

1           **Agricultural fertilization with poultry manure results in persistent**  
2           **environmental contamination with the pathogen *Clostridioides difficile***

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## Summary

32 During a field experiment applying broiler manure for fertilization of agricultural land, we  
33 detected viable *Clostridioides* (formerly, *Clostridium*) *difficile* in broiler feces, manure, dust,  
34 and fertilized soil. A large diversity of toxigenic *C. difficile* isolates was recovered, including  
35 PCR ribotypes common from human disease. Genomic relatedness of *C. difficile* isolates  
36 from dust and from soil, recovered more than two years after fertilization, traced their origins  
37 to the specific chicken farm that had delivered the manure. We present evidence of long-term  
38 contamination of agricultural soil with manure-derived *C. difficile* and demonstrate the  
39 potential for airborne dispersal of *C. difficile* through dust emissions during manure  
40 application. *Clostridioides* genome sequences virtually identical to those from manure had  
41 been recovered from chicken meat and from human infections in previous studies, suggesting  
42 broiler-associated *C. difficile* are capable of zoonotic transmission.

43

## Introduction

44

45

46 The anaerobic gut bacterium *Clostridioides difficile* (formerly, *Clostridium difficile* (Lawson *et al.*, 2016)) is the most frequent infectious cause of antibiotic-associated diarrhea and among  
47 *al.*, 2016)) is the most frequent infectious cause of antibiotic-associated diarrhea and among  
48 the leading culprits of healthcare-associated infections (Martin *et al.*, 2016). However,  
49 modelling studies have suggested that transmission in the community and in the healthcare  
50 system were equally relevant for sustaining *C. difficile* in the human population (Durham *et al.*, 2016; McLure *et al.*, 2019). Patients asymptotically colonized with *C. difficile* upon  
51 *al.*, 2016; McLure *et al.*, 2019). Patients asymptotically colonized with *C. difficile* upon  
52 hospital admission have a six-fold increased risk of suffering a *C. difficile* infection (CDI)  
53 (Zacharioudakis *et al.*, 2015), and even without developing CDI themselves they may  
54 increase the overall burden of nosocomial CDI significantly by spreading the pathogen to  
55 other patients (Longtin *et al.*, 2016; Blixt *et al.*, 2017; Donskey *et al.*, 2018). In addition, CDI  
56 occurs independent from healthcare at increasing incidence (Ofori *et al.*, 2018), but reservoirs  
57 and pathways of transmission outside of the hospital environment are incompletely  
58 understood (Warriner *et al.*, 2017; Rodriguez Diaz *et al.*, 2018).

59

60 Toxigenic *C. difficile* seems widespread in various environments, since it was recovered from  
61 domestic wastewater (Moradigaravand *et al.*, 2018; Numberger *et al.*, 2019) and river  
62 sediments (Zidaric *et al.*, 2010), from retail compost (Lim *et al.*, 2020), soil (Janezic *et al.*,  
63 2016) and root vegetables (Lim *et al.*, 2018; Tkalec *et al.*, 2019). It was also found to colonize  
64 various mammals and birds, including wildlife, pets, and livestock (Weese, 2020). Notably,  
65 fattening pigs have been proposed as a potential source for transmission of *C. difficile* to  
66 humans, since strains with highly related genomes were isolated from both, pigs and farm  
67 workers (Knetsch *et al.*, 2018). *Clostridioides difficile* was also detected in chicken feces and

68 chicken meat repeatedly (Zidaric *et al.*, 2008; Weese *et al.*, 2010; Harvey *et al.*, 2011; Abdel-  
69 Glil *et al.*, 2018; Heise *et al.*, 2021), even though there is no evidence for significant CDI in  
70 birds (Weese, 2020). Livestock manure often contains *C. difficile* even after being treated by  
71 composting or fermentation in biogas plants (Usui *et al.*, 2017; Dharmasena and Jiang, 2018;  
72 Le Maréchal *et al.*, 2020). As a consequence, the disposal of manure or manure-derived  
73 products as fertilizer on agricultural land may lead to environmental contamination with *C.*  
74 *difficile* spores. The survival of *C. difficile* in fertilized agricultural soil and its release with  
75 surface water runoff or dust has as yet not been investigated, in contrast to other manure-  
76 derived pathogens (Blaustein *et al.*, 2015; Thiel *et al.*, 2020).

77

78 The spread of pathogenic bacteria can be tracked by comparing their genome sequences  
79 (Croucher *et al.*, 2015; Besser *et al.*, 2019; Thiel *et al.*, 2020). Within the EnteroBase platform,  
80 we have recently established a publicly accessible database for *Clostridioides* genomic data  
81 that currently (January 2021) contains 20,972 draft genomes and their associated metadata  
82 (Frentrup *et al.*, 2020). Standardized sequence data assembly and quality control in  
83 conjunction with core-genome multilocus sequence typing (cgMLST) and hierarchical  
84 clustering of cgMLST allelic profiles - as implemented in EnteroBase - facilitates the detection  
85 of *C. difficile* spread, since isolates from transmission chains frequently can be identified by  
86 being related at the HC2 level (i.e. constituting chains of genomes with pairwise differences of  
87 maximally two cgMLST alleles) (Frentrup *et al.*, 2020). Moreover, widespread epidemic  
88 strains commonly are related at the HC10 level, and PCR ribotypes correlate well with  
89 clusters at the HC150 level (which we dubbed 'core-genome sequence typing complexes';  
90 CC) (Frentrup *et al.*, 2020).

91

92 In the present study, we detected the persistence of viable *C. difficile* in agricultural soil for  
93 several years following its fertilization with manure from broiler chickens. Genomic  
94 relatedness of *C. difficile* isolates from soil and from dust released during the fertilization  
95 process traced their origins to the specific chicken farm that had delivered the manure.

