion. Cultures were incubated at 37°C anaerobically for 24 hours, then cultures
1054 were plated on fastidious anaerobe agar with 5% (v/v) defibrinated horse blood to quantify
1055 VRE growth.
1056
1057 ***Carbon and nitrogen utilisation assays:***

1058

1059 Nutrients increased in antibiotic-treated faecal cultures were tested for their potential to serve
1060 as individual carbon and/or nitrogen sources for *E. faecium* and *E. faecalis*. We designed an
1061 *Enterococcus* minimal medium to test the individual nutrients, which did not allow any
1062 background growth without a supplemented carbon and nitrogen source. The minimal medium
1063 consisted of the following: potassium phosphate monobasic (4 g/L), potassium phosphate
1064 dibasic (14 g/L), sodium citrate (1 g/L), magnesium sulphate (0.2 g/L), ammonium sulphate (2
1065 g/L), adenosine (0.04 g/L), thymine (0.04 g/L), Kao and Michayluk vitamin solution (Merck,
1066 Cat. No. K3129; 10 mL/L). If the study was performed under anaerobic conditions, fumarate
1067 (5.2 g/L) was added to increase growth of VRE strains. The minimal medium was sterilised
1068 with a 0.22 µm filter and used immediately for aerobic incubations or degassed overnight if
1069 used for anaerobic incubations.

1070

1071 For the carbon assays the minimal medium was supplemented with the RPMI-1640 amino
1072 acid solution (Merck, Cat. No. R7131; 50 mL/L) as nitrogen sources, and each individual
1073 nutrient was supplemented in at a concentration of 0.5% (w/v) to test their utilisation as a
1074 carbon source.

1075

1076 For the nitrogen assays the minimal medium was supplemented with 0.5% (w/v) glucose as a
1077 carbon source. As *E. faecium* and *E. faecalis* had undefined amino acid auxotrophies, nitrogen
1078 utilisation was assessed by comparing growth of the nitrogen mixture lacking one of the
1079 nitrogen sources to growth on the complete nitrogen mixture (leave-one-out experimental
1080 design). The complete “all nitrogen” mixture contained the following: L-alanine (2 g/L), L-
1081 arginine (2.5 g/L), L-asparagine (0.71 g/L), L-aspartate (0.25 g/L), L-cysteine (0.625 g/L), L-
1082 glutamate (0.25 g/L), L-glycine (0.125 g/L), L-histidine (0.1875 g/L), L-isoleucine (0.625 g/L),
1083 L-leucine (0.625 g/L), L-lysine HCl (0.5 g/L), L-methionine (0.1875 g/L), L-phenylalanine
1084 (0.1875 g/L), L-proline (0.25 g/L), L-serine (0.375 g/L), L-threonine (0.25 g/L), L-tryptophan
1085 (0.0625 g/L), L-tyrosine (0.145 g/L), L-valine (0.25 g/L), N-acetylglucosamine (2 g/L), and

1086 uracil (0.5 g/L). Each leave-one-out mixture excluded one of the nitrogen sources found in the
1087 all nitrogen mixture.
1088
1089 For both carbon and nitrogen assays, VRE strains were grown on Reasoner's 2A (R2A) agar
1090 overnight and inoculated into supplemented minimal medium at a starting OD_{600} of ~0.05.
1091 Cultures were incubated either anaerobically or aerobically in a plate reader which measured
1092 OD_{600} every 15 minutes for 24 hours.
1093
1094 ***Mixed nutrient preference assays:***
1095
1096 Nutrient preference was assessed for *E. faecium* (NCTC 12202) and *E. faecalis* (NCTC
1097 12201) by inoculating each strain in a minimal medium supplemented with a mixture of
1098 nutrients that were increased in antibiotic-treated faecal cultures. Decreases in the
1099 concentrations of each nutrient were measured over time. The *Enterococcus* minimal medium
1100 was supplemented with a mix of the following nutrients at 0.015% (w/v) for each nutrient: L-
1101 alanine, L-arginine, L-aspartate, L-glutamate, L-glycine, L-isoleucine, L-leucine, L-lysine, L-
1102 methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-tyrosine, L-valine, L-
1103 arabinose, D-fructose, L-fucose, D-galactose, D-glucose, D-mannose, D-ribose, D-xylose, N-
1104 acetylglucosamine, D-maltose, sucrose, D-trehalose, uracil and succinate.
1105
1106 VRE strains were grown on Reasoner's 2A (R2A) agar overnight and inoculated into the
1107 supplemented minimal medium at a starting OD_{600} of ~0.05. Cultures were incubated either
1108 anaerobically or aerobically in a plate reader which measured OD_{600} every 15 minutes for 24
1109 hours. Samples were collected for ^{1}H-NMR spectroscopy at 0, 4.5, 9, and 24 hours. Nutrient
1110 utilisation was expressed as a percentage of the peak integration value for each nutrient at 0
1111 hours.
1112
1113 ***Analysis of Microbial Growth Assays (AMiGA):***

1114

1115 The growth curve data generated for *E. faecium* and *E. faecalis* strains were analysed using
1116 the AMiGA software (available at https://github.com/firasmidani/amiga) in Python (v3.6.5 or
1117 v3.12.9)[66]. The carbon source utilisation growth curves and mixed nutrient growth curves were
1118 normalised to the no carbon control using the subtraction method. The nitrogen utilisation
1119 growth curves were normalised to the no nitrogen control using the subtraction method.
1120 Differential growth was compared between the substrate and the no carbon control for the
1121 carbon assays, or to the all nitrogen control for the nitrogen assays by performing Gaussian
1122 Process regression in AMiGA. The functional difference and its 95% credible interval were
1123 also calculated between the substrate and no carbon control for the carbon assay, or to the
1124 all nitrogen control for the nitrogen assays.

1125

1126 ***VRE and CRE co-culture assays:***

1127

1128 VRE strains (*E. faecium* NCTC 12202 or *E. faecalis* NCTC 12201) and CRE strains (*E. coli*,
1129 *K. pneumoniae*, or *E. hormaechei*) were co-cultured in a basal minimal medium supplemented
1130 with a mixture of nutrients that are enriched in antibiotic-treated faecal microbiomes. The
1131 composition of the basal minimal medium and the steps used to prepare it are shown in
1132 Supplementary Data 1. The minimal medium was supplemented with a mixture of nutrients
1133 that were enriched in antibiotic-treated faecal microbiomes at a final concentration of 0.015%
1134 (w/v): L-alanine, L-arginine, L-aspartate, L-glutamate, L-glycine, L-isoleucine, L-leucine, L-
1135 lysine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-tyrosine, L-
1136 valine, L-arabinose, D-fructose, L-fucose, D-galactose, D-glucose, D-mannose, D-ribose, D-
1137 xylose, N-acetylglucosamine, D-maltose, sucrose, D-trehalose, uracil and succinate.

1138

1139 The following co-cultures were tested and compared to their growth as monocultures: *E.*
1140 *faecium* + *E. faecalis*, *E. faecium* + *K. pneumoniae*, *E. faecium* + *E. coli*, *E. faecium* + *E.*
1141 *hormaechei*, *E. faecalis* + *K. pneumoniae*, *E. faecalis* + *E. coli*, *E. faecalis* + *E. hormaechei*.

