

1 SUPPLEMENTARY INFORMATION

2 **Article title:** Five-year pan-European, longitudinal surveillance of *Clostridium difficile*
3 ribotype prevalence and antimicrobial resistance: the extended ClosER study

4 **Target journal:** *European Journal Clinical Microbiology & Infectious Diseases*

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7 Pan-European Longitudinal Surveillance of Antibiotic Resistance among Prevalent
8 *Clostridium difficile* Ribotypes' Study Group

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21 Online Resource 1

22 Supplementary Methods

23 Culture and toxin testing

24 Alcohol-shocked faecal specimens or *C. difficile* isolates were inoculated on to cycloserine-
25 cefoxitin-egg-yolk agar (LabM, Heywood, Lancashire, UK) with lysozyme and cultured
26 anaerobically for 48 h at 37°C. Forty-eight hour anaerobic brain-heart infusion broth culture
27 supernatants of each test isolate were added to a Vero cell culture cytotoxicity assay with
28 *Clostridium sordellii* antitoxin (ProLab Diagnostics, Bromborough, UK) neutralisation

29 PCR ribotyping

30 PCR ribotyping using capillary electrophoresis was performed on each isolate by the *C.*
31 *difficile* Ribotyping Network (CDRN) Reference Laboratory at Leeds Teaching Hospitals
32 Trust, Leeds, UK, according to the method of Stubbs *et al.* [12, 23]. Ribotypes (RTs) were
33 assigned against the UK *C. difficile* reference library at Leeds

34 Susceptibility testing

35 Minimum inhibitory concentrations (MICs) of metronidazole, vancomycin, rifampicin,
36 chloramphenicol (Sigma, St Louis, MO, USA); moxifloxacin (Bayer, Leverkusen, Germany),
37 clindamycin, tigecycline (Pfizer, New York, NY, USA), imipenem (MSD, Hoddesdon, UK),
38 and fidaxomicin (Astellas, Tokyo, Japan) were determined for each isolate using a Clinical
39 and Laboratory Standards Institute (CLSI) agar incorporation method with Wilkins–Chalgren
40 agar, as previously described [1, 4, 9]. Susceptibility breakpoints were defined according to
41 CLSI or European Committee on Antimicrobial Susceptibility Testing (EUCAST)
42 recommendations. Fidaxomicin breakpoints were defined according to the manufacturer's
43 stated epidemiological cut-off (ECOFF) value [7].

44 Ribotype diversity scores

45 RT diversity scores for each country were calculated by dividing the total number of RTs
46 detected, irrespective of prevalence, in that country by the number of isolates tested for that
47 country. Numbers closest to 1 indicate the highest RT diversity and those closest to 0
48 correspond to the lowest RT diversity.

49 Antimicrobial susceptibility scores by country

50 For each isolate, MICs were designated susceptible (S), intermediately resistant (I) or fully
51 resistant (R). Each result was assigned a score and a cumulative resistance score (CRS) for
52 all of the nine antimicrobials tested was then generated for each isolate. An isolate that was
53 fully susceptible to 4, intermediately resistant to 2, and resistant to 3 antimicrobials would
54 therefore generate a score of 8 (0+0+0+0+1+1+2+2+2). Isolates were grouped by country
55 and a mean CRS was generated for each country.

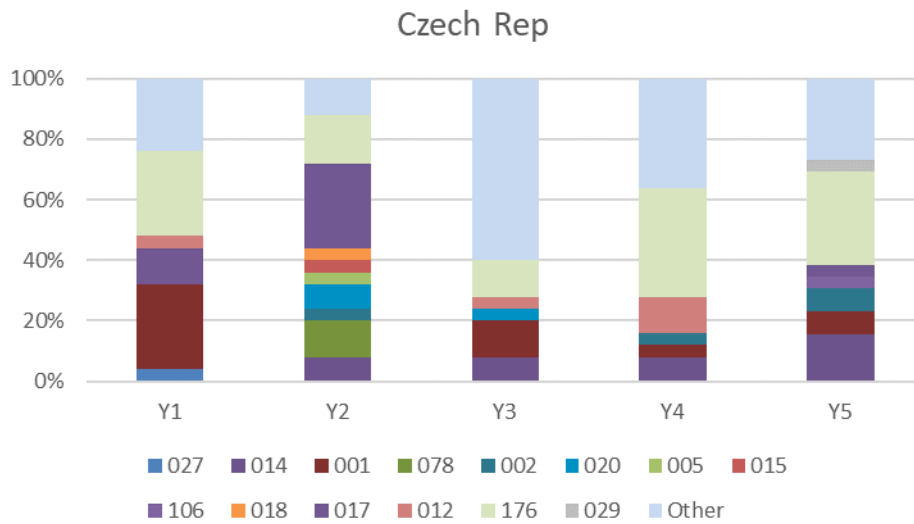
56 Statistical analyses

57 The relationship between RT diversity and mean CRS scores was analysed using the
58 Pearson correlation coefficient (Fisher's Normal Transformation) in Excel Analyse-IT.
59 Fidaxomicin MICs were compared using one-way analysis of variance for independent
60 samples with Tukey's Honest Significant Difference test, at <http://www.vassarstats.net/>.