96

## 97 **Results**

98

99 ***Diversity of C. difficile isolates in chicken manure.*** Chicken manure was sampled at three  
100 different locations, including two farms and a manure trading cooperative. Altogether 146 *C.*  
101 *difficile* isolates were obtained from manure samples by applying an anaerobic enrichment  
102 protocol (Janezic *et al.*, 2018) and their genomes were sequenced. Genomic data indicated  
103 that 98% of the isolates carried both toxin genes, *tcdA* and *tcdB* (Suppl. Figure S1A), and only  
104 three isolates were non-toxigenic. Analysis of genome sequences with Enterobase showed  
105 that manure isolates were related to 13 CCs (i.e. hierarchical clusters at the level HC150),  
106 which we had previously shown to correlate well with PCR ribotypes (RT, Figure 1A) (Frentrup  
107 *et al.*, 2020). The majority of isolates (94%) from Farm 1 were related to CC3 (Table 1, Figure  
108 1B), which corresponds to PCR ribotype 001 (Frentrup *et al.*, 2020), and repeated samplings  
109 showed that this predominance of CC3 at Farm 1 was evident over a period of at least one  
110 year (Suppl. Figure S1B). In contrast, only one isolate (4%) from Farm 2 was CC3, and none  
111 from the manure trader (Figure 1B). Instead, isolates from the latter two suppliers were  
112 distributed among a number of different CCs, the most predominant of which were CC71  
113 (RT014/020), CC88 (RT014), CC2 (RT002), CC86 (RT005), and CC391 (RT081) (Figure 1).

114

115 **Close genomic relationships identify source of environmental *C. difficile*.** A 2.1-hectare  
116 agricultural field was fertilized with 12 tons of poultry manure from Farm 1 (Thiel *et al.*, 2020).  
117 Prior to fertilization, our enrichment approach failed to detect any *C. difficile* in soil from this  
118 field. After fertilization, however, *C. difficile* got enriched and cultivated consistently from soil  
119 samples collected at multiple points in time for up to 143 weeks (Figure 2). Moreover, one  
120 dust sample collected during manure spread by using an aerosol collection device (Thiel *et*  
121 *al.*, 2020) at the edge of the field tested positive for *C. difficile* by enrichment (Figure 2). Of  
122 note, *C. difficile* was detected in soil and dust by enrichment culture only, whereas cultivation  
123 and quantification by direct plating on selective agar medium was not successful. Altogether,  
124 we collected 144 *C. difficile* isolates from fertilized soil and from dust, and from poultry feces  
125 and manure from Farm 1. Bacterial genome sequencing and cgMLST-based hierarchical  
126 clustering analysis with Enterobase (Frentrup *et al.*, 2020) resulted in three HC2 clusters  
127 (HC2\_1232, HC2\_5435, HC2\_5465; Figure 3) and four singletons. Generally, hierarchical  
128 clustering at the level HC2 indicates close genomic relationships of *C. difficile* isolates; it was  
129 previously shown to correlate with events of transmission between hospital patients (Frentrup  
130 *et al.*, 2020).

131

132 Two HC2 clusters (HC2\_1232, HC2\_5435) included genomes from two or more different  
133 sources, including chicken feces collected at Farm 1, manure from Farm 1, dust collected  
134 during the application of manure to the field, and fertilized soil from multiple points in time  
135 (Table 3 and Figure 3). This result confirmed that the *C. difficile* strains that were recovered  
136 during and after fertilization indeed originated from Farm 1, i.e. they had been disseminated  
137 onto the agricultural field through the fertilization process. These close genomic relationships  
138 were found among *C. difficile* isolates from all soil samples, indicating the persistence of

139 viable, manure-derived *C. difficile* in the soil for up to 143 weeks after fertilization (Figure 3).  
140 Likewise, the detection of closely related *C. difficile* in mineral dust showed that viable cells of  
141 the pathogen got aerosolized during the fertilization process and transported in an ascending  
142 dust plume at a distance of at least 20 meters from the applying tractor (Figure 3).

143

144 **PCR ribotypes and antibiotic susceptibilities.** *Clostridioides difficile* isolates (n=19)  
145 selected to represent sources (i.e. manure from the different suppliers, fertilized soil, and  
146 dust) and genomic diversity (at the level of CCs) proved to be phenotypically susceptible to  
147 the antibiotics vancomycin, metronidazole, moxifloxacin, clindamycin, and tetracycline (Table  
148 2). None of the genome sequences (n=278) carried resistance-causing mutations in the  
149 gyrase gene *gyrA* (Zaiß *et al.*, 2010), confirming the lack of fluoroquinolone resistance in our  
150 strain collective (not shown). PCR ribotypes determined in the laboratory were fully  
151 concordant with ribotype predictions based on hierarchical clustering in EnteroBase (Table 2).

152

153 **Closely related clinical and poultry meat isolates.** Hierarchical clustering of cgMLST allelic  
154 profiles in EnteroBase routinely determines genomic relationships at multiple phylogenetic  
155 levels among all >20,000 entries in the *Clostridioides* database (Frentrup *et al.*, 2020).

156 Remarkably, a limited number of genome sequences from several previous studies were  
157 closely related (at HC2 level) to those from Farm 1 (Figure 3). Most notably, virtually identical  
158 *C. difficile* genome sequences had been recovered from retail chicken meat (n=6; Figure 3),  
159 which had been purchased in one region in Germany (Berlin and Brandenburg), but had been  
160 produced in a number of different cutting plants in Germany and the Netherlands (Heise *et al.*,  
161 2021). Additional closely related genomes originated from isolates from human patients  
162 suffering from *C. difficile* infection in Germany (n=2), the Netherlands (n=5) and Hungary

163 (n=1; Figure 3). Of note, these genomic similarities were not due to impaired quality of the  
164 sequence data, since >99% of cgMLST alleles were successfully called for all genome  
165 sequences. Moreover, no genes of the whole-genome MLST set (Frentrup *et al.*, 2020) were  
166 differentially present (not shown), indicating that accessory genomes were virtually identical  
167 among all these isolates, too,

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## Discussion

171

172 ***Chicken manure carried diverse C. difficile, including clinically relevant strains.*** Almost  
173 all *C. difficile* isolates from manure in our study carried the *tcdA* and *tcdB* genes in their  
174 genomes, and hence must be considered fully virulent and able to cause gastrointestinal  
175 disease in humans. This result is in concordance with most previous studies on poultry-  
176 associated *C. difficile* (e.g. (Dharmasena and Jiang, 2018; Berger *et al.*, 2020; Le Maréchal *et*  
177 *al.*, 2020; Heise *et al.*, 2021)) even though there is little evidence that *C. difficile* may cause  
178 disease in birds (Weese, 2020).