1142 Each strain was inoculated into the minimal medium at a concentration of 10^3 CFU/ml and the
1143 cultures were incubated for 24 hours under anaerobic conditions. Pathogen growth was
1144 quantified by plating samples on Brilliance VRE plates to quantify *E. faecium* or *E. faecalis*
1145 growth and on Brilliance CRE plates to quantify *K. pneumoniae*, *E. coli*, or *E. hormaechei*
1146 growth. Growth of each isolate was measured with 6 replicates from two independent
1147 experiments.
1148
1149 ***Statistical analysis:***
1150
1151 GraphPad Prism 10.1.0 (La Jolla, California) or R (v4.2.3) were used to perform the statistical
1152 tests. P values or q values less than 0.05 were considered to be significant. For the *ex vivo*
1153 human faecal culture experiment, the 16S rRNA gene sequencing data (log transformed
1154 absolute abundances) and the ^{1}H-NMR spectroscopy data (peak integrations) were analysed
1155 using the Wilcoxon signed rank test (two-sided) with Benjamini Hochberg false discovery rate
1156 (FDR) correction using the DA.wil function within in the DAtest R package version 2.7.18. For
1157 the log transformed *E. faecium* and *E. faecalis* plate counts, antibiotic-treated faecal culture
1158 counts were compared to water-treated faecal culture counts using a mixed effects model with
1159 Dunnett's multiple comparison, and no faeces counts were compared to water-treated faecal
1160 culture counts using an unpaired t-test (two-sided). For the experiment measuring *E. faecium*
1161 and *E. faecalis* growth in concentrations of antibiotics measured in human faeces, antibiotic-
1162 or water-treated samples were compared to the inoculum using a one-way ANOVA with
1163 Dunnett's multiple comparison test. For the mouse experiment, the ^{1}H-NMR spectroscopy
1164 data (peak integrations) were analysed using an unpaired t-test (two-sided) with Benjamini
1165 Hochberg FDR using the t_test function within the rstatix R package version 0.7.2. The log
1166 transformed *E. faecium* plate counts were compared using Mann–Whitney U test (two-sided).
1167 For the mixed metabolite inhibition assays, a Kruskal-Wallis with Dunn's multiple comparison
1168 test compared the no metabolite control to each concentration of the metabolite mixture at the
1169 same pH. For the individual metabolite inhibition assay, a Kruskal-Wallis test with Dunn's

1170 multiple comparison test compared the no metabolite control to each concentration of the
1171 individual metabolite. IC_{50} values were calculated for each individual metabolite in Graphpad
1172 Prism. The mixed nutrient growth curves, carbon assay growth curves, and nitrogen assay
1173 growth curves were analysed using the AMiGA software in Python (v3.6.5) as described
1174 above. For the supernatant growth tests, comparisons between growth in the spent
1175 supernatants and fresh medium used a Kruskal-Wallis with Dunn's multiple comparison test,
1176 while comparison of unsupplemented vs glucose-supplemented supernatants used a
1177 Wilcoxon signed rank test. For the nutrient preference experiment comparisons were made
1178 between the *E. faecium* or *E. faecalis* cultures and the sterile media controls over time using
1179 a two-way mixed ANOVA followed by pairwise comparisons with Bonferroni correction. For
1180 the *E. faecium* and *E. faecalis* co-culture experiment, log transformed monoculture plate
1181 counts were compared to log transformed co-culture plate counts using an unpaired t-test
1182 (two-sided). For the VRE strain and CRE strain co-culture experiment, log transformed
1183 monoculture plate counts were compared to log transformed co-culture plate counts using an
1184 ANOVA with Dunnett's multiple comparison test.
1185
1186 **DATA AVAILABILITY**
1187
1188 We will provide source data with this paper upon publication. The 16S rRNA gene sequencing,
1189 16S rRNA gene qPCR, and ^{1}H-NMR spectroscopy datasets will be deposited into the Figshare
1190 repository upon publication. The 16S rRNA gene sequencing raw reads will also be deposited
1191 into the ENA upon publication. The SILVA bacterial database version 138.1 can be found at
1192 https://www.arb-silva.de/. The compound database for the Chenomx NMR Suite v9.02 can be
1193 found at https://www.chenomx.com/.
1194

1195 **CODE AVAILABILITY**

1196

1197 MATLAB r2019b was used to import and process ^{1}H-NMR spectra using a custom script which
1198 will be available in the Figshare repository upon publication, using code found in the Zenodo
1199 repository at https://doi.org/10.5281/zenodo.3077413.

1200

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1368

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1370

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1380

1381 **AUTHOR CONTRIBUTIONS**

1382

1383 OGK—formal analysis: equal; investigation: lead; methodology: equal; visualisation: equal;
1384 writing – review & editing: supporting. AYGY— formal analysis: supporting; investigation:
1385 supporting; writing – review & editing: supporting. VH—formal analysis: supporting;
1386 investigation: supporting; writing – review & editing: supporting. JMB—investigation:

supporting. JRM—resources: supporting; writing – review & editing: supporting. BHM—resources: supporting; writing – review & editing: supporting. TBC—investigation: supporting; methodology: supporting; writing – review & editing: supporting. JAKM—conceptualisation: lead; data curation: lead; formal analysis: equal; funding acquisition: lead; methodology: equal; project administration: lead; resources: lead; supervision: lead; visualisation: equal; writing – original draft: lead; writing – review & editing: lead.

COMPETING INTERESTS

JAKM, AYGY and OGK filed a patent application (patent application number 2217266.2) related to this work. BHM received consultancy fees from Finch Therapeutics Group and Ferring Pharmaceuticals outside the submitted work. JRM received consultancy fees from EnteroBiotix Ltd., Cultech Ltd. and Crescent Enterprise Ltd. outside the submitted work. All other authors declare no competing interests.

FIGURE LEGENDS

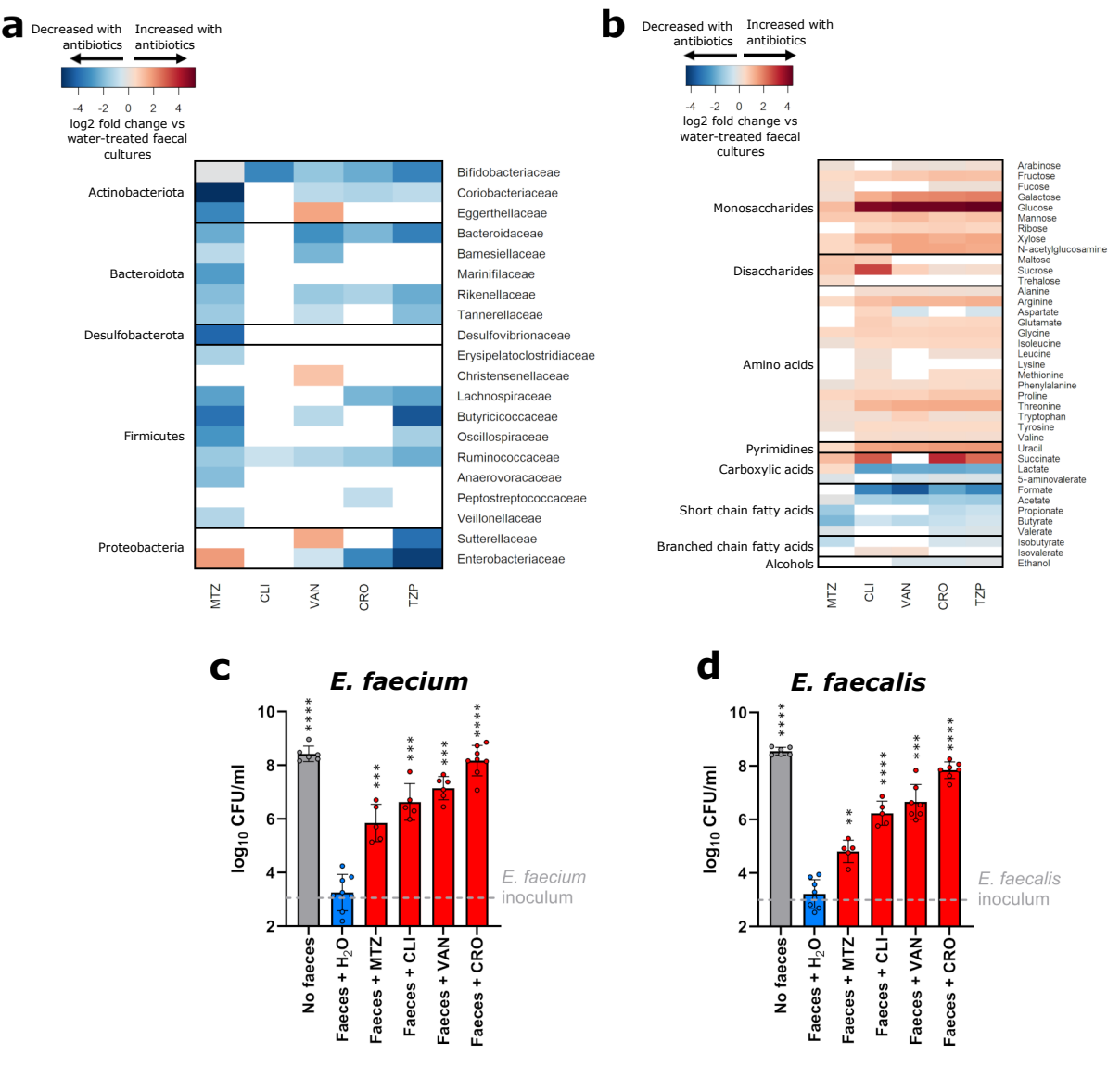
Figure 1: Antibiotic treatment significantly reduced gut commensals, increased nutrients, decreased microbial metabolites, and increased VRE growth. (a) Heatmap illustrating the log2 fold change (antibiotic-treated relative to water-treated) in bacterial taxa that were significantly decreased (shown 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 water-treated) 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