61 Online Resource 2

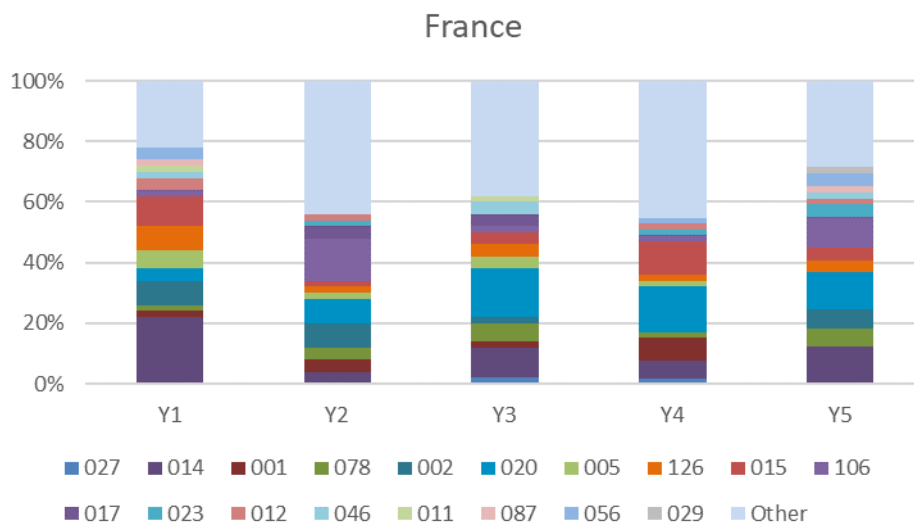
62 Percentage prevalence of the most common *C. difficile* PCR ribotypes for countries with
 63 submissions in all five years of the *ClosER* study

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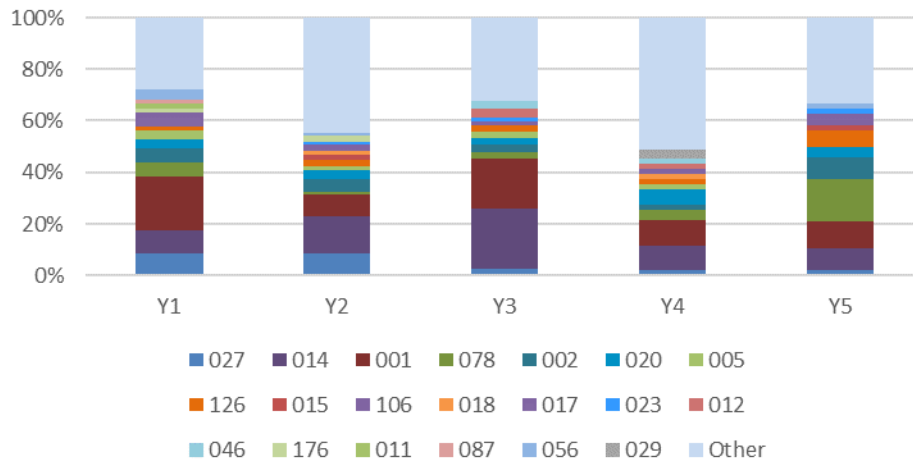
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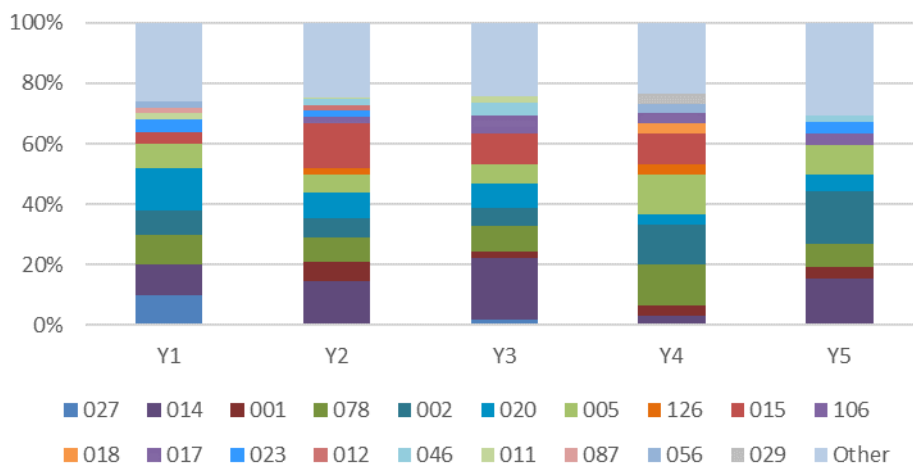
Germany