179

180 In manure samples from three suppliers, we found a total of 13 CCs (core-genome sequence-  
181 type complexes) of *C. difficile*. CCs correlate well with PCR ribotypes (Frentrup *et al.*, 2020)  
182 (Table 2), and ribotypes 001, 014/020 and 005 have been reported from poultry feces (Indra  
183 *et al.*, 2009; Hussain *et al.*, 2016; Abdel-Glil *et al.*, 2018; Le Maréchal *et al.*, 2020) and from  
184 broiler meat (De Boer *et al.*, 2011; Tkalec *et al.*, 2020) in the past. In our manure samples, the  
185 most predominant strains were CC3 (RT001), CC71 (RT014/020) and CC88 (RT014).  
186 Remarkably, these are also among the most prevalent strains causing human *C. difficile*



187 infections in Europe (Davies *et al.*, 2016). However, our isolates from broiler chickens were  
188 not resistant to fluoroquinolones or clindamycin, in contrast to the vast majority of clinical  
189 RT001 isolates from human CDI (Zaiß *et al.*, 2010; Eyre *et al.*, 2018). This striking difference  
190 in antibiotic resistances suggests that *C. difficile* RT001 in chickens constitutes a population  
191 separate from the epidemic RT001 strain causing healthcare-associated CDI in humans, with  
192 limited exchange. This notion was confirmed by hierarchical clustering, which indicated that  
193 all our CC3 *C. difficile* from broiler manure (n=199) were related to a single HC10 cluster  
194 (HC10\_783; Suppl. Table 1) that currently includes only 15 (7%) human-associated *C. difficile*  
195 isolates in EnteroBase. This separation was not observed for RT014/020, which is antibiotic  
196 resistant more rarely (Zaiß *et al.*, 2010; Eyre *et al.*, 2018), and where 13 isolates from broilers  
197 were affiliated to nine different HC10 clusters (Suppl. Table 1), the larger of which included  
198 numerous isolates from diverse host species and geographic origins. Fluoroquinolone and  
199 clindamycin resistance in poultry-associated *C. difficile* has occasionally been reported (from  
200 the USA and Zimbabwe (Harvey *et al.*, 2011; Dharmasena and Jiang, 2018; Berger *et al.*,  
201 2020)). Since macrolides and fluoroquinolones are the two antibiotics most heavily used in  
202 the poultry industry in Europe, and resistance against these drugs is widespread among other  
203 gastrointestinal pathogens from chickens (Roth *et al.*, 2019), lowered susceptibilities might  
204 also have been expected from broiler-associated *C. difficile*, but yet this was not detected in  
205 our samples.

206

### 207 ***Long-term persistence of manure-derived C. difficile in fertilized agricultural soil.***

208 *Clostridioides difficile* has been reported from a wide range of different environmental  
209 samples, including soil (Rodriguez Diaz *et al.*, 2018). To our best knowledge, however, our  
210 study is the first to use genome sequence analysis to trace environmental *C. difficile* back to

211 its source. As one result, we show that *C. difficile* in fertilized soil indeed originated from  
212 chickens in Farm 1. Hence, our field experiment demonstrated that manure-derived *C. difficile*  
213 remained viable in fertilized soil over the entire study period, i.e. for at least 143 weeks, or  
214 almost three years. The continued bacteriological detection of *C. difficile* in all samples  
215 investigated suggested that its survival may be much longer than the sampling period, even  
216 though precise extrapolation was not possible due to the failure of quantitative cultivation. The  
217 observed long-term contamination of the soil certainly was enabled by the ability of *C. difficile*  
218 to produce endospores, which can stay viable for many years (Yang and Ponce, 2011). In  
219 contrast, these bacteria are unlikely to perform much metabolic activity or even proliferate  
220 under ambient conditions in the soil, since their physiology is adapted to life in the intestines  
221 of warm-blooded animals.

222

223 We previously reported that chicken manure carried additional pathogens, including  
224 *Enterococcus faecium* and extended-spectrum beta-lactamase (ESBL) producing *Escherichia*  
225 *coli* (Thiel *et al.*, 2020). However, ESBL *E. coli* died off within a few days during manure  
226 storage (Siller *et al.*, 2020) and enterococci rapidly declined in soil within weeks after  
227 fertilization (Thiel *et al.*, 2020). In the present study, in contrast, we demonstrate that viable *C.*  
228 *difficile* remained detectable in fertilized soil for several years and hence represented a long-  
229 lasting pollution.

230

231 **Potential for long-distance dispersal of *C. difficile*.** Hierarchical clustering indicated that  
232 altogether 13 entries in the *Clostridioides* database shared identical HC2 clusters (HC2\_1232,  
233 HC2\_5465) with isolates from Farm 1, i.e. they had highly similar cgMLST profiles with at  
234 most two allelic differences, despite their origins from unrelated, previous studies. Seven of

235 these isolates had been recovered from retail chicken meat from various cutting plants in  
236 Germany and the Netherlands (Heise *et al.*, 2021), indicating widespread dissemination of *C.*  
237 *difficile* HC2\_1232 by the poultry industry. Furthermore, the occurrence of the same HC2  
238 clone in human CDI in Germany, Hungary and the Netherlands indicates that this strain is  
239 able to cause human disease. Consequently, this *C. difficile* HC2 clone poses a risk of  
240 zoonotic transmission.

241

242 It should be noted that pathogen genomic similarity alone does not prove direct transmission  
243 between remote places, but should be interpreted with particular care in the absence of  
244 additional, epidemiological evidence (Besser *et al.*, 2019). However, several plausible  
245 scenarios for long-distance transport of poultry-associated *C. difficile* exist. Chicken meat  
246 contaminated with *C. difficile* (De Boer *et al.*, 2011; Harvey *et al.*, 2011; Candel-Pérez *et al.*,  
247 2020; Heise *et al.*, 2021) gets distributed to customers through widely ramified retail chains.  
248 Similarly, pork products (i.e., meat or manure) were suspected to promote the long-distance  
249 spread of *C. difficile*, after closely related *C. difficile* genomes had been detected in fattening  
250 pigs and humans across large geographic distances, without any documented  
251 epidemiological connections (Knetsch *et al.*, 2018; Knight *et al.*, 2019). Another potential path  
252 for the long-range dissemination of livestock-associated *C. difficile* may be the transport of  
253 colonized, live animals, e.g. from farms to slaughterhouses (Heise *et al.*, 2021). Potentially  
254 even more important is the globalized structure of the poultry industry, which ships industrially  
255 produced broiler chicks by airfreight for stocking fattening farms globally (Lowder *et al.*, 2009).  
256 It would be interesting to investigate the colonization status of chickens upon their arrival at  
257 fattening farms.