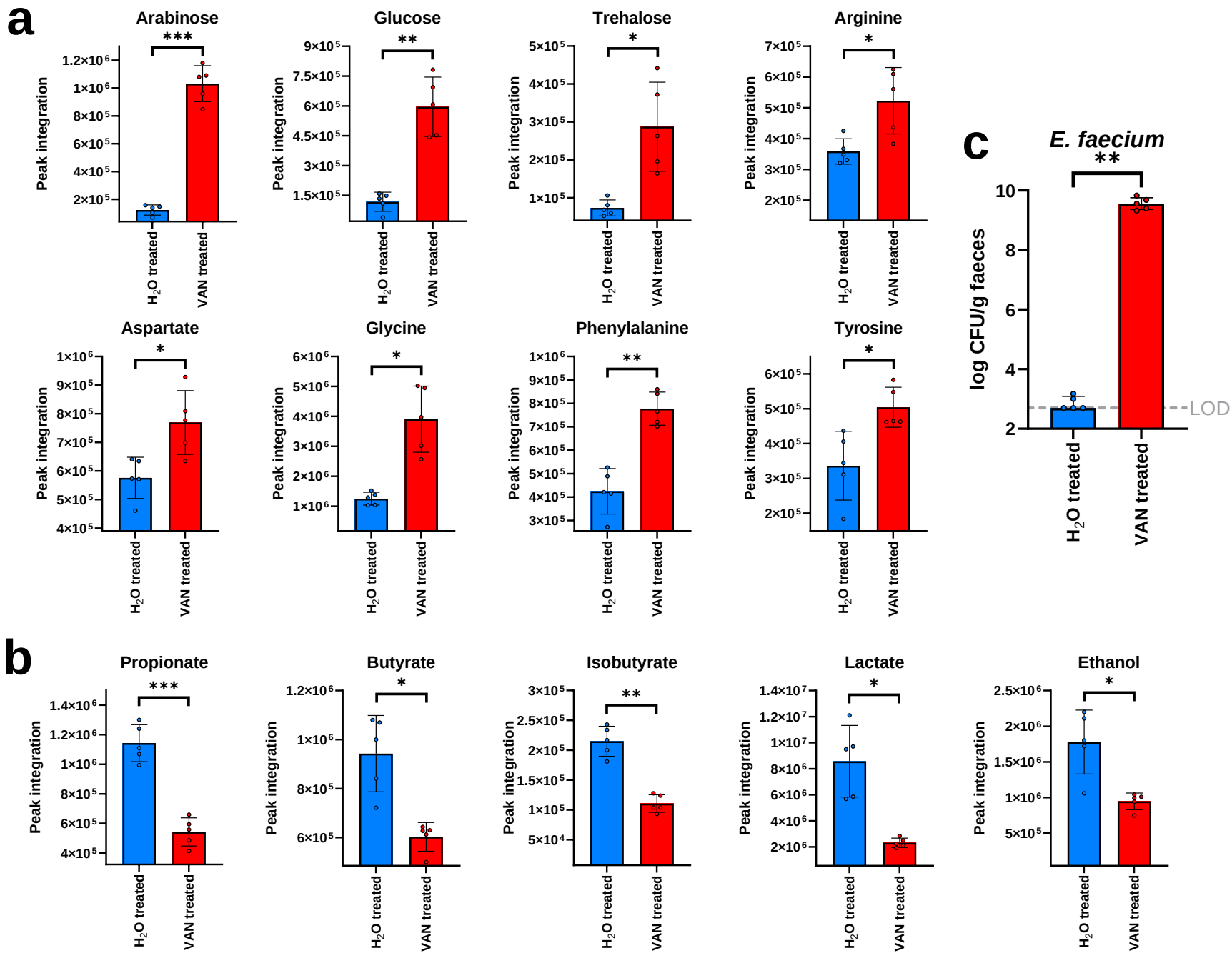
1415 antibiotic treatment were not plotted (shown in white). n = 12 healthy human faecal donors,
1416 Wilcoxon signed rank test (two-sided) with Benjamini Hochberg FDR correction, $p < 0.05$. **(c-**
1417 **d)** Antibiotic treatment significantly promoted the growth of vancomycin-resistant *E. faecium*
1418 (NCTC 12202) and *E. faecalis* (NCTC 12201) in faecal microbiomes. *E. faecium* or *E. faecalis*
1419 were spiked into faecal cultures at 10^3 CFU/ml (horizontal dashed line). n = 5-8 healthy human
1420 faecal donors, with data shown as mean ± SD. Antibiotic-treated faecal culture counts were
1421 compared to water-treated faecal culture counts using a mixed effects model of log
1422 transformed CFU/ml with Dunnett's multiple comparison. No faeces counts were compared to
1423 water-treated faecal culture counts using an unpaired t-test (two-sided) of log transformed
1424 CFU/ml. ** = $P \leq 0.01$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$. MTZ = metronidazole, CLI =
1425 clindamycin, VAN = vancomycin, CRO = ceftriaxone, TZP = piperacillin/tazobactam.
1426
1427 **<u>Figure 2</u>: Nutrients were increased and metabolites were decreased in the faeces of**
1428 **vancomycin-treated mice. (a)** Nutrients that were increased in the faeces of VAN-treated
1429 mice compared to H_2O-treated mice. **(b)** Metabolites that were decreased in the faeces of
1430 VAN-treated mice compared to H_2O-treated mice. Comparison of nutrients and metabolites in
1431 the VAN-treated faeces to the H_2O-treated faeces used an unpaired t-test (two-sided) with
1432 Benjamini Hochberg FDR, data shown as mean ± SD. **(c)** Vancomycin-resistant *E. faecium*
1433 (NCTC 12202) counts were high in the faeces from vancomycin-treated mice and nearly
1434 undetectable in the faeces of water-treated mice. Comparisons of the *E. faecium* plate counts
1435 were made using a Mann–Whitney U test (two-sided) of log10-transformed counts. n = 5 mice
1436 per group from one independent experiment, with data shown as medians ± IQR. * = $P \leq 0.05$,
1437 ** = $P \leq 0.01$, *** = $P \leq 0.001$. VAN = vancomycin, H_2O = water, LOD = limit of detection.
1438
1439 **<u>Figure 3</u>: Individual SCFAs suppressed VRE growth at physiological concentrations.**
1440 Vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis* strains were grown in
1441 tryptic soy broth (pH 6) supplemented with an individual metabolite at low, average or high
1442 concentrations measured in healthy human faeces, concentrations spanning a defined range

