CRISPR-Cas inhibits plasmid transfer and immunizes bacteria against antibiotic resistance acquisition in manure

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Abstract

The horizontal transfer of antibiotic resistance genes among bacteria is a pressing global issue. The bacterial defense system CRISPR-Cas acts as a barrier to the spread of antibiotic resistance plasmids, and CRISPR-Cas-based antimicrobials can be effective to selectively deplete antibiotic-resistant bacteria. While significant surveillance efforts monitor the spread of antibiotic-resistant bacteria in the clinical context, a major, often overlooked aspect of the issue is resistance emergence in agriculture. Farm animals are commonly treated with antibiotics, and antibiotic resistance in agriculture is on the rise. Yet, CRISPR-Cas efficacy has not been investigated in this setting. Here, we evaluate the prevalence of CRISPR-Cas in agricultural *Enterococcus faecalis* strains and its anti-plasmid efficacy in an agricultural niche – manure. Analyzing 1,986 *E. faecalis* genomes from human and animal hosts, we show that the prevalence of CRISPR-Cas subtypes is similar between clinical and agricultural *E. faecalis* strains. Using plasmid conjugation assays, we found that CRISPR-Cas is a significant barrier against resistance plasmid transfer in manure. Finally, we used a CRISPR-based antimicrobial approach to cure resistant *E. faecalis* of erythromycin resistance, but this was limited by delivery efficiency of the CRISPR antimicrobial in manure. However, immunization of bacteria against resistance gene acquisition in manure was highly effective. Together, our results show that *E. faecalis* CRISPR-Cas is prevalent and effective in an agricultural setting and has the potential to be utilized for depleting antibiotic-resistant populations. Our work has broad implications for tackling antibiotic resistance in the increasingly relevant agricultural setting, in line with a One Health approach.

47 **Importance**

48 Antibiotic resistance is a growing global health crisis in human and veterinary medicine.
49 Previous work has shown technologies based on CRISPR-Cas - a bacterial defense system - to
50 be effective in tackling antibiotic resistance. Here we test if CRISPR-Cas is present and
51 effective in agricultural niches, specifically in the ubiquitously present bacterium – *Enterococcus*
52 *faecalis*. We show that CRISPR-Cas is both prevalent and functional in manure, and has the
53 potential to be used to specifically kill bacteria carrying antibiotic resistance genes. This study
54 demonstrates the utility of CRISPR-Cas based strategies for control of antibiotic resistance in
55 agricultural settings.

56

57 **1. Introduction**

58 A widely prevalent adaptive immunity system possessed by bacteria and archaea is Clustered
59 Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas [1,2]. Although it has now been
60 programmed as a tool to edit genomes of a large number of organisms[3–6], in its native
61 environment, it confers bacteria and archaea defense against mobile genetic elements (MGE)
62 like viruses and plasmids[1,2,7,8]. Upon entry of the MGE, a portion of its DNA, known as a
63 protospacer, is integrated into the host genome in the form of a spacer[8]. Later, the spacer is
64 transcribed (called crRNA) and loaded on Cas-encoded nuclease(s) to surveille for future MGE
65 entries. If a region of an incoming MGE is complementary to the crRNA, the MGE is cleaved by
66 the nuclease[9–11]. Since its discovery, a large majority of studies on the CRISPR-Cas mechanism
67 of action have focused on its antiviral activity. However approximately 10% of CRISPR
68 protospacers identified in bacteria and archaea target plasmids, suggesting that CRISPR-Cas
69 naturally provides defense against invading plasmids as well[12]. Indeed, CRISPR-Cas has been
70 shown to act as a natural barrier against conjugative plasmid transfer[13,14].

71

72 A highly relevant clinical organism in the study of CRISPR-Cas function is *Enterococcus*
73 *faecalis. E. faecalis* is a Gram-positive bacterium routinely isolated from a large range of hosts
74 including animals, humans and environmental samples such as plants, sand, and water[15,16,17]. It
75 is an opportunistic pathogen and causes diseases including urinary tract infections, infective
76 endocarditis and bacteremia[18–21]. Owing in part to its significant repertoire of intrinsic antibiotic
77 resistance genes and its ability to efficiently acquire resistance genes from external sources, *E.*
78 *faecalis* is one of the leading causes of hospital-acquired infections[22,23]. *E. faecalis* also
79 colonizes several food animals and strains from these sources have been reported to carry both
80 antibiotic resistance and virulence genes [24,25]. Further, due to the generalist nature of *E.*
81 *faecalis*, transmission of resistant *E. faecalis* from animals to humans (zoonosis) can occur.
82 Indeed, there have been several cases of probable zoonotic transmission leading to major
83 outbreaks in the past[26,27], and enterococci, including *E. faecalis*, are routinely monitored in retail
84 meat as part of the National Antimicrobial Resistance Monitoring System[28].

85

86 Some *E. faecalis* strains encode Type II CRISPR-Cas systems which employ a crRNA-Cas9
87 nuclease-tracrRNA complex to surveille and cleave MGEs[29–31]. *E. faecalis* has two functional
88 CRISPR-Cas loci – CRISPR1-Cas and CRISPR3-Cas. Both have a CRISPR array consisting of
89 36 bp repeats interspersed with 30 bp spacers. Both loci have their own set of *cas* genes and
90 protospacer adjacent motif (PAM) sequences, suggesting distinct modes of action and targets.
91 Almost every *E. faecalis* strain examined so far has also been found to have another CRISPR
92 locus called CRISPR2, however it lacks *cas* genes and is thus not independently functional[32].
93 Since plasmid-mediated transfer of antibiotic resistance genes is a major contributor to
94 resistance transmission in *E. faecalis*[23,33], it is important to test if CRISPR-Cas could act as a
95 barrier against this process, especially in conditions where zoonotic transmission may occur.

96 Additionally, the prevalence and function of CRISPR-Cas in strains of animal origin should be
97 investigated, as most focus has been on human-origin strains. Some recent studies have looked
98 at *E. faecalis* samples isolated from wastewater treatment plants, animal feces and soil.
99 However, these studies have either small sample sizes, low source diversity or unclear origin of
100 strains[34–36].

101

102 Previous work has shown *E. faecalis* CRISPR-Cas systems confer defense against plasmids
103 and can be programmed as antimicrobials against specific antibiotic-resistant strains, both *in*
104 *vitro* and *in vivo* in the mouse gut [14,37]. During in vitro mating experiments, CRISPR-Cas acted
105 as a moderately effective barrier against plasmid transfer, while in vivo its efficiency was
106 strikingly robust[14], suggesting that the conjugation environment impacts CRISPR-Cas function.
107 Given the importance of agriculturally relevant niches in *E. faecalis* biology, it is crucial to test
108 how effective CRISPR-Cas is in this setting. A key niche of this setting is manure. Animal
109 manure, which is frequently used as fertilizers, are a known reservoir of antibiotic resistance
110 gene-encoding pathogenic bacteria including *E. faecalis.* Thus they are potential hubs for
111 zoonotic transmission[38–40], as well as potential critical control points. Since previous work in *E.*
112 *faecalis* has demonstrated the surrounding environmental plays a major role in CRISPR-Cas
113 efficacy[14], discerning its efficacy in manure could be of major significance.

114

115 In this study, we first identified the prevalence of CRISPR-Cas in a large cohort of agricultural *E.*
116 *faecalis* strains. Next, we evaluated CRISPR-Cas efficacy in sterilized manure using previously
117 established model *E. faecalis* strains. Finally, we tested the effectiveness of CRISPR-
118 antimicrobials designed to eliminate antibiotic resistance genes from *E. faecalis* in manure.

