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Antimicrobial susceptibility of anaerobic bacteria

A Europe-wide assessment of antibiotic resistance rates in *Bacteroides* and *Parabacteroides* isolates from intestinal microbiota of healthy subjects

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ABSTRACT

Here, we sought to assess the levels of antibiotic resistance among intestinal *Bacteroides* and *Parabacteroides* strains collected between 2014 and 2016 in Europe and also attempted to compare resistance levels between clinical and commensal isolates. *Bacteroides* and *Parabacteroides* isolates were recovered from faecal samples via the novel *Bacteroides* Chromogenic Agar (BCA) method. Antibiotic susceptibilities were determined by agar dilution for ten antibiotics. The values obtained were then statistically evaluated. Altogether 202 *Bacteroides*/*Parabacteroides* isolates (of which 24, 11.9%, were *B. fragilis*) were isolated from the faecal specimens of individuals taken from five European countries. The percentage values of isolates resistant to ampicillin, amoxicillin/clavulanate, cefoxitin, imipenem, clindamycin, moxifloxacin, metronidazole, tetracycline, tigecycline and chloramphenicol were 96.6, 4.5, 14.9, 2.0, 47.3, 11.4, 0, 66.2, 1.5 and 0%, respectively. These values are close to those reported in the previous European clinical *Bacteroides* antibiotic susceptibility survey except for amoxicillin/clavulanate and clindamycin, where the former was lower and the latter was higher in normal microbiota isolates. To account for these latter findings and to assess temporal effects we compared the data specific for Hungary for the same period (2014–2016), and we found differences in the resistance rates for cefoxitin, moxifloxacin and tetracycline.

1. Introduction

The related genera of *Bacteroides* and *Parabacteroides*, which previously formed the *B. fragilis* group belonging to the *Bacteroidetes* phylum, are important opportunistic anaerobic pathogens. They are also important members of the human and mammalian normal intestinal microbiota that, together with the *Prevotella* and the Firmicutes species, constitute the most common taxa in the gut. With their abundance they are an indispensable part of it and contribute to the healthy function of the gut [1]. Earlier microbiological investigations confirmed their role in colonisation resistance, commensalism, production of nutrients and the maturation of the gut [1]. Also, the application of next-generation sequencing methods has aided the analysis of the role of the gut microbiome in various healthy and diseased states by revealing a contribution to disorders such as obesity, diabetes, inflammatory bowel syndrome and other autoimmune diseases [2]. Studies have confirmed the

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complexity of the intestinal microbiota, but they also showed some consistent patterns (enterotypes) [3]. The gene content of the microbiota exceeds the number of host genes [4] and it exerts complex metabolic, nutritional, microbiological, ecological and immunological interactions. It has been shown experimentally that the *B. fragilis* group contributes to these interactions by digestion capabilities [5,6] and the modulation of the immune system by their capsular polysaccharides [7,8].

However, the mechanisms by which their commensalism and virulence are regulated together are not known. Their potential for pathogenicity is exacerbated by the fact that '*B. fragilis* group species' are the most antibiotic resistant among all pathogenic anaerobes in terms of resistance prevalence and the number of resistance mechanisms [9]. Regular susceptibility surveys have been conducted to estimate antibiotic susceptibility both spatially and temporally. From these studies it is possible to monitor the evolution of their resistance mainly in Europe and the USA, and it has been shown that (i) there are very high resistance rates for penicillins, cephalosporins and tetracyclines (about 70–99%); (ii) intermediate levels of resistance are common for cephamycins, clindamycin, moxifloxacin and some β-lactam/β-lactamase inhibitor combinations which are subject to changes in antibiotic usage and (iii) carbapenems, metronidazole and tigecycline remain very effective and seem almost unaffected by their usage rates [10]. Also, genomic investigations have demonstrated that *Bacteroides* species may be reservoirs of antibiotic resistance genes [11].

We sought to determine the susceptibility rates of the normal *Bacteroides* microbiota in European citizens with possible implications for treatment of infections that may arise from this flora and compare these with the antibiotic resistance rates of isolates from clinical cases. Here, we report our results on the antibiotic susceptibilities of 202 *B. fragilis* group isolates obtained from the normal flora of individuals living in five European countries.

2. Materials and methods

2.1. Subjects

During 2014–2016 stool samples taken from healthy donors ($n = 42$) who did not have an enteric disease and had not received any antibiotic therapy for at least 3 months prior to the tests, were taken in Belgium ($n = 5$), Germany ($n = 5$) Hungary ($n = 12$), Slovenia ($n = 8$) and Turkey ($n = 12$).