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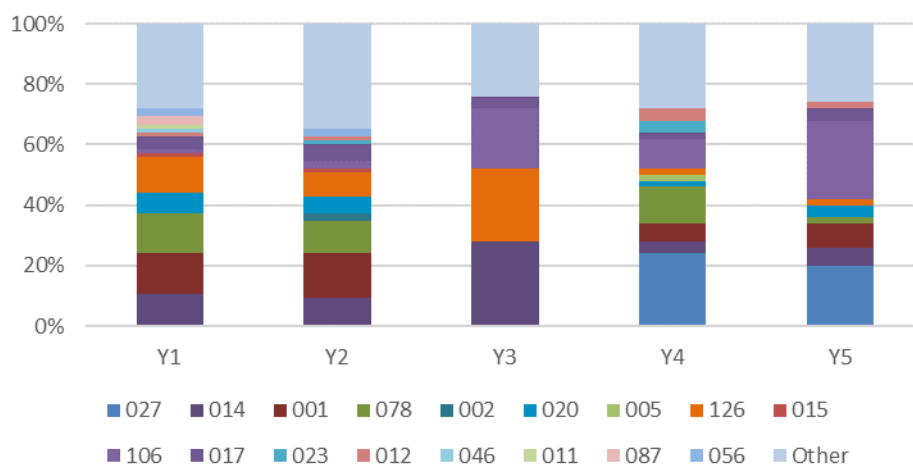
Ireland



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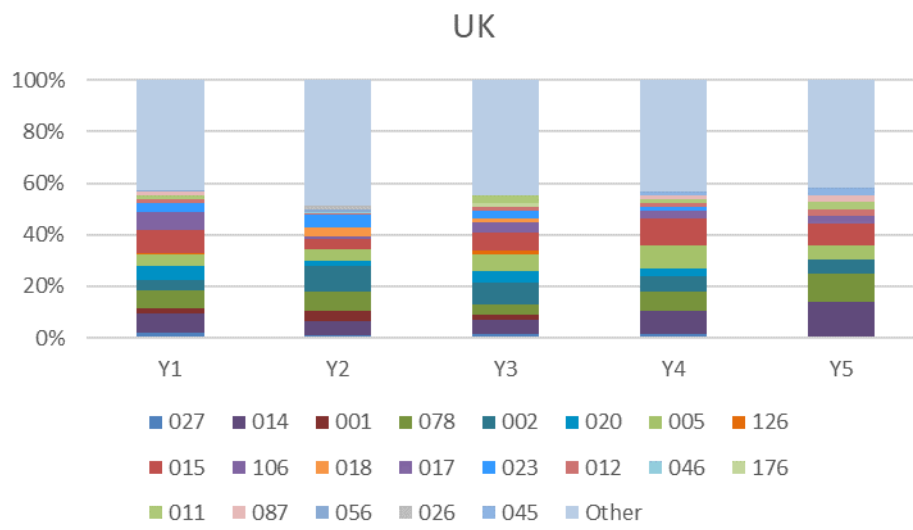
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Spain



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77 Other = all other ribotypes with prevalence <1%

78 Online Resource 3

79 Proportions of sensitive, intermediate and resistant *C. difficile* isolates from Years 1–5 of the *ClosER* study

	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
Sensitive isolates, % (n/N)									
Antimicrobial concentration, mg/L (breakpoint)	≤2	≤2	≤1	≤0.004	≤2	≤2	≤4	≤8	≤0.25
Year 1	97.9 (923/943)	96.7(912/943)	100.0 (943/943)	80.5 (759/943)	58.7 (553/942)	37.6 (354/942)	62.7 (591/943)	92.9 (876/943)	100.0 (943/943)
Year 2	98.1 (935/948)	98.8 (942/948)	100.0 (948/948)	82.1 (783/948)	64.5 (616/948)	29.1 (279/948)	77.3 (739/948)	93.1 (888/948)	100.0 (948/948)
Year 3	96.9 (778/803)	99.8 (801/803)	100.0 (803/803)	86.8 (705/802)	66.0 (531/804)	18.3 (147/803)	78.1 (628/803)	91.5 (735/803)	100.0 (803/803)
Year 4	99.5 (557/560)	100.0 (560/560)	100.0 (560/560)	88.4 (494/559)	70.5 (395/560)	53.2 (305/560)	66.4 (372/560)	96.8 (541/559)	99.3 (559/560)
Year 5	100.0 (242/242)	100.0 (245/245)	99.6 (244/245)	85.3 (209/245)	67.6 (164/244)	54.5 (133/245)	60.2 (148/245)	97.6 (240/245)	100.0 (245/245)