119

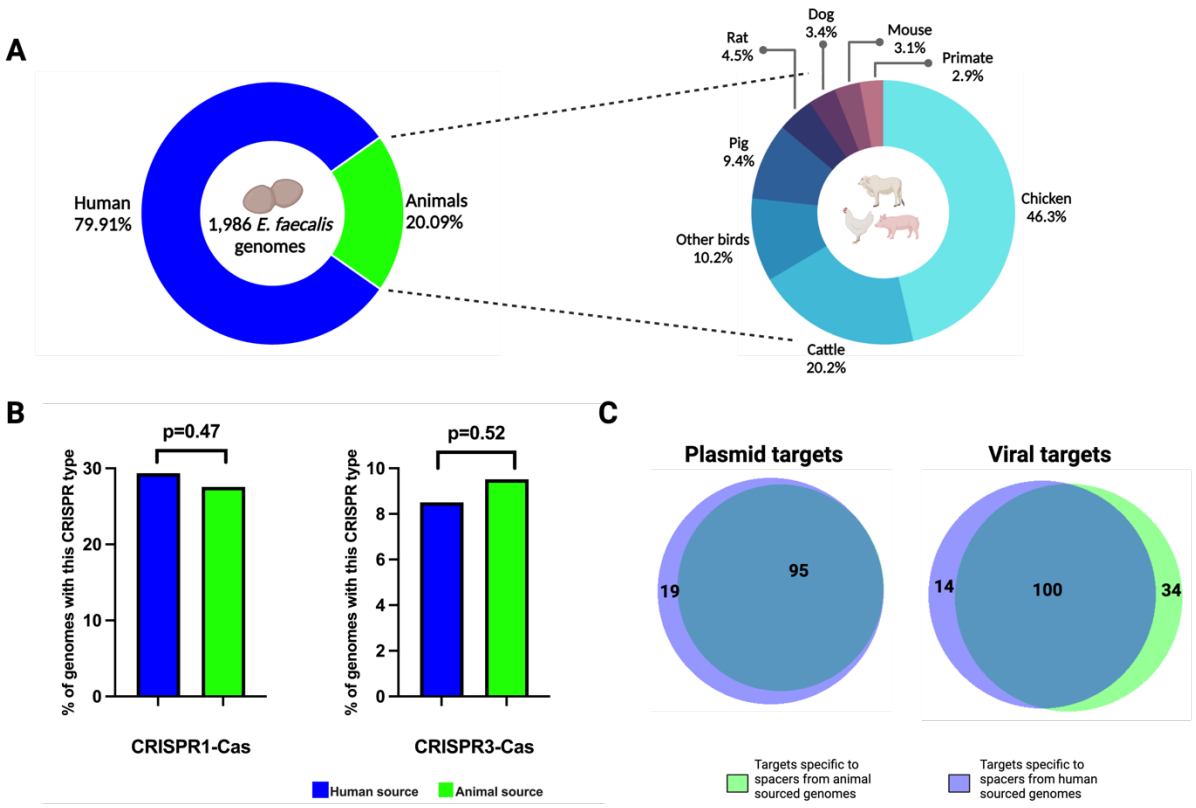
120 **2. Results**

121 **CRISPR-Cas prevalence in agricultural *Enterococcus faecalis* is similar to that in human-**
122 **derived strains**

123 Before testing the efficacy of CRISPR-Cas in a model agricultural setting, we wanted to
124 determine how prevalent CRISPR-Cas is in agriculturally-derived *E. faecalis* and how it
125 compares to its prevalence in strains derived from humans. For this, we downloaded all *E.*
126 *faecalis* genomes from NCBI Refseq available as of September 1, 2021, along with genomes
127 from a recent large-scale OneHealth investigation of Enterococci[25]. We segregated the
128 genomes based on source of isolation – human or animal (the term animal here is an umbrella
129 term for non-human sources like food animals, birds, pets etc.). Together, these comprised
130 1587 genomes sourced from humans (79.91%) and 399 sourced from animals (20.09%) (Fig
131 1A), for a total of 1,986 genomes. To identify CRISPR-Cas loci in these genomes, we performed
132 a BLAST search using the *cas9* sequences of the *E. faecalis* CRISPR1-Cas and CRISPR3-Cas
133 loci as queries, and then searched for bona fide CRISPR arrays within the set of genomes
134 containing *cas9*. We focused on CRISPR1-Cas and CRISPR3-Cas for this analysis since these
135 are the only two independently functional CRISPR arrays in *E. faecalis*. We found that 27.57%
136 of animal-derived strains possessed CRISPR1-Cas, and 9.52% strains possessed CRISPR3-
137 Cas. This was similar to their prevalence in human-derived strains – 29.36% for CRISPR1-Cas
138 and 8.5% for CRISPR3-Cas (Fig 1B). Next, we investigated the CRISPR arrays of these strains
139 to determine whether and to what extent the targets of CRISPR-Cas systems in these two
140 groups differ. For this, we extracted the CRISPR-Cas arrays corresponding to the established *E.*
141 *faecalis* CRISPR loci from the genomes, and collated the spacers across them separately for
142 animal and human derived genomes. For assigning targets to these spacers, we created a
143 database of plasmids isolated from *E. faecalis, E. faecium and E. hirae*, as well as the NCBI
144 viral genome database. With respect to plasmid targets, we found 95 targets for the set of

145 spacers from animal derived *E. faecalis*, and 114 targets for spacers derived from human
146 sources. The Venn diagram in Fig 1C shows that all targets of animal derived strains were also
147 in the list of targets of human derived strains. With respect to viral targets, we found 134 targets
148 for animal derived strains, and 114 targets for human derived strains. Again, a large majority of
149 the targets (100) overlapped between the two groups of targets, suggesting major similarity in
150 CRISPR-Cas targets across the two environments. This suggests that CRISPR-Cas prevalence
151 is consistent across human and animal niches along with major similarities in their targets, with
152 the caveat that our study is limited to the *E. faecalis* strains that have been selected for
153 sequencing by other groups, which may have biases (i.e. prioritization of antibiotic-resistant
154 strains for sequencing).

155



156

157 **Figure 1**

158 **FIG 1** *E. faecalis* strains studied and their CRISPR-Cas abundances and targets. (A) shows the
159 distribution of *E. faecalis* strains from human and animal sources analyzed in this study. (B)
160 shows relative abundances of CRISPR-Cas types in *E. faecalis* strains from animal and human
161 sources. Percentages were calculated as the number of strains in each source category
162 (animal/human) containing a particular CRISPR-Cas type (CRISPR1-Cas or CRISPR3-Cas)
163 divided by the total number of strains in that source category. p-values were calculated using
164 the Z test for equality of two percentages using independent samples. (C) shows Venn
165 diagrams for plasmid and viral targets of spacers derived from animal and human sourced *E.*
166 *faecalis*.

167

168 **CRISPR-Cas blocks plasmid transfer in manure, and manure-specific effects on CRISPR**
169 **defense are observed**

170 We next tested the hypothesis that *E. faecalis* CRISPR-Cas is effective in an agricultural niche
171 (manure). We tested the efficacy of CRISPR-Cas in blocking the dissemination of pAM714 from
172 *E. faecalis* OG1SSp to *E. faecalis* T11RF (Fig 2). For this, we performed conjugation assays in
173 three conditions - solid brain heart infusion (BHI) agar, liquid BHI broth, and liquified autoclaved
174 dairy manure (Fig 3). Two different recipients were used, T11RF with a functional CRISPR-Cas
175 system, and T11RF∆*cas9* which lacks functional CRISPR-Cas defense against pAM714 (Table
176 1)[13,14]. For these experiments, donor and recipient cells were mixed together and either spread
177 on BHI agar to form a biofilm, or inoculated into liquid BHI, or manure. After 18 hours incubation,
178 agar plate biofilms were harvested and resuspended in buffer, and liquid cultures (liquid BHI
179 and manure) were sampled as well. These were then plated for determination of colony forming
180 units (CFU) of donors, recipients, and transconjugant cells. This timepoint is referred to as
181 passage 0.