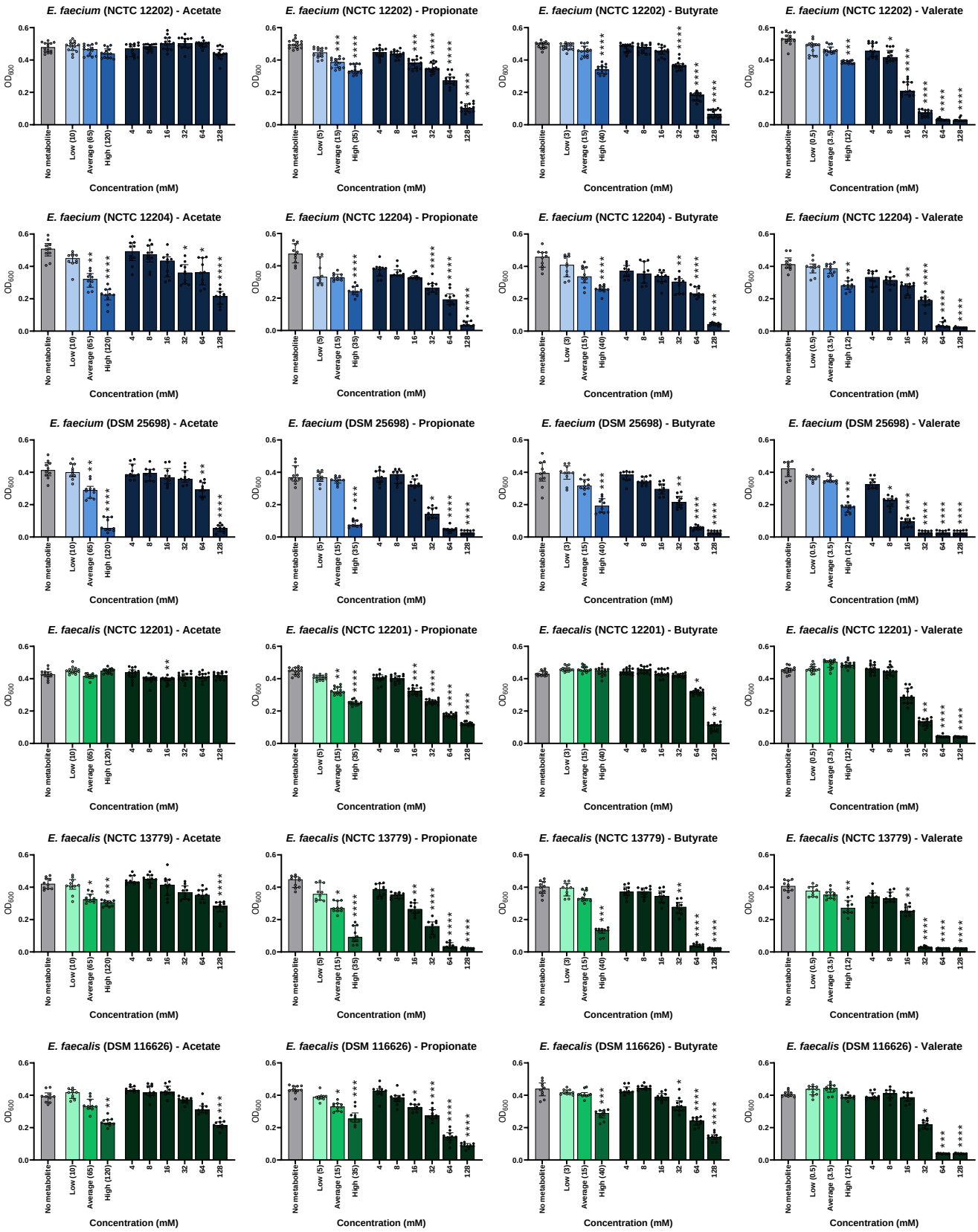
1443 (4-128 mM), or unsupplemented (no metabolite control). Cultures were incubated under
1444 anaerobic conditions overnight. Growth of each isolate was measured with 10-14 replicates in
1445 two independent experiments. Kruskal-Wallis test with Dunn's multiple comparison test (each
1446 metabolite was compared to the no metabolite control). * = P ≤ 0.05, ** = P ≤ 0.01,
1447 *** = P ≤ 0.001, **** = P ≤ 0.0001. Data are presented as medians ± IQR. Optical density at
1448 600 nm, OD_{600}.
1449
1450 **<u>Figure 4</u>: VRE growth was highly suppressed by mixtures of SCFAs at high**
1451 **concentrations.** Vancomycin-resistant *E. faecium* and vancomycin-resistant *E. faecalis*
1452 strains were grown in tryptic soy broth supplemented with a mixture of propionate, butyrate,
1453 and valerate ("PBV") or a mixture of acetate, propionate, butyrate, and valerate ("APBV") at
1454 concentrations mimicking the high concentrations measured in human faeces, or
1455 unsupplemented ("No metabolites" control). Broth was adjusted to pH 6, 6.5, or 7 to mimic the
1456 pH of the healthy large intestine, and cultures were incubated under anaerobic conditions
1457 overnight. Data shown as medians ± IQR, with 12 replicates from 3 independent experiments.
1458 Kruskal-Wallis with Dunn's multiple comparison test comparing the no metabolites control to
1459 each metabolite mixture at the same pH. * P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001,
1460 **** = P ≤ 0.0001. OD_{600} = optical density at 600 nm.
1461
1462 **<u>Figure 5</u>: VRE used nutrients enriched in antibiotic-treated faecal microbiome as sole**
1463 **carbon and nitrogen sources.** AMiGA-predicted growth curves for vancomycin-resistant *E.*
1464 *faecium* and vancomycin-resistant *E. faecalis* strains cultured in minimal medium
1465 supplemented with a mixture of nutrients under anaerobic or aerobic conditions. The bold lines
1466 show the predicted mean of growth while the shaded bands show the predicted 95% credible
1467 intervals. n = 6 replicates in two independent experiments. OD = optical density.
1468
1469 **<u>Figure 6</u>: Faecal culture supernatants suppressed VRE growth through nutrient**
1470 **depletion.** Minimal medium was supplemented with a mixture of nutrients that were increased

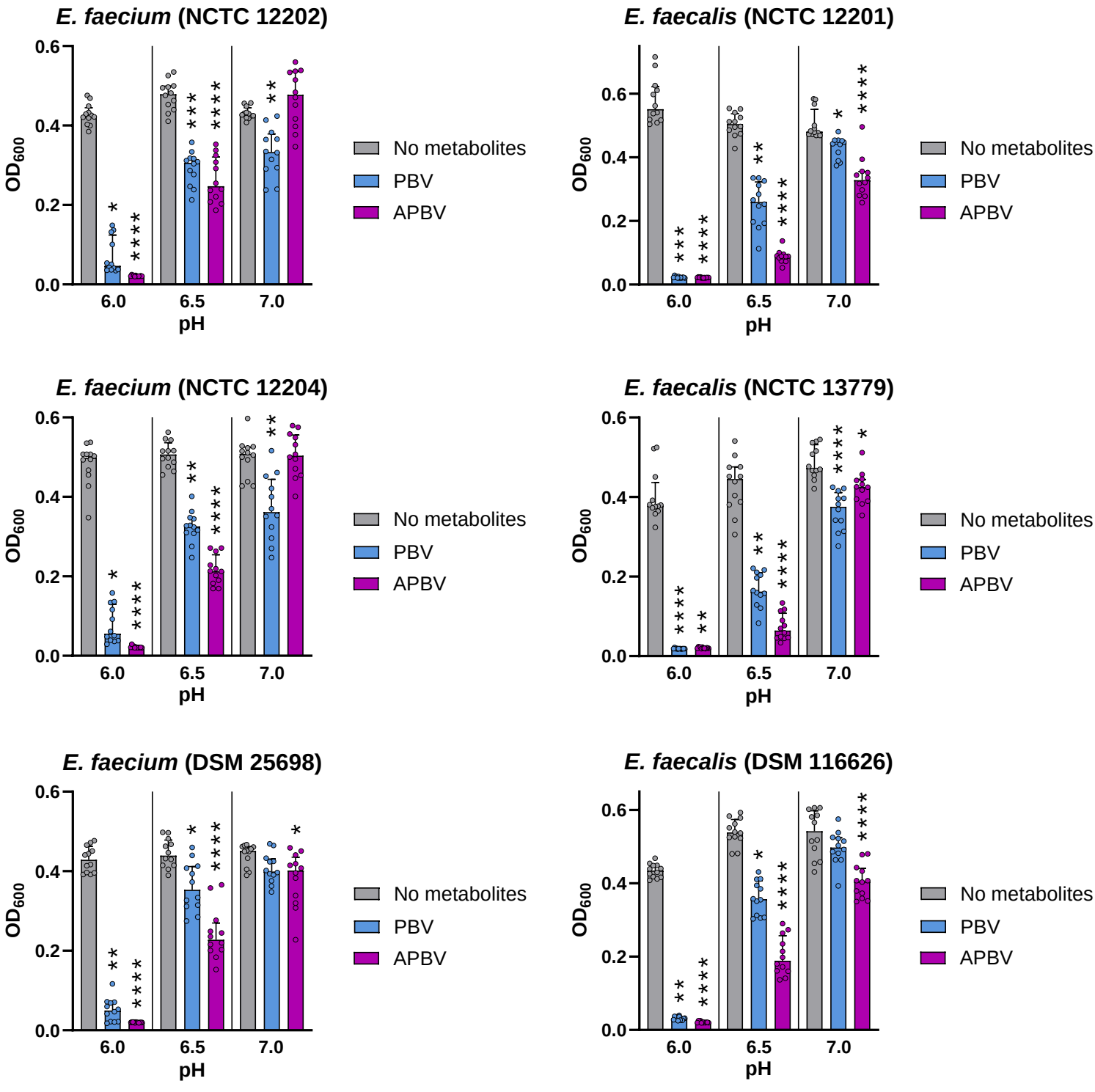
1471 with antibiotics and inoculated with either fresh healthy human faeces (n = 3 donors for 9
1472 replicates from 3 independent experiments), a single VRE strain (*E. faecium* NCTC 12202 or
1473 *E. faecalis* NCTC 12201), or left uninoculated (n = 9 replicates from 3 independent
1474 experiments). Spent supernatants were filter sterilised and then inoculated with a single VRE
1475 strain. As a control, spent supernatants were also supplemented with 0.5% glucose to confirm
1476 nutrient depletion. VRE growth was measured after overnight incubation using plate counts.
1477 Comparisons between growth in the spent supernatants and fresh medium were assessed
1478 using a Kruskal-Wallis with Dunn's multiple comparison test. Comparison of unsupplemented
1479 vs glucose-supplemented supernatants was assessed through a Wilcoxon signed rank test.
1480 ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001. Data are presented as medians ± IQR.
1481
1482 **<u>Figure 7</u>: VRE growth was supported by individual monosaccharides and disaccharides**
1483 **as carbon sources.** AMiGA-predicted growth curves for vancomycin-resistant *E. faecium* and
1484 vancomycin-resistant *E. faecalis* strains cultured in minimal medium supplemented with single
1485 carbon sources under anaerobic or aerobic conditions. The bold lines show the predicted
1486 mean of growth while the shaded bands show the predicted 95% credible intervals. n = 6
1487 replicates in 2–3 independent experiments. OD = optical density.
1488
1489 **<u>Figure 8</u>: Amino acids served as nitrogen sources to support VRE growth.** AMiGA-
1490 predicted growth curves for vancomycin-resistant *E. faecium* and vancomycin-resistant *E.*
1491 *faecalis* strains cultured in minimal medium supplemented with a mixture of all nitrogen
1492 sources ("all nitrogen sources"), a leave-one-out mixture lacking a single nitrogen source, or
1493 lacking all nitrogen sources ("no nitrogen sources") under anaerobic or aerobic conditions.
1494 The bold lines show the predicted mean of growth while the shaded bands show the predicted
1495 95% credible intervals. n = 6 replicates in 2-3 independent experiments. OD = optical density.
1496
1497 **<u>Figure 9</u>: VRE preferred specific nutrients when grown in nutrient mixtures found in**
1498 **antibiotic-treated faecal microbiomes.** Percentage of nutrients remaining in vancomycin-