258

259 In addition, here we show that mineral dust from agricultural operations may carry  
260 aerosolized, manure-derived *C. difficile*. This dust may stay airborne for several days and  
261 during this time may get transported over several hundred kilometers, depending on  
262 atmospheric conditions (Faust *et al.*, 2020; Thiel *et al.*, 2020). Poultry manure is particularly  
263 prone to aerosolization due to its high dry-matter content (Kabelitz *et al.*, 2020; Thiel *et al.*,  
264 2020) and therefore, its application for fertilization of agricultural fields likely contributes to the  
265 airborne dispersal of chicken-associated *C. difficile* over long distances. Aerosolized *C.*  
266 *difficile* is considered a potential source of human infection when inhaled (Best *et al.*, 2010),  
267 similar to other enteric pathogens (Jahne *et al.*, 2015). Hence, *C. difficile* in agricultural dust  
268 may represent a risk of airborne zoonotic transmission. Taken together, our results  
269 corroborate the relevance of a 'One Health' approach for curbing the spread of *C. difficile*  
270 between human, livestock, and environmental reservoirs.

271

272

273

## Experimental procedures

274

275 **Manure samples.** To capture the diversity of *C. difficile* isolates in manure samples, three  
276 samplings were performed on three different sites. Manure samples from two broiler fattening  
277 farms and one manure trading cooperative were investigated. In addition, chicken feces were  
278 sampled by collecting 30 chicken droppings from each of 11 stables in Farm 1. Farm 1 is an  
279 intensive poultry-fattening farm in Brandenburg, Germany, housing about 19,000 animals per  
280 stable on wood pellets. Manure from this farm was sampled three times (May 30th 2017,  
281 November 8th 2017 and May 19th 2018). In Farm 2, which is located in Saxony-Anhalt,

282 Germany, manure was collected in four different stables on August 14th 2017. Manure from  
283 the trader was sampled on March 27th 2017.

284

285 **Field experiment.** In a field experiment, 12 tons of chicken manure from Farm 1 (see above)  
286 were applied to a 2.1-hectare agricultural field, which had not been fertilized with animal  
287 manure for 15 years. Details of this experiment have been published previously (Thiel *et al.*,  
288 2020). Briefly, dust particles that were released during the fertilization process were collected  
289 by impingement into 5 mL phosphate-buffered saline (PBS) at a height above ground of 1.50  
290 m and at a distance from the tractor of 20, 50, and 100 m, respectively. Soil samples were  
291 taken on three representative sites on the field site prior to fertilization, directly after, and two,  
292 four, seven, ten, 14, 19 and 143 weeks later.

293

294 **Isolation of *C. difficile* isolates.** Ten g of poultry feces, manure and soil samples were mixed  
295 with 90 g Luria-Bertani broth (Roth) each and subsequently homogenized for 30 s with a bag  
296 mixer (Interscience). After sedimentation of coarse particles (30 min, room temperature),  
297 supernatants and impingement suspensions from the aerosol collector were diluted to  
298 extinction with PBS and subsequently streaked on ChromID *C. difficile*-agar (Biomérieux).  
299 After incubation at 37°C for 24 h, *C. difficile* colonies were identified by species-specific PCR  
300 (locus TR10) (Zaiß *et al.*, 2009). In addition, for enrichment cultures, 0.5 mL of suspensions  
301 were added to 10 mL brain heart infusion (BHI) broth (Roth) supplemented with 0.1%  
302 taurocholic acid (Sigma), 0.1% cysteine (Sigma) and *C. difficile* selective Supplement (Oxoid)  
303 in Hungate tubes (Janezic *et al.*, 2018). After seven days of incubation at 37°C, an ethanol  
304 shock was performed by adding an equal amount of absolute ethanol to 0.5 mL culture and  
305 incubation for 1 h at room temperature. The culture was centrifuged at 2,500 x g for 5

306 minutes, the resulting cell pellet was resuspended in 200  $\mu$ L PBS, and 100  $\mu$ L were plated on  
307 ChromID *C. difficile*-agar and incubated at 37°C for 24 h. Again, bacterial colonies were  
308 tested by *C. difficile*-specific PCR (Zaiß *et al.*, 2009).

309

310 **Antibiotic susceptibility testing.** Isolates from agar-plates were transferred to anaerobic  
311 BHI broth (Roth) in Hungate tubes and grown for two days at 37°C. Subsequently, the culture  
312 was diluted 1:5 with PBS and 100  $\mu$ l was spread on Columbia blood-agar (Oxoid). For each  
313 antimicrobial agent, an E-test strip was applied to the agar surface, followed by 24 hours of  
314 incubation at 37°C. The tests were interpreted visually by reading the minimum inhibitory  
315 concentration (MIC). MICs were determined for vancomycin, metronidazole, moxifloxacin  
316 (Biomérieux), clindamycin and tetracycline (Liofilchem). For interpretation, MIC breakpoints  
317 for antibiotic resistance were applied according to Pirš *et al.* (Pirš *et al.*, 2013): metronidazole,  
318  $\geq 2$   $\mu$ g/mL; vancomycin,  $\geq 2$   $\mu$ g/mL; moxifloxacin,  $\geq 4$   $\mu$ g/mL; clindamycin,  $\geq 8$   $\mu$ g/mL;  
319 tetracycline,  $\geq 16$   $\mu$ g/mL.

320

321 **PCR ribotyping.** PCR ribotyping of *C. difficile* isolates was performed as reported previously  
322 (Indra *et al.*, 2008), applying capillary electrophoresis and the Webribo database  
323 (<https://webribo.ages.at/>).

324

325 **Whole genome sequencing.** Genomic DNA was extracted by using the DNeasy Blood &  
326 Tissue kit (Qiagen), libraries were prepared as described previously (Steglich *et al.*, 2018) and  
327 sequenced on an Illumina NextSeq 500 machine using a Mid-Output kit (Illumina) with 300  
328 cycles. Illumina sequencing reads were uploaded to Enterobase  
329 (<http://enterobase.warwick.ac.uk/>) and assembled with the embedded standardized pipeline

330 (Frentrup *et al.*, 2020). Thirty-two sequences did not pass the quality check in Enterobase  
331 (Frentrup *et al.*, 2020) and were excluded from further analyses. For 278 genomes, cgMLST  
332 allelic profiles (>99% complete) were determined and cgMLST-based hierarchical clustering  
333 performed using Enterobase tools. To visualize genomic relatedness, rapid-neighbor-joining  
334 and minimum-spanning trees were calculated applying GrapeTree (Zhou *et al.*, 2018;  
335 Frentrup *et al.*, 2020). PCR ribotypes were predicted based on genomic relatedness at the  
336 level HC150 (i.e. hierarchical clusters of genome sequences with pairwise differences of  
337 maximally 150 cgMLST alleles; for details see (Frentrup *et al.*, 2020)).