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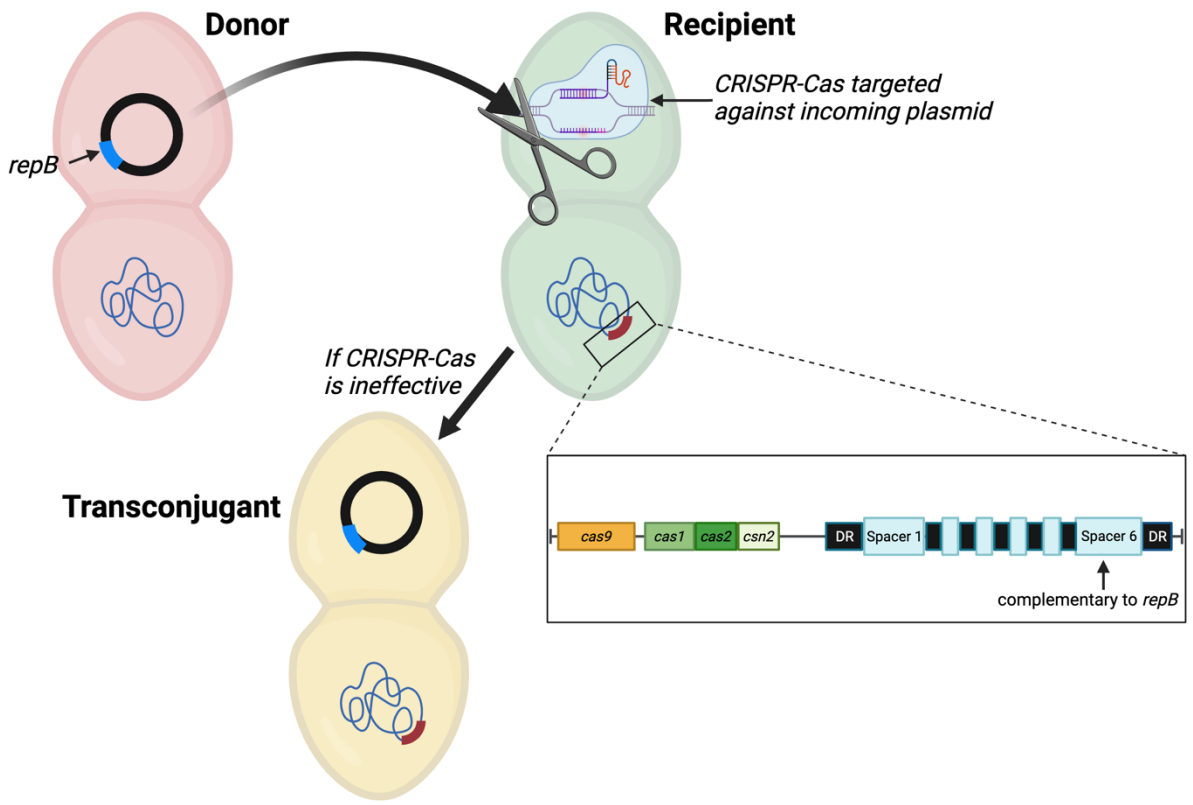

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184 **Figure 2**

185 **FIG 2** A schematic representation of the concept. The donor strain OG1SSp contains a
186 pheromone-responsive plasmid which harbors a *repB* gene (blue). The recipient strain T11RF
187 encodes a Type-II CRISPR-Cas system (red) containing a spacer with perfect complementarity
188 to *repB*. If CRISPR-Cas is effective in a particular niche, it will block the entry of the incoming
189 plasmid. If not, the plasmid enters the recipient, which is then termed a transconjugant.

190

191

192

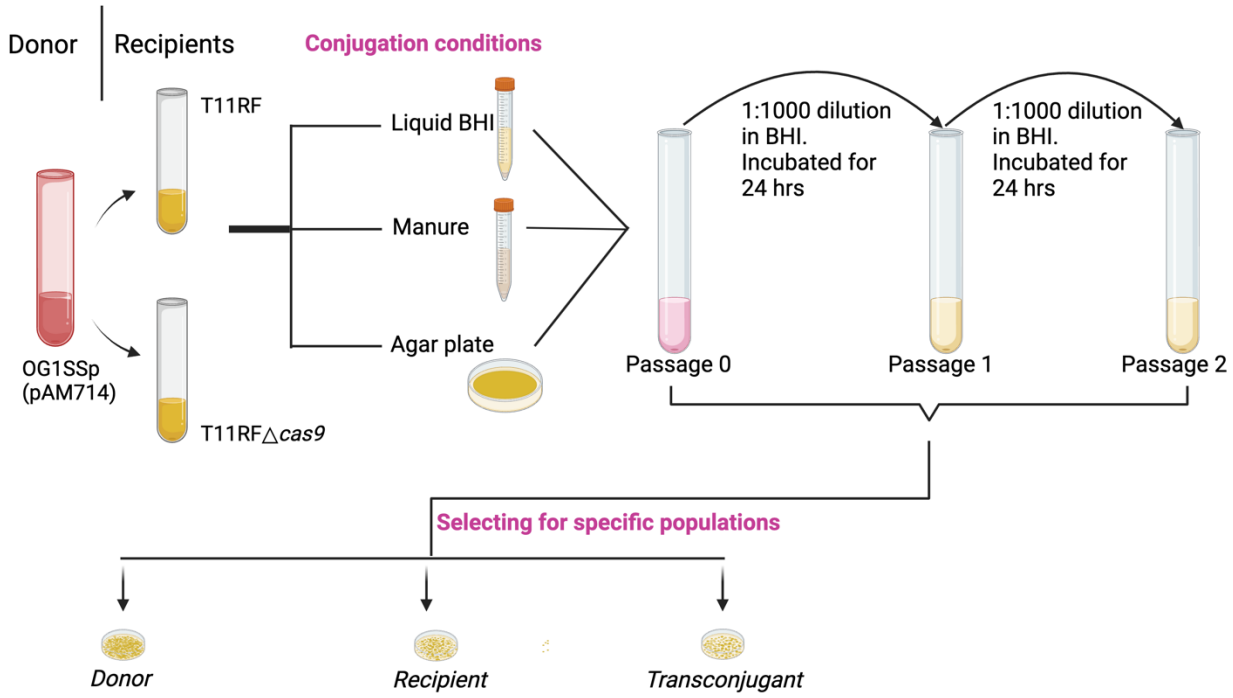
193

194 **Table 1** *E. faecalis* strains used in this study

Strain	Description	Reference(s)
T11RF	Rifampin-fusidic acid-resistant derivative of strain T11	[41,42]
T11RFΔcas9	T11RF with an in-frame deletion of *cas9*	[41]
OG1SSp(pAM714)	Spectinomycin-streptomycin-resistant derivative of strain OG1 harboring pAM714, conferring erythromycin resistance via Tn*917* insertion upstream of the par locus; *cyl*+	[43,44]
CK135RF	Spontaneous fusidic acid-resistant derivative of CK135	[37]

195

196 In passage 0, we found transconjugants in the range of 10^4–10^7 CFU/mL across the three
197 environments and both recipients, suggesting widespread plasmid transfer across all 6
198 combinations (Fig 4). However, there was a significant difference in pAM714 conjugation
199 frequency (number of transconjugants divided by the number of donors) on BHI agar in the
200 absence vs presence of *cas9*, with a higher conjugation frequency in the absence of functional
201 CRISPR-Cas (Fig 5a). This was expected since it has been reported previously, demonstrating
202 that CRISPR-Cas is a moderately effective barrier against plasmid transfer on solid agar[14]. This
203 effect was also seen in liquid BHI medium, and importantly, also in manure, although the
204 difference in conjugation frequency in manure for T11RF versus T11RFΔ*cas9* recipients was
205 less than the other conditions at this time point.