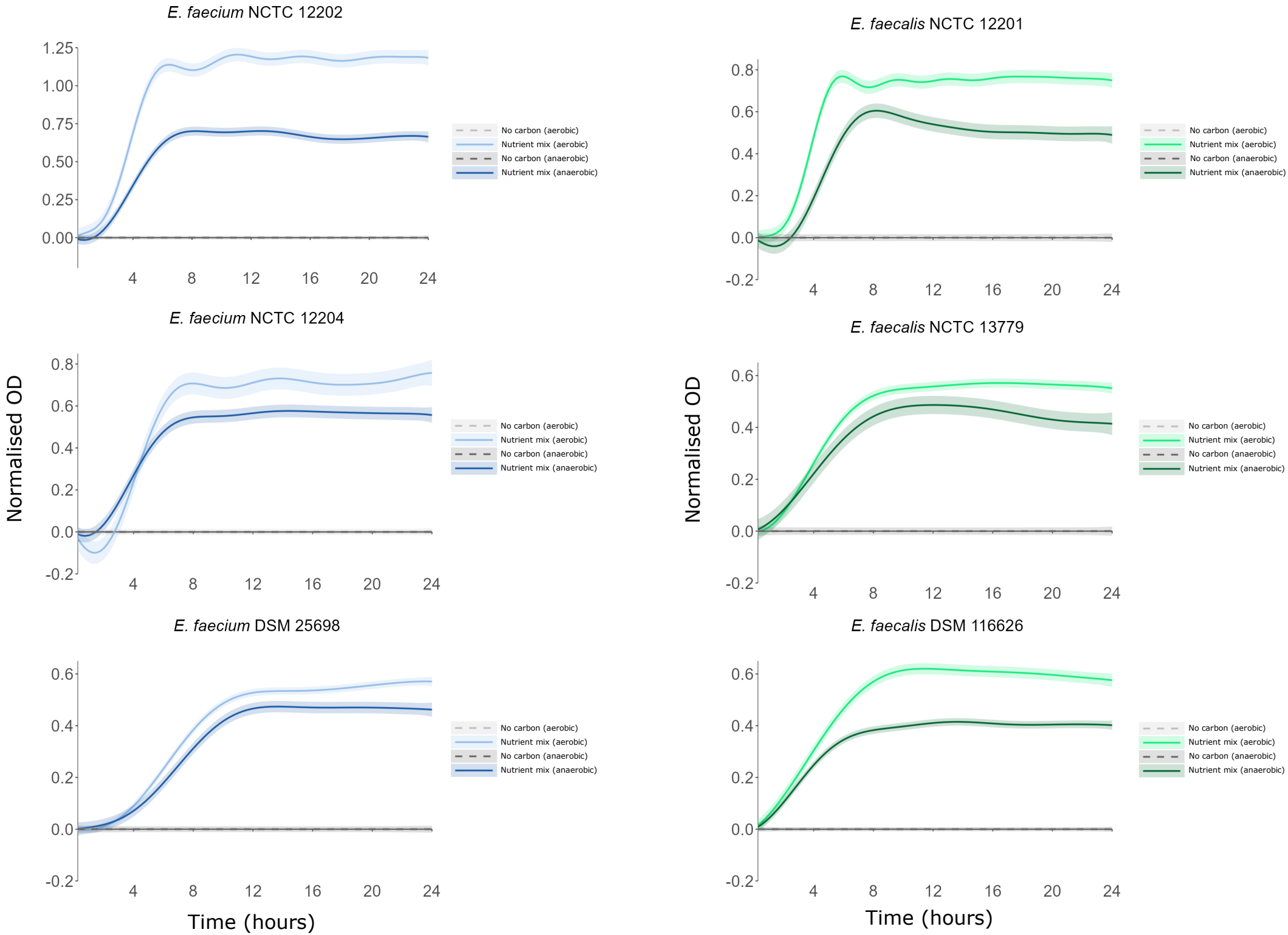
1499 resistant *E. faecium* (NCTC 12202) and *E. faecalis* (NCTC 12201) cultures grown in a minimal
1500 medium supplemented with a mixture of nutrients, under aerobic or anaerobic conditions. ^{1}H-
1501 NMR spectroscopy peak integrations were used to measure nutrient concentrations.
1502 Comparisons were made using a two-way mixed ANOVA followed by pairwise comparisons
1503 with Bonferroni correction. n = 3 replicates per group, with data shown as mean ± SD.
1504 * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001.
1505
1506 **<u>Figure 10</u>: VRE and CRE achieved high growth in co-culture, but VRE growth was**
1507 **slightly suppressed. (a)** *E. faecium* (NCTC 12202) and *E. faecalis* (NCTC 12201) were co-
1508 cultured in a minimal medium supplemented with a mixture of nutrients that were enriched in
1509 antibiotic-treated faecal microbiomes. **(b)** *E. faecium* (NCTC 12202) or *E. faecalis* (NCTC
1510 12201) were co-cultured with *K. pneumoniae*, *E. coli*, or *E. hormaechei* in a minimal medium
1511 supplemented with a mixture of nutrients that were enriched in antibiotic-treated faecal
1512 microbiomes. Each strain was inoculated into the minimal media at a concentration of 10^3
1513 CFU/ml (represented as the grey dashed line) and the cultures were incubated for 24 hours
1514 under anaerobic conditions. Samples were plated on Brilliance VRE plates to quantify *E.*
1515 *faecium* or *E. faecalis* growth and on Brilliance CRE to quantify *K. pneumoniae*, *E. coli*, or *E.*
1516 *hormaechei* growth. Growth of each isolate was measured with 6 replicates in two independent
1517 experiments. Comparison of *E. faecium* and *E. faecalis* monoculture growth to co-culture
1518 growth was made using an unpaired t-test (two-sided). Comparison of VRE strain and CRE
1519 strain monoculture growth to co-culture growth was made using ANOVA with Dunnett's
1520 multiple comparison test. * = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001, **** = P ≤ 0.0001. Data are
1521 presented as mean values ± SD.