338

339 Sequences of the *gyrA* gene (cgMLST locus CD630\_00060) were scanned for the mutations  
340 Thr-82-Ile and Asp-71-Glu, which are associated with fluoroquinolone resistance in *C. difficile*  
341 (Zaiß *et al.*, 2010).

342

343 All genome sequencing data were submitted to the European Nucleotide Archive  
344 ([www.ebi.ac.uk/ena](http://www.ebi.ac.uk/ena)) under the study accession number PRJEB42049. A list of all analyzed  
345 genomes can be found in Supplementary Table1.

346

347 **Detection of toxin genes.** DNA from selected isolates (n=19) was tested for the presence of  
348 toxin genes *tcdA*, *tcdB*, *cdtA* and *cdtB* by PCR (Persson *et al.*, 2008). The presence or  
349 absence of toxin genes *tcdA* and *tcdB* was determined for all genomes in this study (n=275)  
350 based on allelic numbers for toxin gene loci in Enterobase (i.e., allele number 0 was  
351 interpreted as absence of gene).

352

353

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359

360

### **Conflict of interest.**

361 The authors declare no conflict of interest.

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552 **Figure legends**

553

554 **Figure 1.** Rapid-neighbour-joining phylogenetic trees based on cgMLST allelic differences  
555 between *C. difficile* isolates (n=146) from manure samples. The scale bar indicates the  
556 branch length corresponding to sequence differences at 200 cgMLST loci. **A** Colors indicate  
557 CCs (core-genome sequence-typing complexes); RT, PCR ribotype. **B** Colors indicate origins  
558 of manure.

559

560 **Figure 2.** Distribution of isolates recovered from chicken feces and manure from Farm 1 and  
561 from samples collected during the field experiment (n=144). Colors represent CCs, which  
562 were determined based on cgMLST allelic profiles in EnteroBase.

563

564 **Figure 3. A** Rapid-neighbor-joining phylogenetic tree based on cgMLST allelic profiles from all  
565 isolates (n=144) sampled during the field experiment. Colors indicate HC2 clusters. The scale  
566 bar indicates the branch length corresponding to sequence differences at 200 cgMLST loci. **B**  
567 Minimum-spanning trees for two HC2 clusters. Numbers on branches indicate the number of  
568 cgMLST allelic differences and the colors represent the source from which the isolates were  
569 extracted from.

570

571 **Supplementay Figure S1.** Rapid-neighbour-joining phylogenetic trees based on cgMLST  
572 allelic differences between *C. difficile* isolates (n=146) from manure samples (compare Figure  
573 1). The scale bar indicates the branch length corresponding to sequence differences at 200  
574 cgMLST loci. **A** Colors indicate the complement of toxin genes *tcdA* and *tcdB*. **B** Colors  
575 indicate sampling dates.

**Table 1. Core genome sequence type complexes (CCs)**

<b>CC</b>	<b>PCR Ribotype</b>	<b>Farm1</b>	<b>Farm2</b>	<b>trader</b>
2	002	4 (4.3 %)	1 (4.2 %)	7 (25 %)
3	001	88 (93.6 %)	1 (4.2 %)	0
71	014/020	0	10 (41.7 %)	3 (10.7 %)
86	005	1 (1.1 %)	2 (8.3 %)	4 (14.3 %)
88	014	0	2 (8.3 %)	10 (35.7 %)
391	081	0	5 (20.8 %)	0
5408	029	0	1 (4.2 %)	0
5410	novel	0	2 (8.3 %)	0
207	003	1 (1.1 %)	0	0
34	014	0	0	1 (3.6 %)
596	011/049	0	0	1 (3.6 %)
645	029	0	0	1 (3.6 %)
1643	011/049	0	0	1 (3.6 %)



**Table2.** Genotypes and antibiotic susceptibilities of 19 selected *C. difficile* isolates

				MIC** [ $\mu\text{g/ml}$ ]					Gene content (predicted*** PCR)			
Isolate	Source	Origin	CC (RT predicted* PCR)	VAN	MTZ	MXF	CLI	TET	<i>tcdA</i>	<i>tcdB</i>	<i>cdtA</i>	<i>cdtB</i>
CD-17-00892	manure	trader	88 (RT014 RT014)	0.5	0.094	0.75	1.5	0.016	++	++	-	-
CD-17-01035	manure	trader	1643 (RT011/049 RT049)	0.5	0.125	0.75	3	0.047	++	++	-	-
CD-17-01037	manure	trader	34 (RT014 RT014)	0.5	0.125	0.75	2	0.032	++	++	-	-
CD-17-01039	manure	trader	645 (RT029 RT029)	0.75	0.125	0.75	0.5	0.047	++	++	-	-
CD-17-01040	manure	trader	596 (RT011/049 RT049)	0.5	0.125	0.75	3	0.047	++	++	-	-
CD-17-01068	manure	Farm 1	207 (RT003 RT003)	0.38	0.094	1	0.5	0.023	++	++	-	-
CD-17-01070	dust	field experiment	3 (RT001 RT001)	0.5	0.094	0.5	1.5	0.032	++	++	-	-
CD-17-01381	manure	Farm 2	71 (RT014/020 RT014)	0.75	0.125	0.75	1.5	0.75	++	++	-	-
CD-17-01390	manure	Farm 2	391 (RT081 RT081)	0.5	0.125	0.75	2	0.032	++	++	-	-
CD-17-01395	manure	Farm 2	5408 (n.a. RT029)	0.75	0.125	1	3	0.032	-	-	-	-
CD-17-01424	manure	Farm 2	5410 (n.a. novel)	0.38	0.032	1	2	0.023	-	-	-	-
CD-17-01524	manure	Farm 1	86 (RT005 RT005)	1	0.19	0.75	6	0.032	++	++	-	-
CD-18-00685	manure	Farm 1	2 (RT002 RT002)	0.5	0.064	1	2	0.023	++	++	-	-
CD-19-00355	fert. soil wk. 7	field experiment	2 (RT002 RT002)	0.5	0.125	0.75	2	0.064	++	++	-	-
CD-19-00409	fert. soil wk. 14	field experiment	71 (RT014/020 RT014)	0.75	0.125	1	4	0.032	++	++	-	-
CD-19-00417	fert. soil wk. 14	field experiment	3 (RT001 RT001)	0.5	0.19	0.5	1.5	0.047	++	++	-	-
CD-19-00426	fert. soil wk. 19	field experiment	2 (RT002 RT002)	0.38	0.25	1	4	0.047	++	++	-	-
CD-19-00513	manure	Farm 1	3 (RT001 RT001)	0.75	0.75	0.5	4	0.047	++	++	-	-
CD-20-00542	fert. soil wk. 143	field experiment	3 (RT001 RT001)	0.75	0.75	0.75	6	0.047	++	++	-	-