	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
TOTAL	98.3 (3435/3496)	90.3 (3160/3499)	99.98 (3498/3499)	84.4 (2950/3497)	64.5 (2256/3498)	34.8 (1218/3498)	70.8 (2478/3499)	68.7 (2404/3497)	99.97 (3498/3499)
Intermediate isolates, % (n/N)									
Antimicrobial concentration, mg/L	4	4		0.008-16	4	4	8	16	
Year 1	2.0 (19/943)	2.4 (23/943)		6.0 (57/943)	1.8 (17/942)	12.4 (117/942)	30.1 (284/943)	3.5 (33/943)	
Year 2	1.3 (12/948)	0.6 (6/948)		3.7 (30/948)	0.9 (9/948)	13.7 (129/948)	19.7 (187/948)	2.6 (25/948)	
Year 3	2.6 (21/803)	0.1 (1/803)		1.5 (5/802)	0.5 (4/804)	17.4 (140/803)	19.7 (158/803)	5.1 (41/803)	
Year 4	0.4 (2/560)			1.4 (8/559)	0.5 (3/560)	10.357 (58/560)	31.4 (176/560)	0.9 (5/559)	
Year 5				2.9 (7/245)	0.8 (2/244)	17.1 (42/245)	35.8 (88/245)	1.2 (1/246)	
TOTAL	1.5 (54/3496)	0.86 (30/3499)		3.1 (107/3497)	1.0 (35/3498)	13.9 (486/3498)	24.7 (893/3499)	3.0 (105/3497)	

	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
Resistant isolates, % (n/N)									
Antimicrobial concentration, mg/L	≥8	≥8	>1 (reduced susceptibility)	≥16	≥8	≥8	≥16	≥32	>0.25 (reduced susceptibility)
Year 1	0.1 (1/943)	0.8 (8/943)		13.5 (127/943)	39.5 (372/942)	49.8 (471/942)	7.2 (68/943)	3.6 (34/943)	
Year 2	0.1 (1/948)			13.7 (130/948)	34.1 (323/948)	56.6 (540/948)	2.3 (34/943)	3.7 (35/948)	
Year 3	0.5 (4/803)	0.1 (1/803)		11.6 (92/802)	33.5 (268/804)	64.3 (516/803)	2.2 (17/803)	3.4 (27/803)	
Year 4	0.2 (1/560)			10.2 (57/559)	28.9 (162/560)	35.18 (197/560)	2.1 (12/560)	2.3 (13/559)	0.2 (1/560)
Year 5			0.4 (1/245)	11.8 (29/245)	31.6 (77/244)	28.6 (70/245)	4.1 (88/245)	1.2 (3/246)	
TOTAL	0.2 (6/3496)	0.03 (9/3499)	0.02 (1/3499)	12.4 (432/3497)	40.1 (1401/3498)	51.3 (1794/3498)	6.3 (219/3499)	3.2 (112/3497)	0.03 (1/3499)

80 M, metronidazole; V, vancomycin; FDX, fidaxomicin; RIF, rifampicin; MXF, moxifloxacin; CLINDA, clindamycin; IMI, imipenem; CHLOR, chloramphenicol;
81 TIG, tigecycline

82 Online Resource 4

83 MICs (mg/L) of prevalent *C. difficile* PCR ribotypes over Years 1–5 of the ClosER study

Year	Ribotype	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
1	027	1.41	0.70	0.04	0.472	22.16	3.55	7.13	4.91	0.04
2	027	1.47	0.81	0.07	0.303	22.71	4.82	6.94	5.15	0.04
3	027	1.24	0.71	0.08	0.351	18.35	6.34	6.69	5.72	0.03
4	027	0.89	0.66	0.03	0.512	25.49	2.18	7.10	4.81	0.03
5	027	1.00	0.53	0.02	14.136	4.00	1.46	6.62	7.51	0.04
1	001	0.47	0.83	0.01	0.007	12.18	48.63	5.18	8.74	0.04
2	001	0.33	0.64	0.01	0.006	14.06	41.84	3.48	6.84	0.04
3	001	0.38	0.66	0.02	0.006	13.45	54.40	5.08	7.91	0.03
4	001	0.50	0.72	0.01	0.003	28.13	62.98	6.08	7.38	0.05
5	001	0.31	0.62	0.01	0.039	2.11	16.00	3.79	6.82	0.04
1	078	0.27	0.63	0.04	0.003	3.39	2.85	3.18	5.07	0.05
2	078	0.21	0.69	0.05	0.003	2.63	4.93	3.04	4.21	0.04
3	078	0.19	0.62	0.05	0.003	2.51	6.92	3.09	4.93	0.04
4	078	0.15	0.77	0.02	0.002	2.75	2.71	3.53	5.61	0.04