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207 **Figure 3**

208 **FIG 3** A schematic of the experimental design. The donor strain (OG1SSp(pAM714)) was mixed
209 with two versions of the recipient (T11RF, T11RFΔ*cas9*) in three conditions – liquid BHI,
210 manure and BHI agar plate. After 18 hours, the mixture (Passage 0) was plated to quantify
211 donor (spectinomycin, streptomycin, and erythromycin selection), recipient + transconjugants
212 (rifampin and fusidic acid selection) and transconjugant (rifampin, fusidic acid, and erythromycin
213 selection) populations. At the same time, Passage 0 was also diluted into fresh liquid BHI
214 (1:1000 dilution) and incubated for 24 hours to give Passage 1. The Passage 1 culture was
215 similarly used to generate Passage 2.

216

217 We also performed two successive passages of these initial conjugation mixtures. For passage
218 1, we diluted the passage 0 conjugation mixture 1:1000 into fresh BHI broth and incubated it at
219 37°C for 24 hours, followed by plating on selective agars. Transferring the conjugation mixture
220 from manure exposure to liquid BHI was a way to model transition between two nutritional
221 environments. Here, we found the conjugation frequencies in the presence of CRISPR-Cas as
222 opposed to its absence, continued to be significantly lower across all three conditions
223 ($p<0.0001$, $p<0.0001$, $p=0.0009$ for manure, agar and BHI respectively) (Fig 5b). This
224 demonstrates that CRISPR-Cas continued to be effective at blocking plasmid transfer in this
225 sample set.

226

227 Next, passage 1 conjugation mixtures were diluted and incubated as described above to obtain
228 passage 2 cultures. By the end of passage 2, the transconjugant populations in agar and liquid
229 BHI media were almost completely absent (limit of detection of 10^2 CFU/mL), irrespective of
230 CRISPR-Cas. As a result, no differences in conjugation frequencies across the two conditions
231 were observed (Fig 5c). Surprisingly, for conjugation mixtures that originated from manure,
232 there was a sizeable transconjugant population following passage 2, but only in the absence of
233 functional CRISPR-Cas (Fig 4, top right). As a result, the conjugation frequency in the absence
234 of CRISPR-Cas was significantly higher than in the presence of CRISPR-Cas (Fig 5c). These
235 results suggest a manure-specific effect on either CRISPR-Cas function and/or the
236 competitiveness of T11RF Δ*cas9*(pAM714) transconjugants.

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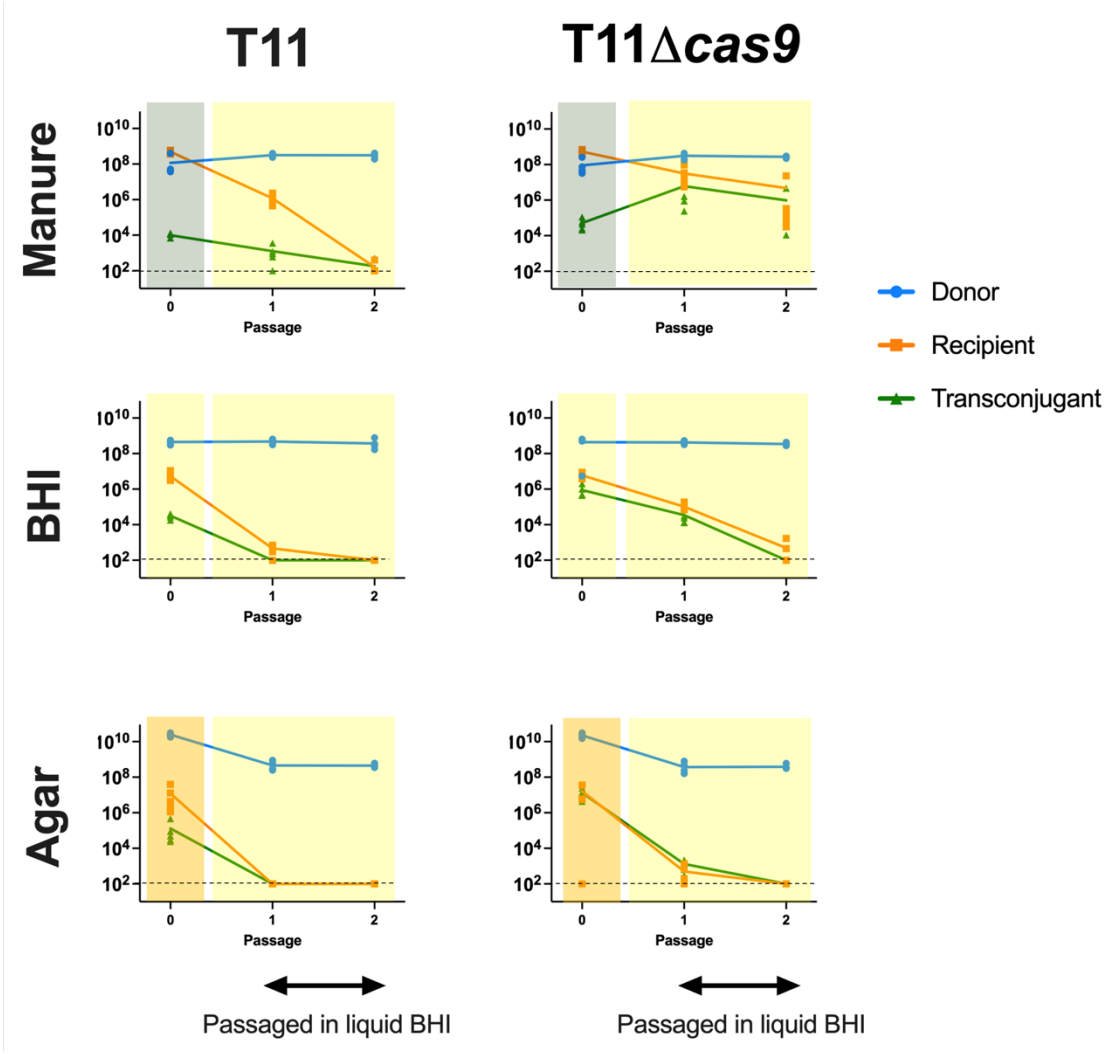