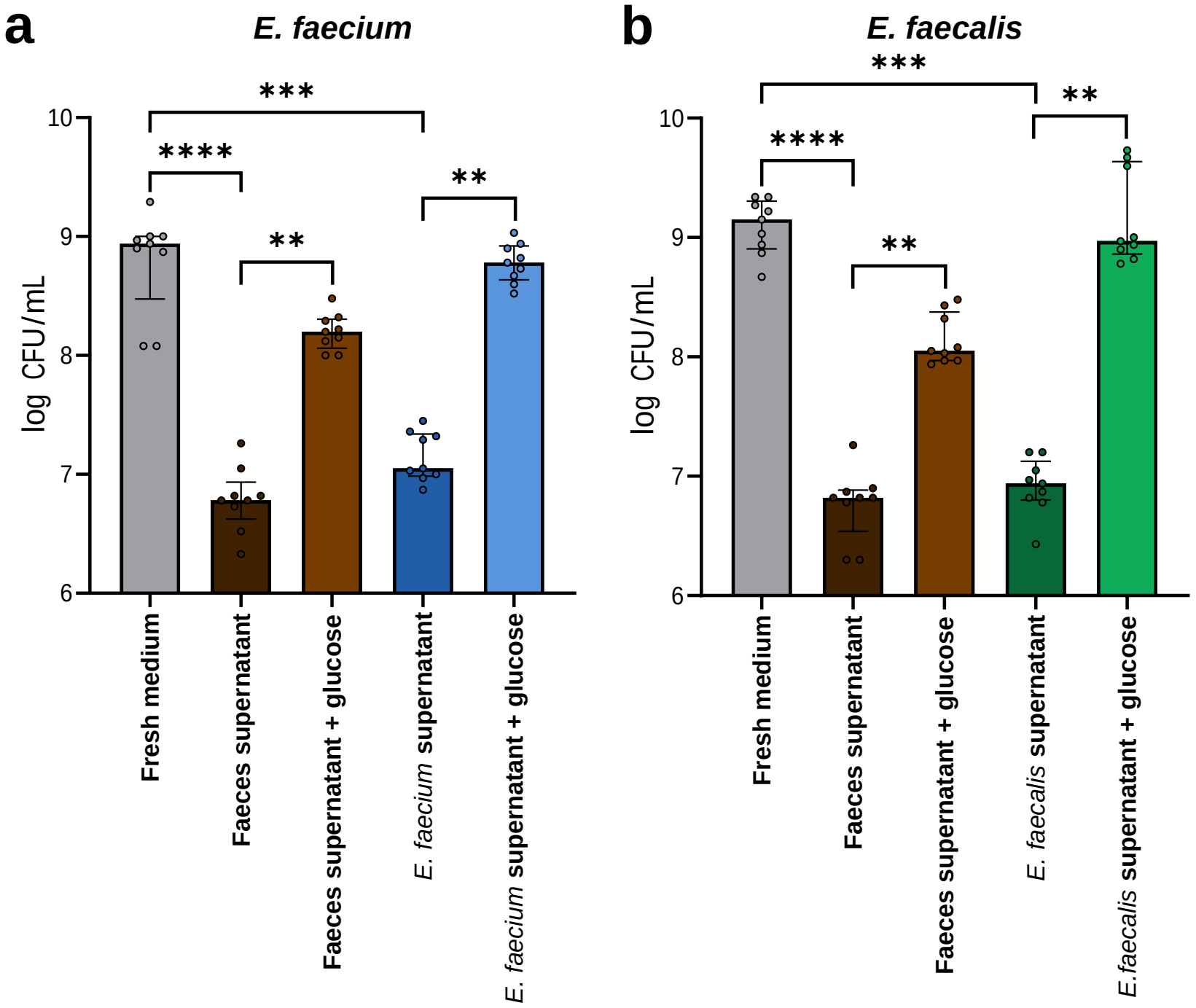

a
Decreased with antibiotics
Increased with antibiotics
-4 -2 0 2 4
log2 fold change vs water-treated faecal cultures
Actinobacteriota
Bacteroidota
Desulfobacterota
Firmicutes
Proteobacteria
Bifidobacteriaceae
Coriobacteriaceae
Eggerthellaceae
Bacteroidaceae
Barnesiellaceae
Marinifilaceae
Rikenellaceae
Tannerellaceae
Desulfovibrionaceae
Erysipelatoclostridiaceae
Christensenellaceae
Lachnospiraceae
Butyricicoccaceae
Oscillospiraceae
Ruminococcaceae
Anaerovoracaceae
Peptostreptococcaceae
Veillonellaceae
Sutterellaceae
Enterobacteriaceae
MTZ
CLI
VAN
CRO
TZP
b
Decreased with antibiotics
Increased with antibiotics
-4 -2 0 2 4
log2 fold change vs water-treated faecal cultures
Monosaccharides
Disaccharides
Amino acids
Pyrimidines
Carboxylic acids
Short chain fatty acids
Branched chain fatty acids
Alcohols
Arabinose
Fructose
Fucose
Galactose
Glucose
Mannose
Ribose
Xylose
N-acetylglucosamine
Maltose
Sucrose
Trehalose
Alanine
Arginine
Aspartate
Glutamate
Glycine
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Proline
Threonine
Tryptophan
Tyrosine
Valine
Uracil
Succinate
Lactate
5-aminovalerate
Formate
Acetate
Propionate
Butyrate
Valerate
Isobutyrate
Isovalerate
Ethanol
MTZ
CLI
VAN
CRO
TZP
c
E. faecium
log10 CFU/ml
10
8
6
4
2
E. faecium inoculum
No faeces
Faeces + H2O
Faeces + MTZ
Faeces + CLI
Faeces + VAN
Faeces + CRO
d
E. faecalis
log10 CFU/ml
10
8
6
4
2
E. faecalis inoculum
No faeces
Faeces + H2O
Faeces + MTZ
Faeces + CLI
Faeces + VAN
Faeces + CRO