Year	Ribotype	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
5	078	0.17	0.72	0.02	0.002	3.10	2.15	4.63	4.63	0.04
1	014	0.27	0.65	0.06	0.002	2.41	5.15	4.31	5.87	0.05
2	014	0.25	0.60	0.06	0.003	1.95	4.83	3.16	4.90	0.04
3	014	0.22	0.63	0.06	0.003	2.00	8.20	3.77	5.97	0.04
4	014	0.18	0.59	0.04	0.002	2.07	3.27	4.07	5.33	0.04
5	014	0.17	0.52	0.02	0.002	1.91	2.39	4.51	4.51	0.03
1	020	0.31	0.73	0.07	0.002	2.63	6.43	4.80	5.66	0.05
2	020	0.27	0.64	0.06	0.002	2.07	4.06	2.96	5.09	0.05
3	020	0.22	0.65	0.06	0.002	1.68	6.94	3.42	5.75	0.04
4	020	0.23	0.56	0.03	0.002	2.18	5.19	4.62	5.82	0.04
5	020	0.18	0.50	0.03	0.002	3.23	4.69	4.45	5.22	0.04
1	126	0.23	0.66	0.04	0.003	9.37	15.69	3.62	5.38	0.06
2	126	0.23	0.60	0.05	0.003	5.79	25.40	3.10	4.59	0.05
3	126	0.17	0.62	0.04	0.003	4.58	15.47	3.05	5.52	0.04
4	126	0.15	0.74	0.02	0.002	5.38	2.21	2.69	5.38	0.04
5	126	0.20	1.00	0.02	0.003	3.56	7.13	4.00	4.49	0.06
1	002	0.24	0.65	0.05	0.002	2.13	6.80	4.00	5.89	0.04

Year	Ribotype	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
2	002	0.25	0.74	0.06	0.003	1.86	5.40	3.04	4.74	0.03
3	002	0.19	0.74	0.07	0.002	2.25	7.54	3.55	5.17	0.03
4	002	0.17	0.79	0.04	0.004	1.76	3.91	3.67	4.97	0.03
5	002	0.17	0.76	0.02	0.002	1.59	2.00	4.49	4.32	0.03
1	015	0.20	0.62	0.04	0.001	1.58	3.15	5.30	5.30	0.05
2	015	0.23	0.70	0.04	0.003	1.54	1.65	3.23	4.00	0.04
3	015	0.19	0.60	0.04	0.002	1.21	3.31	4.13	5.15	0.03
4	015	0.14	0.72	0.04	0.002	1.70	1.64	4.72	5.38	0.04
5	015	0.20	0.56	0.02	0.002	1.78	1.26	5.66	4.00	0.03
1	005	0.26	0.82	0.05	0.003	2.50	4.28	5.12	5.00	0.05
2	005	0.23	0.83	0.05	0.002	1.62	4.70	3.10	4.19	0.05
3	005	0.22	0.87	0.06	0.003	1.60	5.59	3.42	5.47	0.04
4	005	0.16	0.73	0.03	0.002	1.21	1.88	3.21	4.83	0.04
5	005	0.14	0.50	0.01	0.002	2.83	1.00	4.00	3.56	0.03
1	106	0.65	0.82	0.07	0.002	8.48	6.17	6.54	6.92	0.04
2	106	0.40	0.90	0.04	0.004	5.22	14.38	4.95	7.58	0.05
3	106	0.37	0.71	0.08	0.003	5.44	8.00	4.67	4.67	0.04

Year	Ribotype	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
4	106	0.16	0.63	0.02	0.005	8.00	1.85	4.67	4.76	0.03
5	106	0.26	0.55	0.04	0.002	4.27	4.88	5.56	5.94	0.04
1	023	0.19	0.72	0.06	0.003	1.83	0.83	3.65	6.48	0.05
2	023	0.25	0.75	0.07	0.002	1.39	1.34	3.59	4.46	0.04
3	023	0.22	0.76	0.07	0.002	1.74	1.00	3.25	5.66	0.04
4	023*	0.18	0.35	0.02	0.002	1.41	0.59	2.38	4.00	0.03
5	023	0.15	0.50	0.05	0.003	1.68	0.59	4.76	4.00	0.04
1	018	0.41	2.00	0.04	2.072	35.33	4.42	5.56	5.04	0.04
2	018	0.31	0.69	0.05	1.111	11.89	7.43	5.80	4.76	0.04
3	018	0.20	0.67	0.03	1.425	11.31	8.00	5.34	5.04	0.04
4	018	0.19	1.00	0.03	0.002	0.92	2.38	3.67	4.36	0.04
5	018	not isolated								
1	356	0.61	2.28	0.04	18.871	50.80	8.55	5.86	4.88	0.04
2	356	0.57	0.57	0.05	32.000	32.00	13.93	6.06	4.59	0.05
3	356	0.13	1.00	0.03	32.000	16.00	32.00	4.00	4.00	0.03
4	356	not isolated								
5	356	not isolated								