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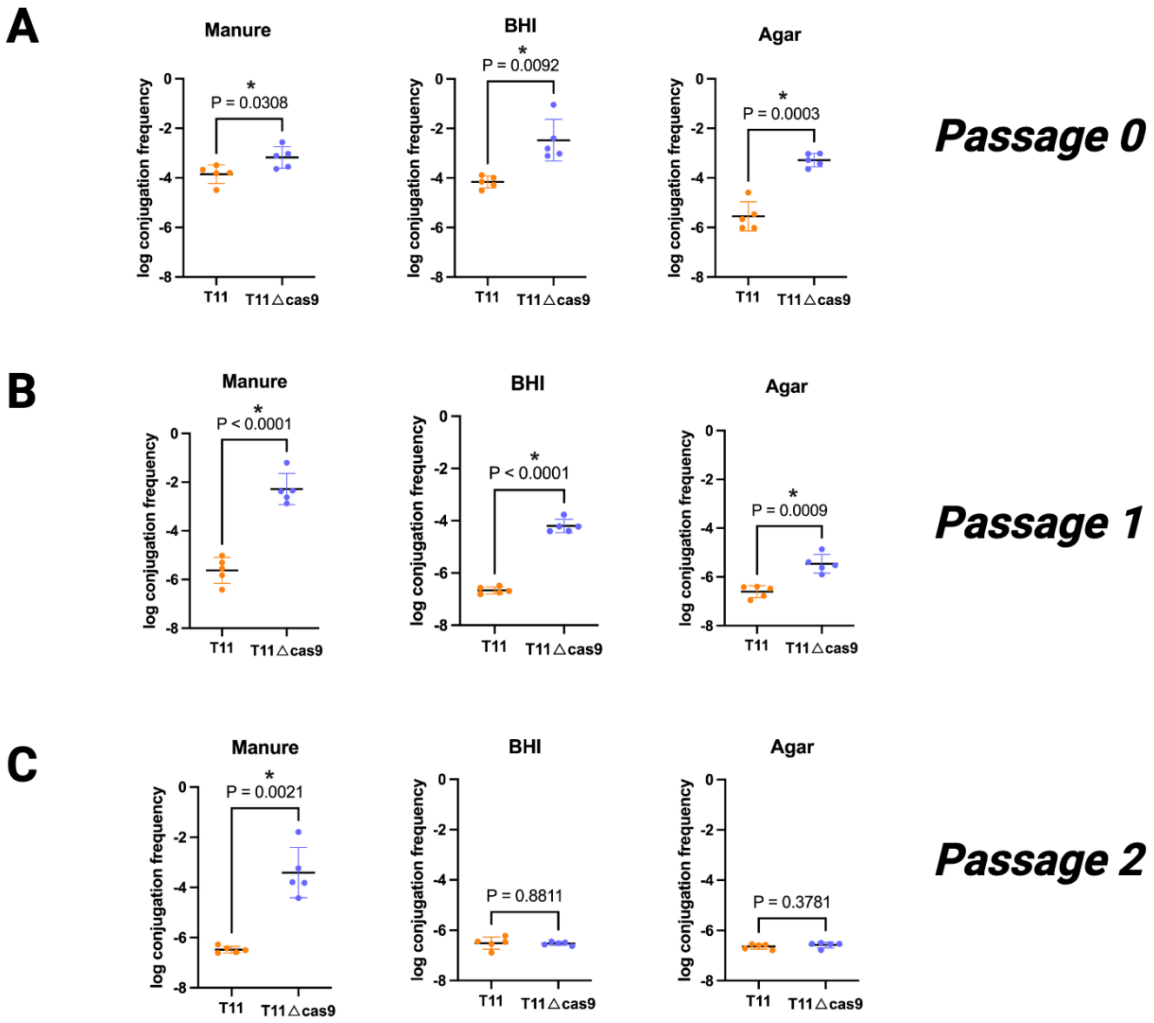
239

240 **Figure 4**

241 **FIG 4** Bacterial populations across time and niche. Each graph shows the number of donors,
242 recipients, and transconjugants in different environments. The conjugation experiments shown
243 on the left had T11RF as the recipient and those on right had T11RFΔ*cas9*. Colors in the
244 background represent the condition in which the bacteria were growing: manure (dark green),
245 liquid BHI (yellow) and solid agar plate (light orange). Dashed lines represent a CFU/ml count of
246 10^2 which is the limit of detection.



247

248 **Figure 5**

249 **FIG 5** CRISPR-Cas conjugation frequency under different conditions. Each point represents log
250 of conjugation frequency (calculated as number of transconjugants divided by number of
251 donors) corresponding to each of the three initial conjugation conditions (manure, BHI broth,
252 BHI agar). A represents Passage 0 (right after initial conjugation reaction); B and C represent
253 subsequent overnight passages - Passage 1 and Passage 2. Student's t-test was used to
254 calculate p-values after determining that log conjugation frequency values followed a normal
255 distribution.

256

257 **CRISPR antimicrobials appear effective in manure, but are limited by low conjugation**
258 **frequency**

259 Having shown that CRISPR-Cas can function in an agriculturally-relevant *in vitro* setting, we
260 next wanted to see if CRISPR-Cas-based antimicrobials can be used to selectively deplete
261 antibiotic-resistant *E. faecalis* in that niche. Previous studies have tested their antimicrobial
262 efficacy in solid agar and the murine gastrointestinal tract[37]. Solid agar is a popular choice for
263 conjugation experiments due to the simplicity of its make-up and experimental convenience, but
264 for CRISPR-based antimicrobials to be widely used, they need to be tested in settings that
265 better mimic the *in situ* environment. Manure is one such environmental niche, since it plays an
266 important role in the emergence and dissemination of resistant strains in agricultural settings.
267 For this experiment, we used a previously designed CRISPR antimicrobial referred to as
268 pKH88[37]. pKH88 is a derivative of the pheromone-responsive plasmid, pPD1, and has been
269 modified to express *cas9* and spacers targeting either *tetM* (for tetracycline resistance) or *ermB*
270 (for erythromycin resistance). For our experiments, we delivered pKH88[sp-*tetM*] or pKH88[sp-
271 *ermB*] from the *E. faecalis* strain CK135RF (donor strain), which is a derivative of the strain
272 OG1. Our target (recipient) strain was OG1SSp(pAM714), which encodes *ermB* on pAM714.
273 Figure 6 shows a schematic diagram of our approach. In this scenario, our goals were to deliver
274 pKH88[sp-*ermB*] to OG1SSp(pAM714), and then either (a) kill or inhibit the recipients' growth by
275 Cas9-mediated DNA damage, or (b) promote the loss of pAM714 via Cas9-mediated DNA
276 damage. We expected no effects of delivering pKH88[sp-*tetM*] to OG1SSp(pAM714), as
277 OG1SSp(pAM714) does not encode *tetM*; pKH88[sp-*tetM*] is our control.