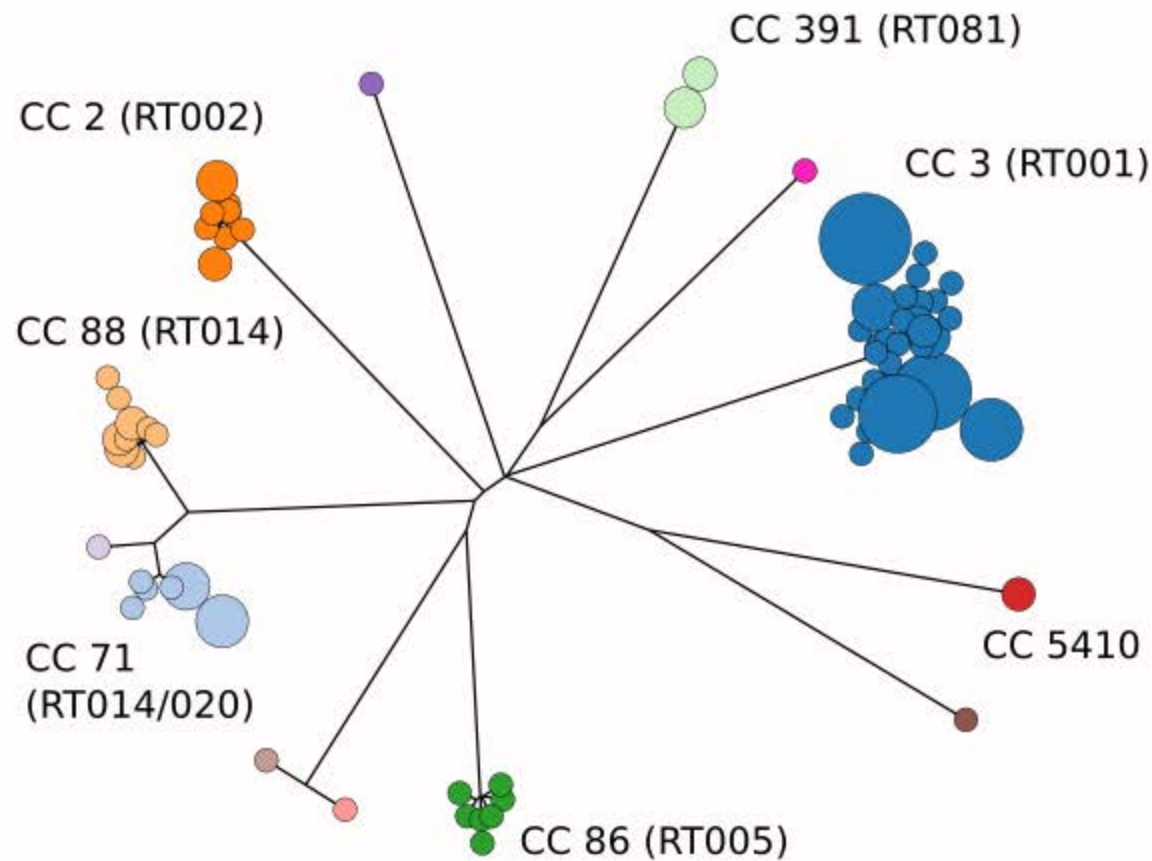
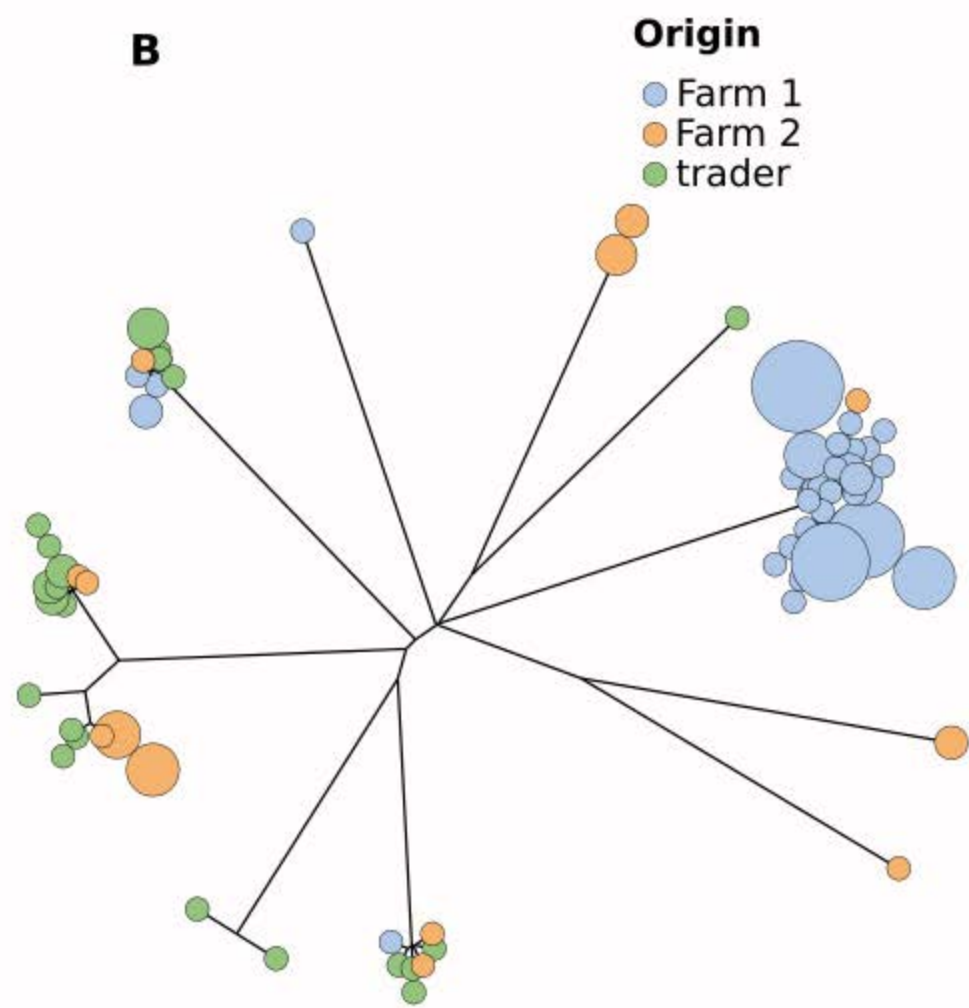
\* Ribotypes were predicted based on hierarchical clustering in EnteroBase