Year	Ribotype	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
1	012	0.27	0.72	0.05	0.005	2.40	51.42	5.36	9.60	0.08
2	012	0.39	0.91	0.05	0.002	1.81	55.17	3.81	6.56	0.08
3	012	0.29	0.79	0.06	0.002	2.52	69.12	4.00	11.76	0.06
4	012	0.21	0.74	0.03	0.007	2.83	43.07	4.64	9.28	0.04
5	012	0.20	0.50	0.04	0.050	1.59	6.35	1.59	3.17	0.06
1	017	0.27	0.65	0.02	0.925	18.22	43.34	5.91	7.66	0.04
2	017	0.22	0.61	0.02	1.187	13.59	39.24	9.81	14.75	0.05
3	017	0.22	0.72	0.05	0.420	9.19	50.80	10.56	14.59	0.05
4	017	0.13	0.74	0.02	0.505	9.75	11.89	4.42	6.56	0.05
5	017	0.20	0.50	0.01	0.201	7.13	57.02	8.98	8.98	0.04
1	198	1.19	0.39	0.04	0.006	5.66	0.77	7.34	4.00	0.03
2	198	1.64	0.55	0.08	0.015	23.78	12.49	7.61	4.88	0.03
3	198	1.68	0.50	0.10	0.004	24.68	4.00	8.00	8.00	0.04
4	198	2.52	0.79	0.07	0.225	28.51	3.56	7.13	5.66	0.04
5	198	not isolated								
1	176	2.18	0.65	0.02	1.009	26.91	2.00	5.66	3.08	0.04
2	176	0.77	0.65	0.05	0.848	17.45	1.41	7.34	3.67	0.03

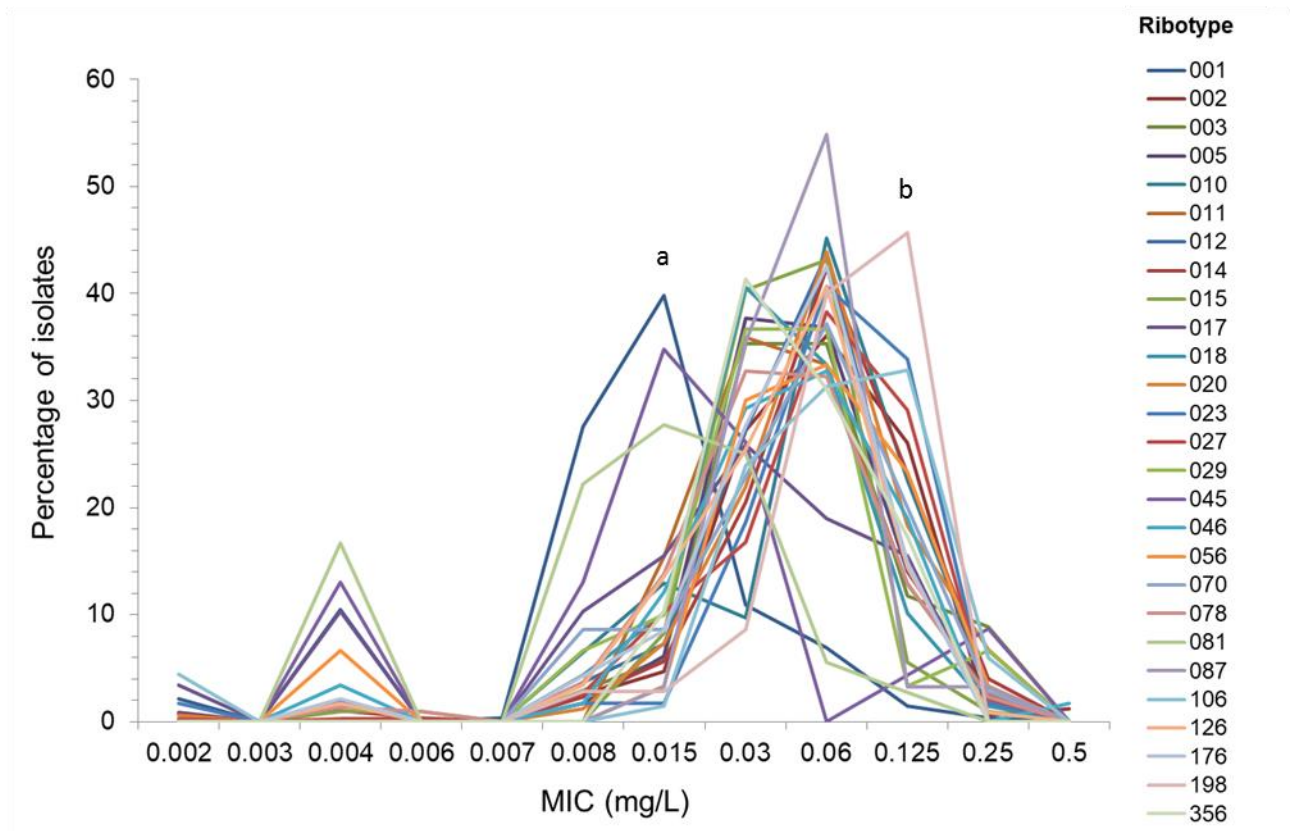
Year	Ribotype	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG
3	176	2.11	1.00	0.08	0.601	30.34	4.69	9.90	4.22	0.04
4	176	1.00	0.50	0.04	32.000	16.00	0.68	8.00	4.00	0.03
5	176	1.41	0.59	0.03	32.000	16.00	0.25	10.37	4.00	0.03
1	all isolates	0.37	0.76	0.04	0.008	4.79	5.72	4.75	5.82	0.05
2	all isolates	0.33	0.70	0.05	0.009	3.90	7.61	3.88	5.39	0.04
3	all isolates	0.29	0.70	0.05	0.007	3.73	9.18	4.17	6.06	0.04
4	all isolates	0.24	0.67	0.03	0.006	3.35	4.11	4.37	5.61	0.04
5	all isolates	0.21	0.59	0.02	0.006	2.57	2.62	4.74	4.97	0.04
1–5	all isolates	0.46	0.70	0.04	0.007	3.88	6.21	4.32	5.67	0.04