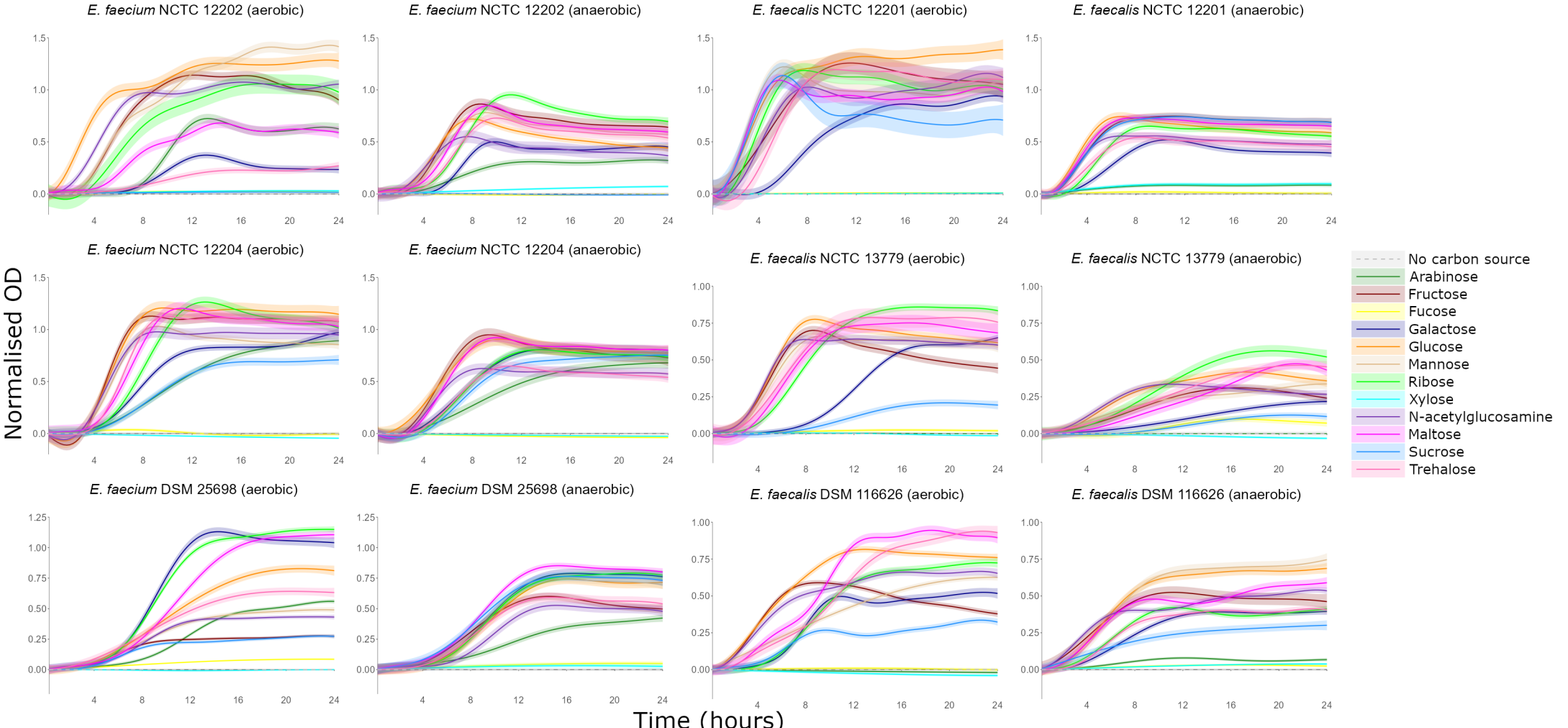

a
Arabinose
Glucose
Trehalose
Arginine
Aspartate
Glycine
Phenylalanine
Tyrosine
Peak integration
H2O treated
VAN treated
b
Propionate
Butyrate
Isobutyrate
Lactate
Ethanol
c
E. faecium
log CFU/g faeces
LOD

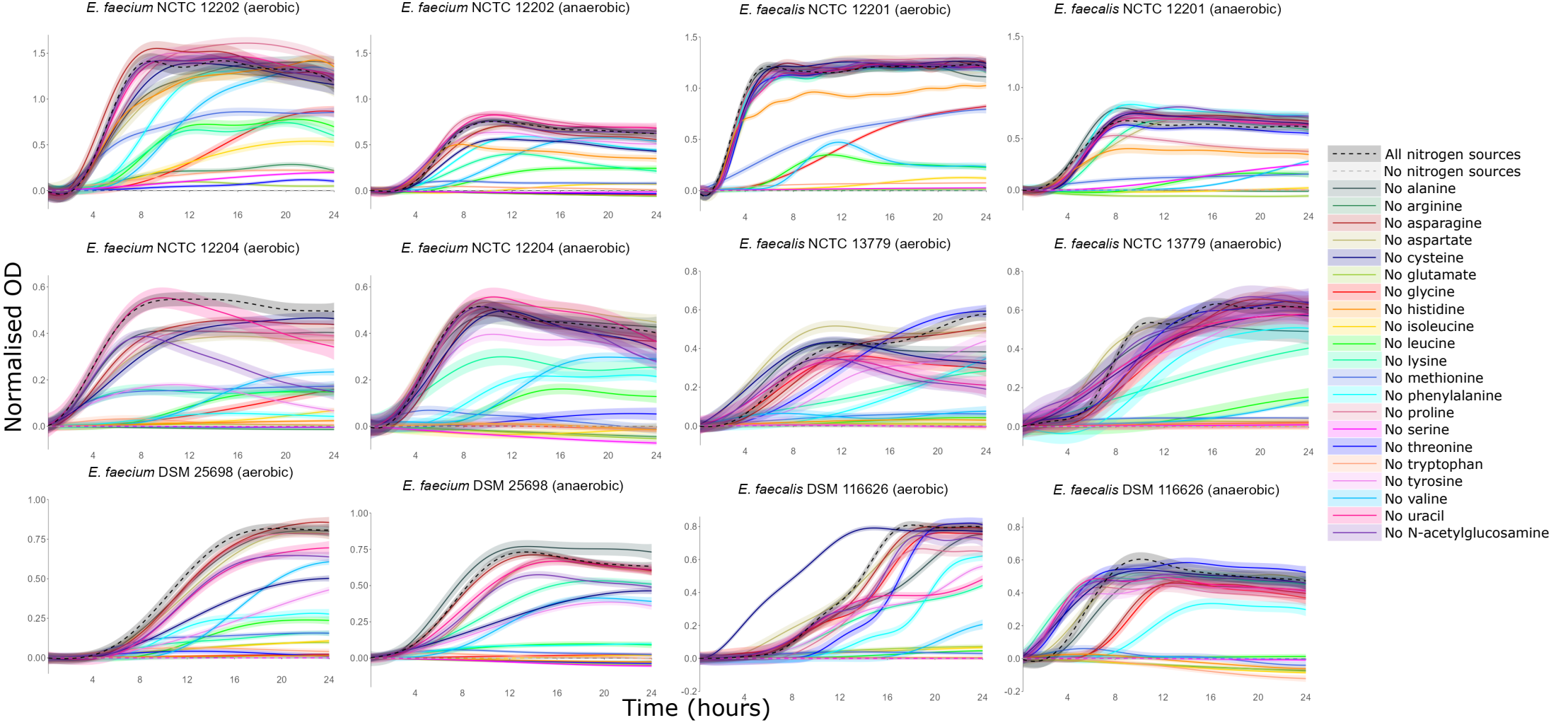

E. faecium (NCTC 12202) - Acetate
E. faecium (NCTC 12202) - Propionate
E. faecium (NCTC 12202) - Butyrate
E. faecium (NCTC 12202) - Valerate
E. faecium (NCTC 12204) - Acetate
E. faecium (NCTC 12204) - Propionate
E. faecium (NCTC 12204) - Butyrate
E. faecium (NCTC 12204) - Valerate
E. faecium (DSM 25698) - Acetate
E. faecium (DSM 25698) - Propionate
E. faecium (DSM 25698) - Butyrate
E. faecium (DSM 25698) - Valerate
E. faecalis (NCTC 12201) - Acetate
E. faecalis (NCTC 12201) - Propionate
E. faecalis (NCTC 12201) - Butyrate
E. faecalis (NCTC 12201) - Valerate
E. faecalis (NCTC 13779) - Acetate
E. faecalis (NCTC 13779) - Propionate
E. faecalis (NCTC 13779) - Butyrate
E. faecalis (NCTC 13779) - Valerate
E. faecalis (DSM 116626) - Acetate
E. faecalis (DSM 116626) - Propionate
E. faecalis (DSM 116626) - Butyrate
E. faecalis (DSM 116626) - Valerate
OD600
Concentration (mM)
No metabolite
Low (10)
Average (65)
High (120)
Low (5)
Average (15)
High (35)
Low (3)
High (40)
Low (0.5)
Average (3.5)
High (12)
4
8
16
32
64
128

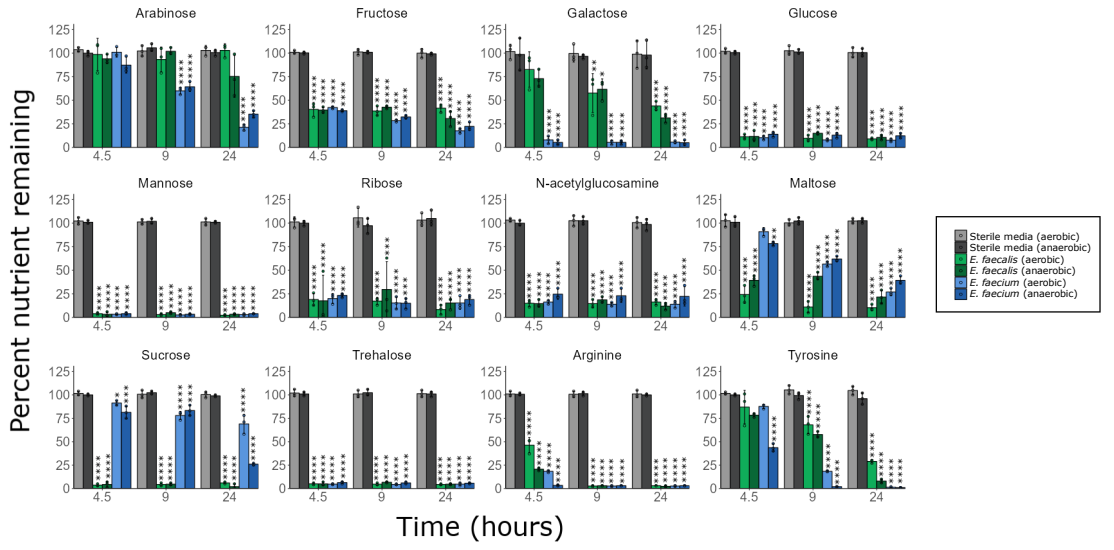

E. faecium (NCTC 12202)
E. faecalis (NCTC 12201)
E. faecium (NCTC 12204)
E. faecalis (NCTC 13779)
E. faecium (DSM 25698)
E. faecalis (DSM 116626)
OD600
0.0
0.2
0.4
0.6
6.0
6.5
7.0
pH
No metabolites
PBV
APBV