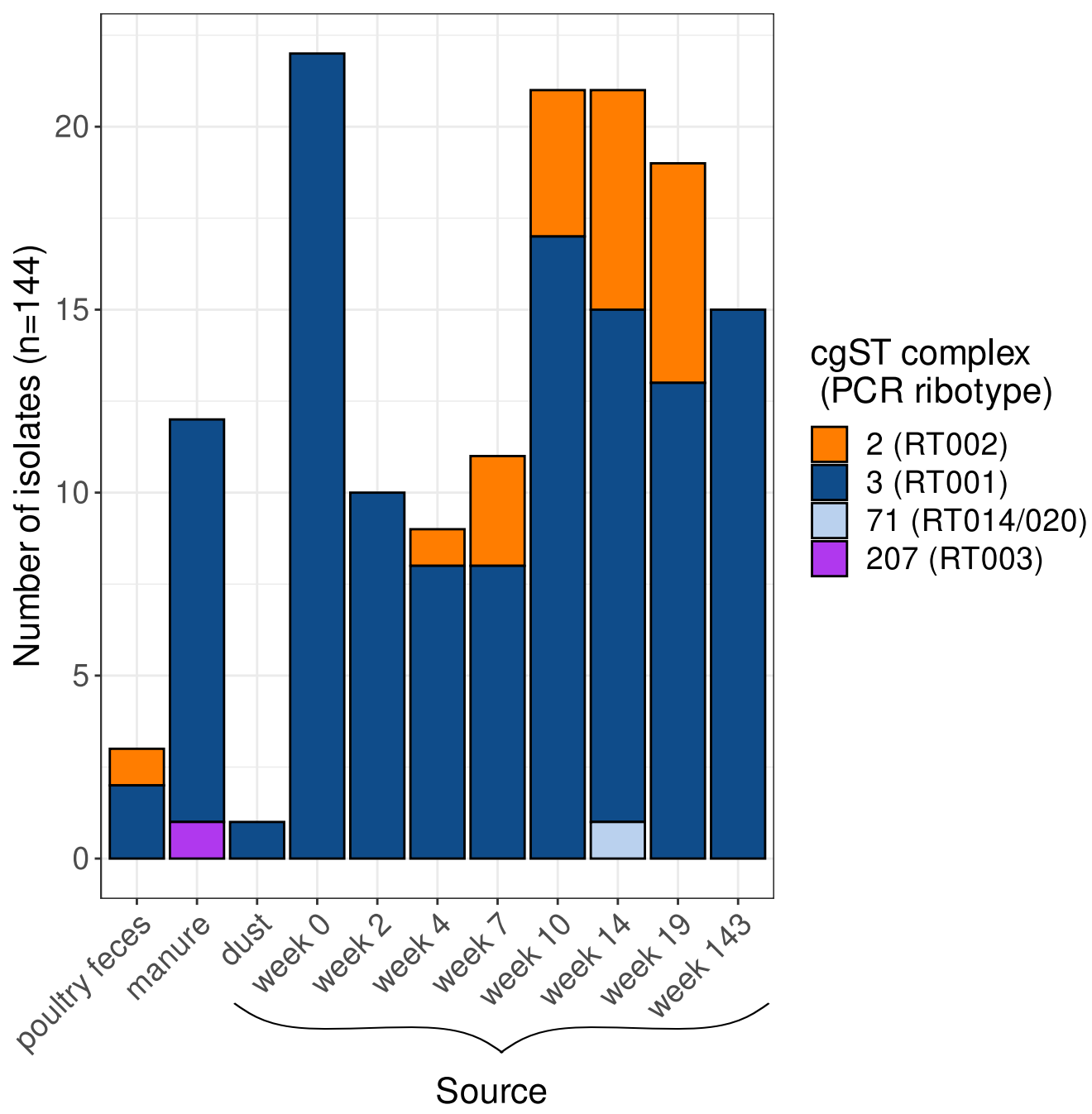
\*\* VAN: vancomycin; MTZ: metronidazole; MXF: moxifloxacin; CLI: clindamycin; TET: tetracycline; *tcdA*: gene encoding for toxin A; *tcdB*: gene encoding for toxin B; *cdtA* and *cdtB*: genes encoding for the binary toxin