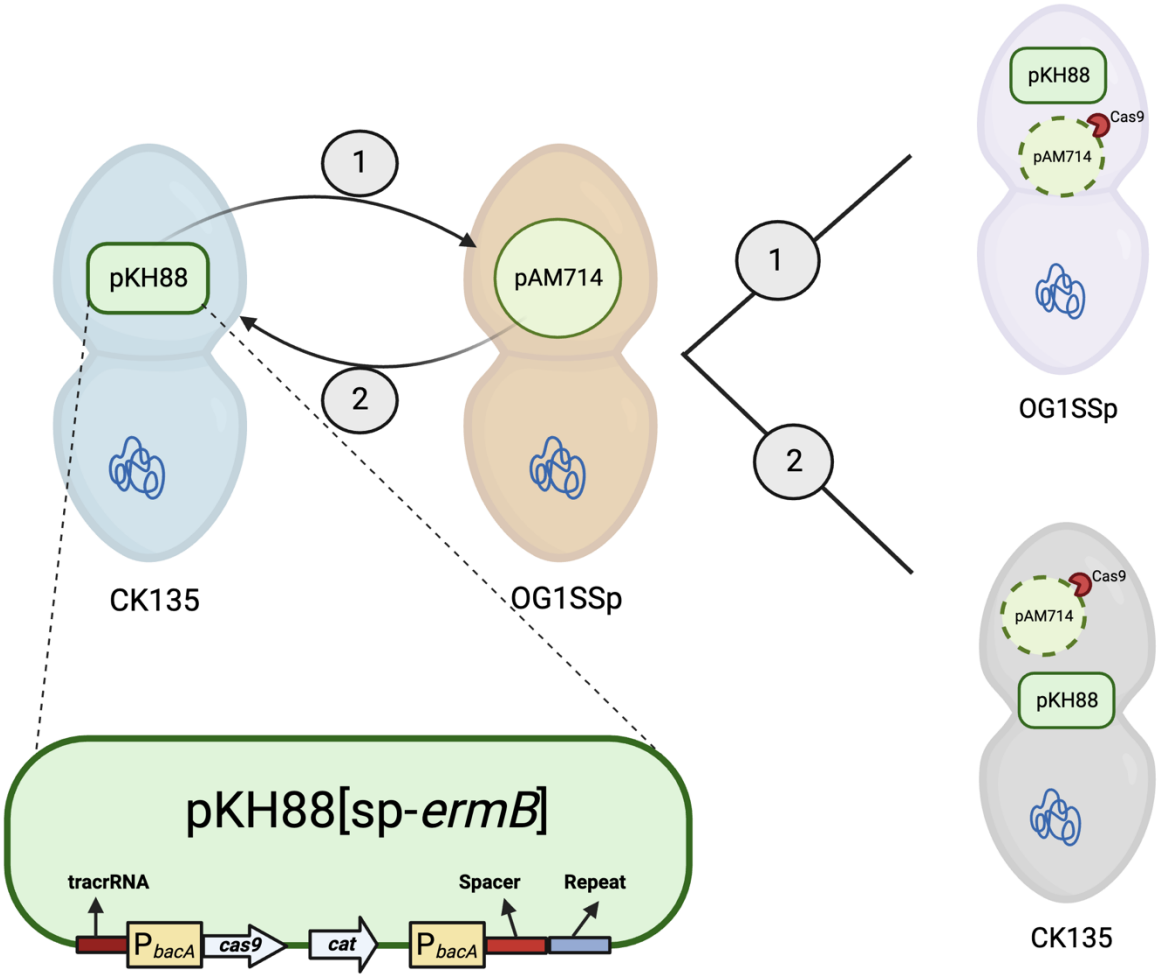


Figure 6

FIG 6 A schematic representation of the CRISPR antimicrobial. The strain CK135RF contains the pheromone-responsive plasmid pKH88, which encodes *cas9* and a CRISPR guide RNA under a constitutively active *bacA* promoter. The guide RNA consists of a spacer that targets either the *ermB* gene or the *tetM* gene, and a repeat sequence. The *cat* gene, encoding chloramphenicol resistance, is inserted in the plasmid only for tracking purposes. The strain OG1SSp contains the plasmid pAM714 which harbors the *ermB* gene. In scenario 1, the CRISPR antimicrobial pKH88[sp-*ermB*] enters OG1SSp and cleaves pAM714. In scenario 2,

287 OG1SSp(pAM714) donates pAM714 to CK135RF(pKH88[sp-*ermB*]) and the CRISPR
288 antimicrobial cleaves the incoming plasmid.

289

290 With this setup, we tested the hypothesis that the pKH88[sp-*ermB*] CRISPR antimicrobial
291 plasmid could selectively deplete erythromycin resistance from *E. faecalis* populations cultured
292 in manure. CRISPR antimicrobial donor strains and OG1SSp(pAM714) recipients were mixed
293 and plated on solid agar or inoculated into liquid BHI or manure, and passages 0, 1 and 2 were
294 assessed for donor, recipient, and transconjugant CFU/mL as described above. We observed a
295 significant decrease in the erythromycin resistant population by passage 2 for agar plate
296 conjugations (Fig 7a), in line with previously published results[37]. However, a similar decrease in
297 the erythromycin-resistant population was not observed for liquid BHI or manure conditions in
298 any of the passages (Fig 7a). To investigate a possible cause for this, we measured the
299 transconjugant population in each case. We found that the transconjugant populations were
300 substantially lower in liquid BHI and manure across all passages (10^2 (limit of detection) to 10^7),
301 as compared to agar (10^9 to 10^{10}) (Fig 7b). A possible reason for this could be the absence of a
302 solid surface for the bacteria to form a biofilm in the case of liquid BHI and manure, which
303 promotes conjugation[45]. However, in both conditions and across all three passages (with the
304 exception of passage 0 in liquid BHI), there were fewer transconjugants in the case of the
305 experimental antimicrobial pKH88-*ermB* as compared to the control pKH88[sp-*tetM*] (Fig 7b),
306 and this reduction was significant in passages 1 and 2 in manure. Assuming similar plasmid
307 transfer levels for both pKH88[sp-*ermB*] and pKH88[sp-*tetM*], this could be explained as the
308 action of the antimicrobial: OG1SSp(pAM714) recipients that received pKH88[sp-*ermB*] had
309 their pAM714 cleaved due to CRISPR-Cas targeting and hence were not detected in
310 transconjugant-selective plates. This suggests that the antimicrobial was effective in manure
311 when conjugation did occur.

312

313 To further test the efficacy of the antimicrobial, we looked at the subset of the transconjugant
314 population that was erythromycin-resistant. These would be OG1SSp(pAM714) recipient cells
315 that received the pKH88[sp-*ermB*] or [sp-*tetM*] antimicrobial plasmid and also maintained
316 erythromycin resistance. We found that in almost every passage for both manure and agar, this
317 cell population was significantly lower in the case of the experimental antimicrobial pKH88[sp-
318 *ermB*] as compared to the control pKH88[sp-*tetM*] (Fig 7c). This again points to the
319 effectiveness of the antimicrobial.