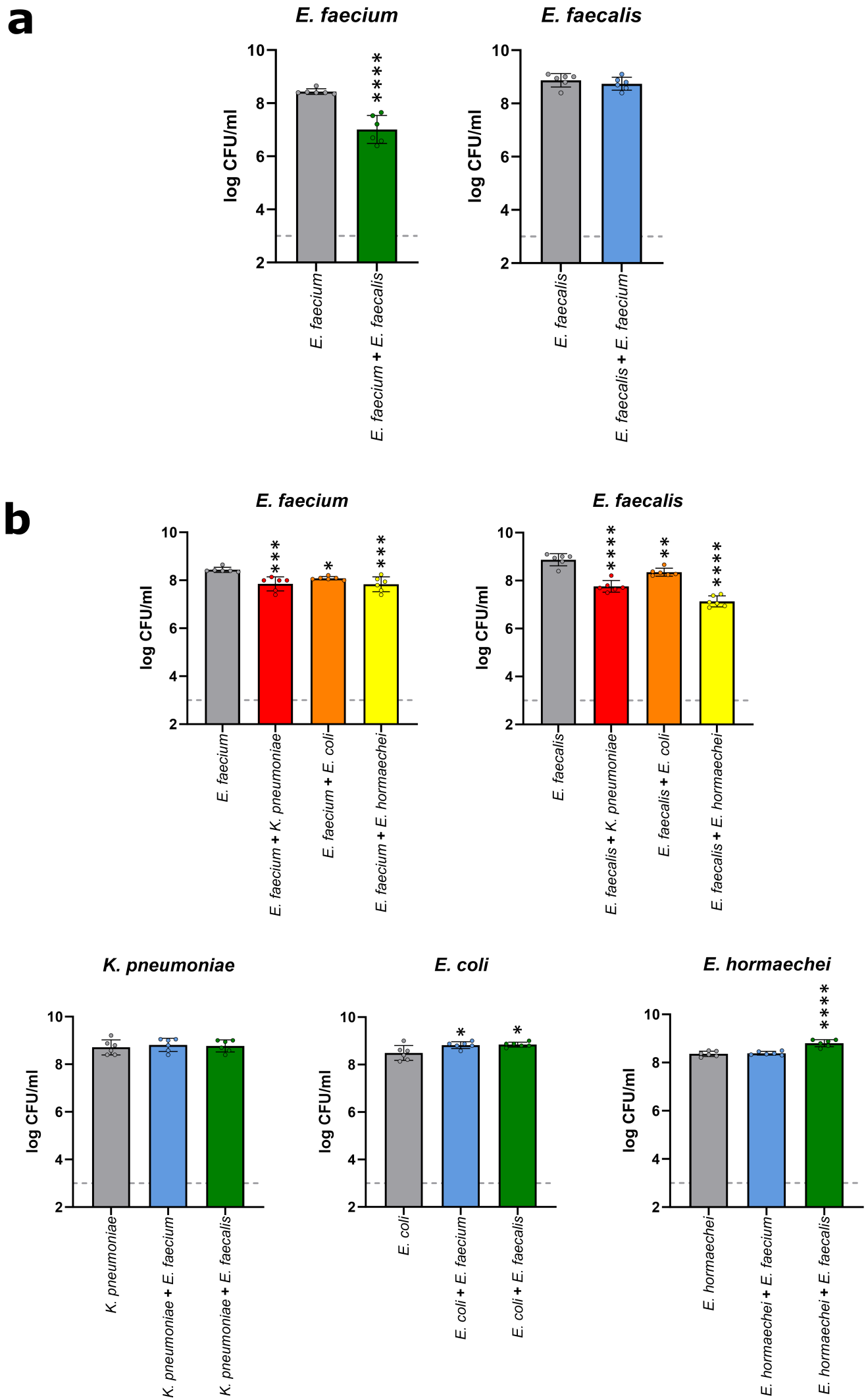

E. faecium NCTC 12202
E. faecalis NCTC 12201
E. faecium NCTC 12204
E. faecalis NCTC 13779
E. faecium DSM 25698
E. faecalis DSM 116626
Normalised OD
Time (hours)
No carbon (aerobic)
Nutrient mix (aerobic)
No carbon (anaerobic)
Nutrient mix (anaerobic)


a
E. faecium
***
****
**
**
10
9
8
7
6
log CFU/mL
Fresh medium
Faeces supernatant
Faeces supernatant + glucose
E. faecium supernatant
E. faecium supernatant + glucose
b
E. faecalis
***
**
****
**
10
9
8
7
6
log CFU/mL
Fresh medium
Faeces supernatant
Faeces supernatant + glucose
E. faecalis supernatant
E.faecalis supernatant + glucose


E. faecium NCTC 12202 (aerobic)
E. faecium NCTC 12202 (anaerobic)
E. faecalis NCTC 12201 (aerobic)
E. faecalis NCTC 12201 (anaerobic)
E. faecium NCTC 12204 (aerobic)
E. faecium NCTC 12204 (anaerobic)
E. faecalis NCTC 13779 (aerobic)
E. faecalis NCTC 13779 (anaerobic)
E. faecium DSM 25698 (aerobic)
E. faecium DSM 25698 (anaerobic)
E. faecalis DSM 116626 (aerobic)
E. faecalis DSM 116626 (anaerobic)
Normalised OD
Time (hours)
No carbon source
Arabinose
Fructose
Fucose
Galactose
Glucose
Mannose
Ribose
Xylose
N-acetylglucosamine
Maltose
Sucrose
Trehalose


E. faecium NCTC 12202 (aerobic)
E. faecium NCTC 12202 (anaerobic)
E. faecalis NCTC 12201 (aerobic)
E. faecalis NCTC 12201 (anaerobic)
E. faecium NCTC 12204 (aerobic)
E. faecium NCTC 12204 (anaerobic)
E. faecalis NCTC 13779 (aerobic)
E. faecalis NCTC 13779 (anaerobic)
E. faecium DSM 25698 (aerobic)
E. faecium DSM 25698 (anaerobic)
E. faecalis DSM 116626 (aerobic)
E. faecalis DSM 116626 (anaerobic)
Normalised OD
Time (hours)
All nitrogen sources
No nitrogen sources
No alanine
No arginine
No asparagine
No aspartate
No cysteine
No glutamate
No glycine
No histidine
No isoleucine
No leucine
No lysine
No methionine
No phenylalanine
No proline
No serine
No threonine
No tryptophan
No tyrosine
No valine
No uracil
No N-acetylglucosamine


Arabinose
Fructose
Galactose
Glucose
Mannose
Ribose
N-acetylglucosamine
Maltose
Sucrose
Trehalose
Arginine
Tyrosine
Percent nutrient remaining
Time (hours)
Sterile media (aerobic)
Sterile media (anaerobic)
E. faecalis (aerobic)
E. faecalis (anaerobic)
E. faecium (aerobic)
E. faecium (anaerobic)


a
E. faecium
E. faecalis
log CFU/ml
10
8
6
4
2
****
E. faecium
E. faecium + E. faecalis
E. faecalis
E. faecalis + E. faecium
b
E. faecium
E. faecalis
***
*
***
****
**
****
E. faecium
E. faecium + K. pneumoniae
E. faecium + E. coli
E. faecium + E. hormaechei
E. faecalis
E. faecalis + K. pneumoniae
E. faecalis + E. coli
E. faecalis + E. hormaechei
K. pneumoniae
E. coli
E. hormaechei
K. pneumoniae
K. pneumoniae + E. faecium
K. pneumoniae + E. faecalis
E. coli
E. coli + E. faecium
E. coli + E. faecalis
E. hormaechei
E. hormaechei + E. faecium
E. hormaechei + E. faecalis