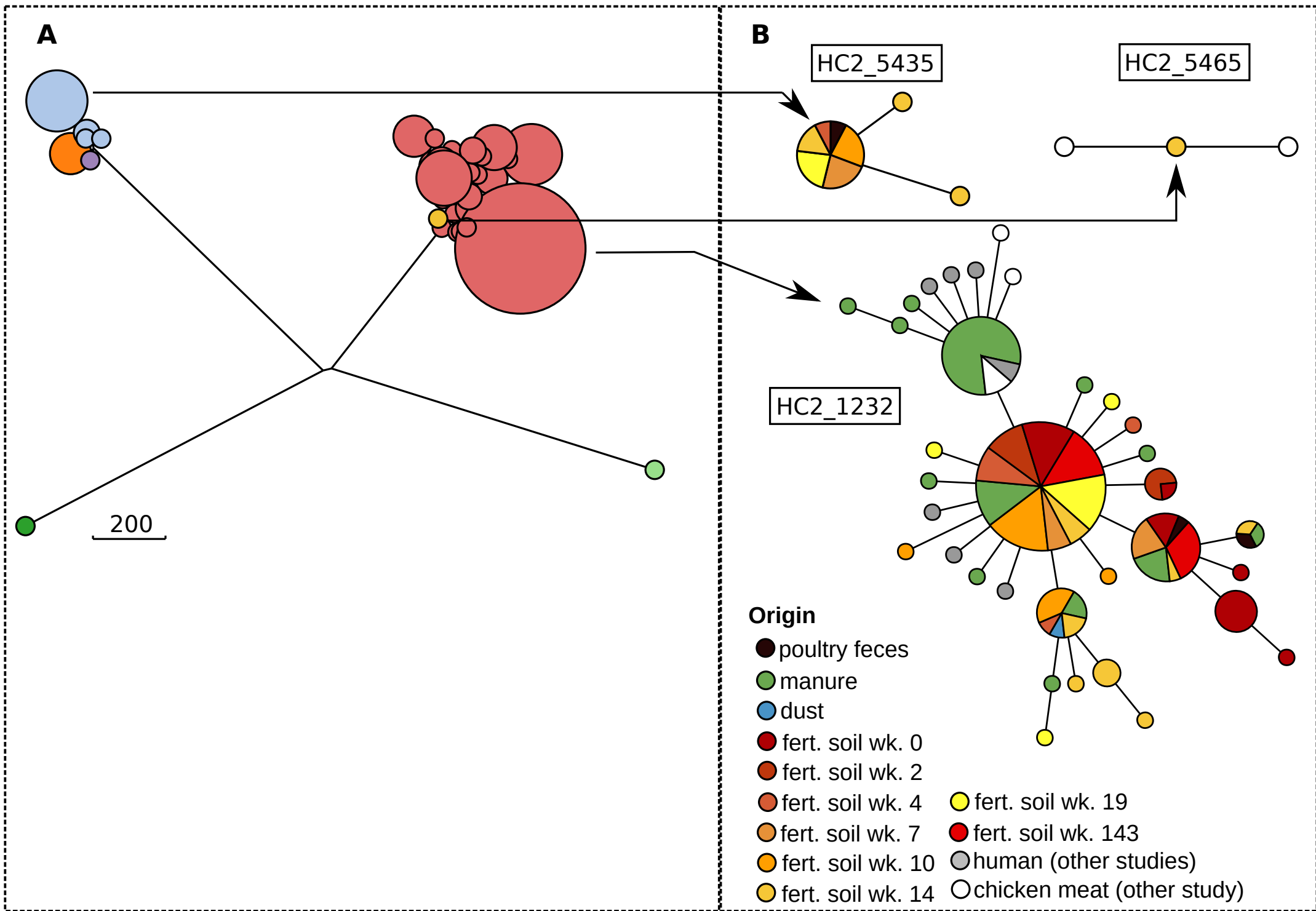
\*\*\* Toxin gene prediction was based on corresponding wgMLST loci (+=present; -=absent)

**Table 3.** HC2 clusters

<b>Source</b>	<b>HC2_1232</b>	<b>HC2_4410</b>	<b>HC2_5435</b>	<b>HC2_5465</b>	<b>HC2_12193</b>	<b>HC2_12207</b>	<b>HC2_12213</b>
poultry feces	2	0	1	0	0	0	0
manure	11	1	0	0	0	0	0
dust	1	0	0	0	0	0	0
fert. soil wk. 0	22	0	0	0	0	0	0
fert. soil wk. 2	10	0	0	0	0	0	0
fert. soil wk. 4	8	0	1	0	0	0	0
fert. soil wk. 7	8	0	3	0	0	0	0
fert. soil wk. 10	17	0	3	0	1	0	0
fert. soil wk. 14	13	0	4	1	2	0	1
fert. soil wk. 19	13	0	3	0	2	1	0
fert. soil wk. 143	15	0	0	0	0	0	0

**A****B**





**tcdA/tcdB genes**

● present

○ absent

**Sampling timepoints**

● November2017

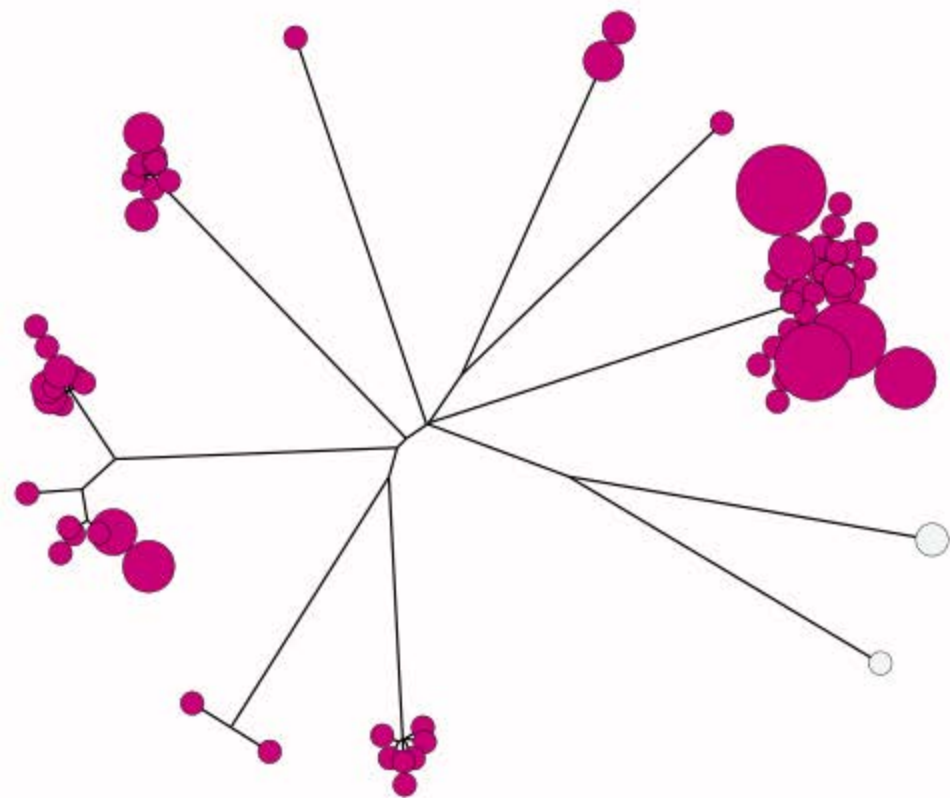
● March2017

● August2017

● May2017

● May2018

**A**



200

**B**