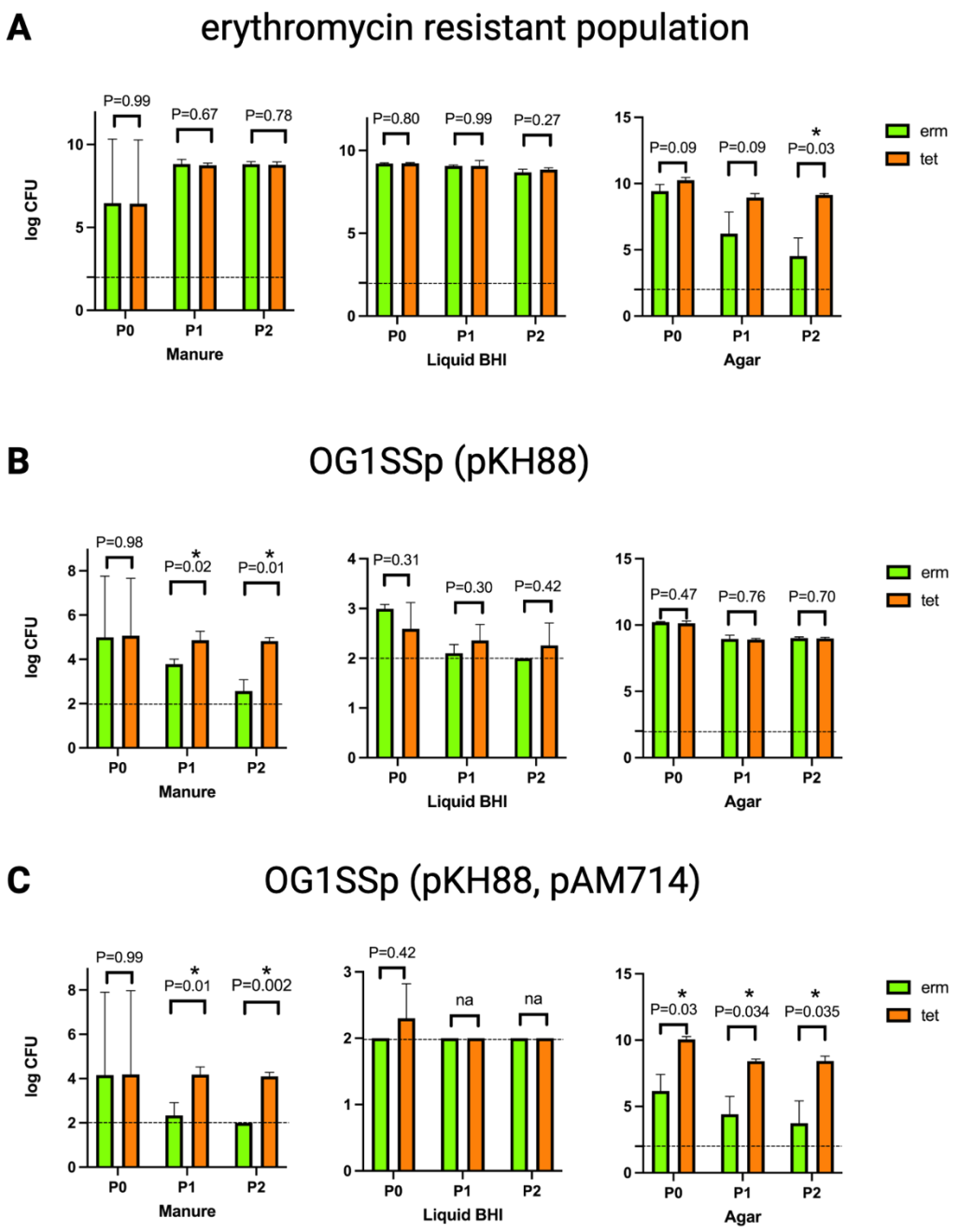


Figure 7

FIG 7 Evaluating CRISPR antimicrobial efficiency. (A) shows the number of total erythromycin-resistant bacteria in the mixture, across the three conditions, 3 passages and with the control (tet, in brown) or experimental (erm, in green) CRISPR antimicrobial. A lower number on this

325 graph signifies depletion of the erythromycin-resistant population. (B) shows the number of
326 transconjugants i.e. the recipient (OG1SSp) cells that have accepted the antimicrobial plasmid
327 pKH88. (C) shows the number of OG1SSp(pKH88) transconjugants that still maintain the
328 pAM714 plasmid. All values were log transformed and t-test with Welch's correction was used to
329 generate p-values. 'na' represents cases where values in both groups were the same and
330 hence a t-test could not be performed. Dashed lines represent a CFU/ml of 10^2 which is the limit
331 of detection.

332

333 Finally, we tested if the presence of the CRISPR antimicrobial could immunize the donor
334 CK135RF(pKH88) bacteria against acquiring the *ermB* gene from OG1SSp(pAM714). For this
335 we calculated the number of CK135RF(pKH88) bacteria that were erythromycin-resistant, which
336 could be the result of pAM714 transferring from OG1SSp to CK135RF. We observed a stark
337 difference in this population between the experimental and control antimicrobial. The number of
338 erythromycin-resistant CK135RF(pKH88) was significantly lower in the case of the *erm* targeting
339 antimicrobial as compared to the one that targeted the *tet* gene (Fig 8). This shows that the
340 pKH88[sp-*ermB*] CRISPR antimicrobial immunized its host CK135RF against acquisition of
341 erythromycin resistance. This effect was seen in both manure and agar.

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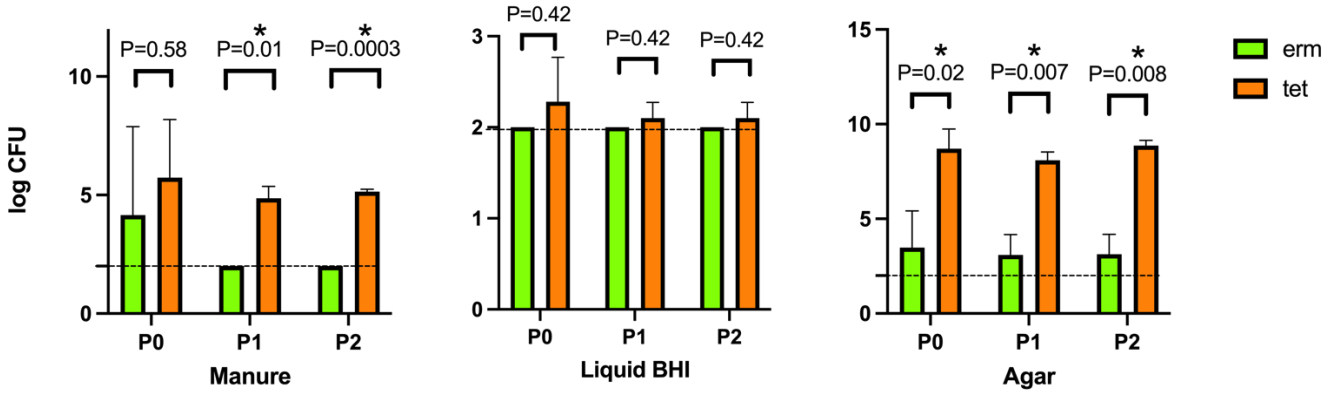

344

345 **Figure 8**

346 **FIG 8** Immunization of bacteria against antibiotic resistance acquisition in manure. Bars
347 represent number of CK135RF cells that have become erythromycin-resistant, likely via
348 accepting pAM714 from OG1SSp, in addition to carrying pKH88. A lower number represents
349 immunization via the antimicrobial plasmid pKH88. All values were log transformed and t-test
350 with Welch's correction was used to generate p-values. Dashed lines represent a CFU/ml of 10^2
351 which is the limit of detection.

352

353 **3. Materials and Methods**

354 *Bacteria and reagents used:* *E. faecalis* strains were grown in brain heart infusion (BHI) broth or
355 on agar plates. Antibiotic concentrations used were as follows: rifampin, 50 µg/ml; fusidic acid,
356 25 µg/ml; spectinomycin, 500 µg/ml; streptomycin, 500 µg/ml; erythromycin, 50 µg/ml;
357 tetracycline, 10 µg/ml; chloramphenicol, 15 µg/ml.

358 *Database collection and CRISPR-Cas prevalence analysis:* NCBI Genome was used to
359 download *E. faecalis* genomes publicly available as of September 1, 2021. Based on the host
360 (source of isolation), the downloaded genomes were categorized as originating from human or

361 animal sources. Genomes with hosts that did not match either of these categories were
362 excluded. A recent study provided a significant number of additional genomes to our study that
363 were not part of NCBI Genome (Refseq)[25]. Genomes were subject to filtering based on genome
364 size to eliminate anomalies. The average genome size was calculated, and genomes with sizes
365 less than half or greater than 1.5 times of this average size were discarded (following Refseq
366 recommendation). Strains were queried on BLAST against the *cas9* sequences of CRISPR1-
367 Cas (GCF_000172575.2) and CRISPR3-Cas (GCF_000157475.1) to discover strains with
368 potentially active CRISPR-Cas (percent identity >= 98.5%). CRISPRCasFinder was used to
369 identify CRISPR arrays in these genomes, and the known repeat sequences of CRISPR 1,2,3
370 were used to filter bona-fide CRISPR arrays from the result[30,46].