84 M, metronidazole; V, vancomycin; FDX, fidaxomicin; MIC, minimum inhibitory concentration; RIF, rifampicin; MXF, moxifloxacin; CLINDA, clindamycin; IMI,
85 imipenem; CHLOR, chloramphenicol; TIG, tigecycline. MICs are reported as geometric means

86 Online Resource 5

87 Fidaxomicin MIC (mg/L) distributions for the most prevalent (n>30 isolates) ribotypes across
88 all years of the study

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91 MIC, minimal inhibitory concentration; RT, ribotype. ^aRT001 geometric mean MICs were significantly
92 lower than the geometric mean fidaxomicin MIC for all ribotypes. ^bRT198 geometric mean MICs were
93 significantly higher than the geometric mean fidaxomicin MICs for all ribotypes

94

95 Online Resource 6

96 MICs of the nine antimicrobials tested for all RT344 isolates

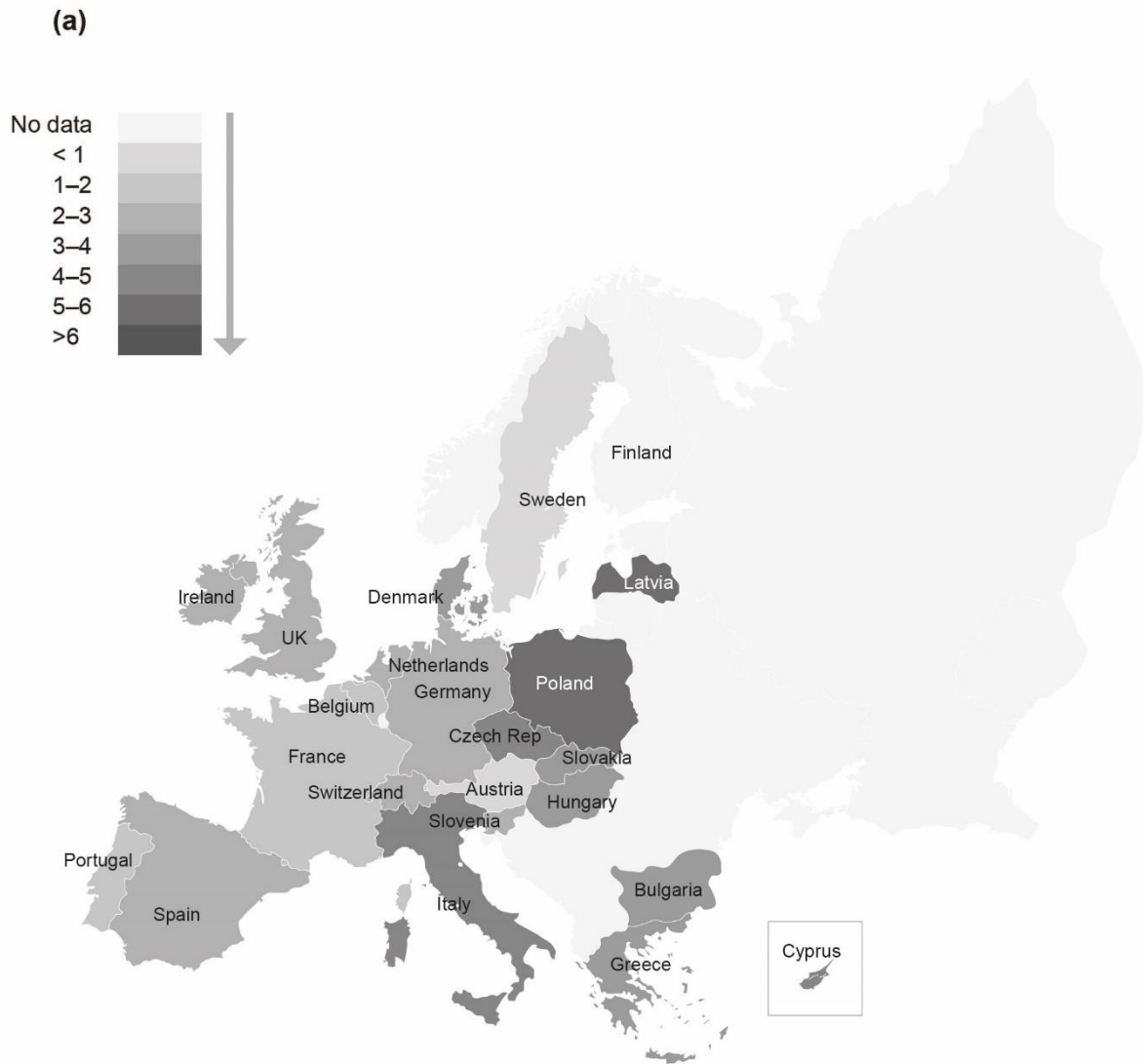
ID	M	V	FDX	RIF	MXF	CLINDA	IMI	CHLOR	TIG	Country
L,16.7569611	0.125	0.25	0.004	0.001	1	0.5	2	4	0.03	France
L,16.7570015	0.125	0.5	0.015	0.002	2	0.125	2	4	0.03	Slovenia
L,16.7570242	0.125	1	0.03	0.001	8	0.5	4	4	0.03	France
L,15.7104276	0.125	0.5	0.06	0.002	2	16	4	8	0.06	Greece
L,16.7569682	0.25	1	0.5	0.001	2	8	4	8	0.06	Belgium
L,16.7570132	0.125	1	>4	0.002	16	16	16	4	0.03	France

97 M, metronidazole; V, vancomycin; FDX, fidaxomicin; MIC, minimum inhibitory concentration; RIF,
 98 rifampicin; MXF, moxifloxacin; CLINDA, clindamycin; IMI, imipenem; CHLOR, chloramphenicol; TIG,
 99 tigecycline. MICs are reported as geometric means

100

101 Online Resource 7

102 Distribution of mean cumulative antimicrobial resistance scores for *Clostridium difficile*
103 isolates across Europe in a) Year 1, b) Year 2, c) Year 3, d) Year 4 and e) Year 5 of the
104 ClosER study

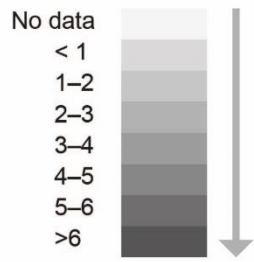


105

106

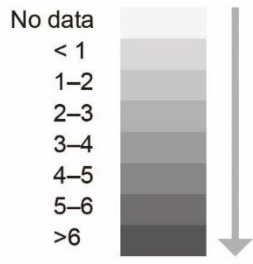
107

(b)

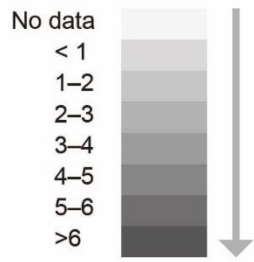


108

(c)

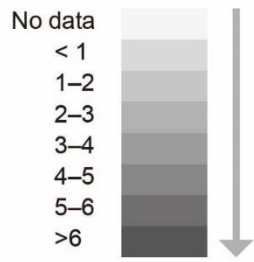


(d)



111

(e)



112

113

Online Resource 8

Cumulative resistance score versus ribotype diversity score for each country, Years 1–5 of the *ClosER* study