371 *CRISPR target analysis:* To investigate CRISPR-Cas targets, we used CRISPR-CasFinder to
372 extract CRISPR arrays for all genomes. Next, using the direct repeats of CRISPR1-Cas,
373 CRISPR2 and CRISPR3-Cas[30], we tested whether the arrays extracted were indeed from one
374 of these known loci by matching the known direct repeats with that of the extracted array,
375 allowing for a Levenshtein distance of up to 3. For the set of bona fide arrays in both animal and
376 human groups, we combined all the spacers across the three CRISPR-Cas subtypes. Next, we
377 created a plasmid database by using PLSDB to gather plasmids isolated from *E. faecalis, E.*
378 *faecium* and *E. hirae*, along with plasmids extracted from recently published *E. faecalis* strains
379 isolated from gut and urine[47–49]. The viral database was created by downloading the viral part of
380 the nr/nt database from NCBI, comprising ~45,000 sequences. We then performed a BLAST
381 search for the spacer sets against both these databases, with word size parameter as 8, and
382 hits with at least 93% sequence identity and 93% query coverage were accepted as bona fide
383 protospacers. The code for this analysis is available at https://github.com/chahatupreti/Agro-
384 CRISPR.

385 *Manure microcosm:* Dairy cattle manure was obtained from a settling basin in Kimberley, Idaho,
386 and autoclaved to destroy resident flora. The solid manure was filtered through a cheesecloth to
387 remove large fecal solids, and 1 mL of the resultant liquid manure was used per conjugation
388 reaction.

389 *Conjugation experiments:* Donors and recipients were grown overnight (from freezer stocks) in
390 liquid BHI medium, followed by a 1:10 subculture. The subsample was statically incubated at
391 37°C for 1.5 hours after which time optical density (OD) measurement at 600 nm was taken to
392 ensure similar ODs for both donor and recipient. For each microcosm, 100 µl of the donor
393 culture and 900 µl of a recipient culture were centrifuged, 90% of the supernatant was removed,
394 and then cells were resuspended. Three experimental conditions were used to study
395 conjugation outcomes – solid brain heart infusion (BHI) agar plate, liquid BHI and liquid manure.
396 Conjugation reactions were incubated for 18 hours at 37°C. After this, the conjugation mixture
397 was collected, serial dilutions were performed and plated on agar plates with appropriate
398 antibiotic selection. Where appropriate, the recipient population was calculated by subtracting
399 CFU/mL of transconjugants from that of recipients+transconjugants. Plates were incubated at
400 37°C for 72 hours followed by counting the number of colony forming units/mL (CFU/mL).
401 Conjugation frequency was calculated as $\frac{CFU/mL\ of\ transconjugants}{CFU/mL\ of\ donors}$. For multi-passage
402 experiments, a 1:1000 dilution subculture was prepared and allowed to incubate in liquid BHI for
403 24 hours before another set of plating. For CRISPR antimicrobial experiments, the pheromone
404 cAD1 (500 ng/mL) was added to the conjugation mixture to increase conjugation.

405

406

407 **4. Discussion**

408 In this study, we assessed the prevalence and function of CRISPR-Cas in agriculturally relevant
409 niches. We discovered that the prevalence of both CRISPR1-Cas and CRISPR3-Cas (the two
410 independently functional CRISPR-Cas types in *E. faecalis*) was similar when comparing strains
411 of animal origin to those of human origin. These two CRISPR-Cas systems differ in terms of
412 their protospacer-adjacent motif (PAM) used by the Cas nuclease to cleave the protospacer in
413 the invading genome, along with differences in the corresponding *cas* genes[13]. The strong
414 similarity in their prevalence suggests that mechanistic differences between the two subtypes do
415 not have an impact on their distributions in *E. faecalis* from diverse hosts. Investigating the
416 targets of the CRISPR systems showed that there was also remarkable similarity in the plasmid
417 and viral targets of these systems across the two environments.

418

419 We also assessed how effective CRISPR-Cas is an agricultural niche, using dairy manure in a
420 microcosm design. Previous studies reported that CRISPR-Cas efficacy varies with the
421 environment it is tested in, with differences observed *in vitro* versus *in vivo*[14]. Our data show *E.*
422 *faecalis* CRISPR-Cas is able to block plasmid transfer following exposure to manure. The effect
423 of manure on CRISPR function is observable even after two post-manure passages in BHI
424 medium, something that was not observed in the control BHI broth and agar microcosms. We
425 speculate that even though the manure was autoclaved and most of its solids removed before
426 using, it still contained factors that could be altering *E. faecalis* physiology. Specifically,
427 T11RFΔ*cas9* remains at high levels in BHI-passaged cultures post-manure exposure (Figure 4).
428 This is not observed for T11RF post-manure, or any of the cultures originating in BHI. Teasing
429 apart the mechanism by which this happens and the relative contributions of manure, CRISPR-
430 Cas, and pAM714 would be the next step. One approach could be to simplify the complex
431 manure environment – manure could be fractionated by size and we could study if components
432 larger or smaller than a particular size can fully capture the effect seen here. We could also

433 target specific biomolecules in manure – lipids, proteins, DNA – by digesting them and seeing
434 its effect on CRISPR-Cas efficacy. Another approach would be to test a different recipient strain
435 to see how manure affects its CRISPR functioning and its growth/stability in culture. Since V583
436 is genetically highly similar to T11 (except for the mobile elements) while also being a highly
437 drug-resistant strain, it would be a good choice of recipient to test this effect.

438

439 Overall, the microcosm studies provided clear evidence of *E. faecalis* CRISPR-Cas functioning
440 in an agricultural (manure) environment and highlighted future challenges to be considered in
441 experiments exploring potential field applications of the concept. First, our manure model is
442 highly simplified – we autoclaved it to remove all resident microbiota and then filtered and
443 strained it to remove most of the solids. The former was done to avoid complications that could
444 arise from the resident microbiota affecting CRISPR-Cas function, and the latter to help
445 standardize quantification of manure amounts. The solids, if present, could provide additional
446 surface for the bacteria to form biofilms on, which would promote conjugation and hence,
447 possibly, enhance the effect of CRISPR antimicrobials. Although we saw CRISPR antimicrobials
448 have some effect, we were unable to bring up the conjugation rates to a sufficient extent to
449 robustly test the effectiveness of the antimicrobial. It is possible that the large size of our
450 antimicrobial plasmid was the reason for its low conjugation rate; engineering the antimicrobial
451 using a smaller plasmid could potentially help improve its conjugation rate and performance in
452 agricultural niches. Secondly, the experiments could be performed with the resident microbiota
453 intact by using a combination of antibiotic-selective and species-selective plates. Thirdly, after
454 letting the bacteria conjugate in the three respective microcosms, we subsequently passaged
455 the conjugation mixture in liquid BHI in all three conditions, as a way to model transition across
456 different nutritive environments which can happen as strains are transmitted between different
457 hosts and niches. An alternative approach could be to passage each of the three arms in the

458 same microcosm that they were exposed to during the initial period of conjugation (solid agar,
459 liquid BHI and manure).

460

461 Our work has several important implications both in terms of basic CRISPR biology and tackling
462 antibiotic resistance. Our analysis looking at CRISPR-Cas prevalence and targets in *E. faecalis*
463 is perhaps the largest of its kind, and the insight that the distribution of CRISPR-Cas subtypes
464 and targets is invariant across animal and human sources points to a lack of CRISPR-mediated
465 host colonization preference. The *E. faecalis* microcosm studies offer a proof-of-concept on the
466 potential for CRISPR-Cas to limit horizonal gene transfer in dairy manures. Finally, while
467 conjugation efficiency limited the ability of our CRISPR antimicrobial to spread in manure to
468 antibiotic-resistant target cells, we did successfully immunize an *E. faecalis* strain against
469 antibiotic resistance acquisition, which was observed to be more robust in manure compared to
470 liquid BHI (Figure 8). Overall, our study demonstrates that CRISPR-Cas is a barrier to antibiotic
471 resistance dissemination in the manure environment and identifies key areas for future study of
472 CRISPR-Cas efficacy in the agricultural setting.

473

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