2.2. Bacterial isolates

The following protocol was used for the isolation of *Bacteroides* and *Parabacteroides* strains from stool samples. Approximately 5 mg (one small loopful) of faecal material was suspended in 1 ml of brain heart infusion (BHI) broth and then diluted 10^2- and 10^4-fold by sequentially adding 50 μL–4950 μl of BHI broth and plating 100 μl aliquots on the surface of the novel selective *Bacteroides* chromogenic agar (BCA) plates with (BCA-A) or without (BCA-B) 4 mg/L meropenem [12]. The plates were incubated anaerobically at 37 °C for 48 h. Afterwards the approximate numbers of colonies grown were estimated and 3–8 colonies with different colony morphologies were selected and subcultured on anaerobic Schaedler and Columbia Blood agars and incubated with standard anaerobiosis (48 h) and aerobiosis (overnight), respectively. The strains isolated in Belgium, Germany, Slovenia and Turkey were transported to the Szeged laboratory in ESwab 480C transport tubes (COPAN Diagnostics Inc., USA) at ambient temperature by courier firms in compliance with international safety regulations for transport of biological materials. After referral to Szeged, Hungary, strains were subcultured immediately, species identifications were carried out via the MALDI-TOF MS method (Microflex LP instrument and Biotyper 3.1 software package, Bruker Daltonics, Bremen, Germany) [13]. Identifications were accepted if the log scores were ≥2.0. If the log scores were <2.0, then 16S rDNA sequencing was applied to determine species identification. We also used MALDI-TOF MS to determine which genetic divisions (Division I – carbapenemase/*cfiA*-negative or Division II – carbapenemase/*cfiA*-positive) the *B. fragilis* strains belonged to Refs. [14]. The long-term storage of the *Bacteroides* or *Parabacteroides* isolates was achieved in BHI broth containing 20% glycerol at −70 °C.

The regular cultivation of the isolates in Szeged, Hungary, was performed on Columbia agar supplemented with 5% sheep blood, 0.6 g/L cysteine and 1 mg/L vitamin K_1 in an anaerobic cabinet (Concept 400, Ruskinn Technology Ltd., Bridgend, UK) with an atmosphere of 70% N_2, 10% H_2 and 5% CO_2.

Overall, 202 *Bacteroides/Parabacteroides* isolates (9 *B. caccae*, 6 *B. cellulosilyticus*, 7 *B. clarus/stercoralis*, 1 *B. coprocola*, 4 *B. eggerthii*, 1 *B. faecis*, 4 *B. finegoldii*, 24 *B. fragilis*, 3 *B. intestinalis*, 2 *B. nordii*, 48 *B. ovatus/xylanisolvens*, 7 *B. stercoris*, 19 *B. thetaiotaomicron*, 14 *B. uniformis*, 36 *B. vulgatus/dorei*, 12 *P. distasonis*, 4 *P. johnsonii* and 1 *P. merdae*) were collected and the species distribution by country is given in the Supplementary data (see Table S1).

2.3. Determination of antibiotic susceptibilities

The standard agar dilution technique was used to measure antibiotic susceptibilities as recommended by the CLSI using antibiotic-supplemented Brucella agar inoculated with around 5×10^5 cells [10,15]. The following antibiotics were tested: ampicillin, amoxicillin/clavulanate, cefoxitin, imipenem, clindamycin, moxifloxacin, metronidazole, tetracycline, tigecycline and chloramphenicol. For quality control, *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were included. Where available, EUCAST resistance breakpoints were applied (>2, >8, >8, >4 and > 4 mg/L for ampicillin, amoxicillin/clavulanate, imipenem, clindamycin and metronidazole) [16]. Where EUCAST breakpoints were unavailable, CLSI breakpoints (>32, >4, >8, >8 and > 16 mg/L for cefoxitin, moxifloxacin, tetracycline, tigecycline and chloramphenicol) [15] were applied to categorize resistance.

2.4. 16S rDNA sequencing

For PCR amplification, template DNA preparations were prepared by the boiling method. Stated briefly, one small loopful of colonies grown anaerobically on Columbia blood agar plates was suspended in 100 μl of sterile distilled water to give a 0.5 McFarland density, incubated at 99.5 °C in a dry bath for 12 min and centrifuged at 14,000 rpm for 2 min then the supernatant stored at −25 °C until use. For 16S rDNA sequencing the E8F (AGAGTTTGATCCTGGCTCAG) and the E533R (TIACCGIIICTICTGGCAC) primers (0.7 μM) were used in 50 μl PCR volumes containing 25 μl PCR master mix (2x, DreamTaq, Fermentas) and 5 μl template DNA using the following cycling conditions: 95 °C 2 min 30 s; 95 °C 14 s, 56 °C 20 s, 72 °C 1 min 35x; 72 °C 7 min. The products were cleaned using a PCR Cleanup Kit and processed via the BigDye Terminator v3.1 Cycle Sequencing Kit with the 3500 Series Genetic Analyzer (LifeTechnologies). The nucleotide sequences obtained were blasted to records of type strains in GenBank for species identification [17].

2.5. Statistical evaluation

The data set collected in this study was analysed via Spearman rank correlation or compared with the data of the clinical isolates obtained earlier by χ^2-tests (resistance data) or variance analyses

(distribution parameters) via a non-parametric variance analysis (Kruskal-Valis method) and using the Sigmaplot 12 software package (Sigmaplot, Germany). The significance level was set to 0.05.

3. Results and discussion

3.1. The isolation of intestinal Bacteroides and Parabacteroides strains from the stool samples of healthy subjects

The cultivation of *Bacteroides/Parabacteroides* isolates from stool samples using BCA plates yielded a total of 202 isolates from five European countries (the complete distribution is shown in Table S1, and non-targeted isolates are listed in the Supplementary material). The approximate numbers of CFU for *Bacteroides* and *Parabacteroides* calculated for 1 g of faecal material among Hungarian patients were 2.6×10^7 (SD = 3.18×10^7) and 1.41×10^9 (SD = 1.83×10^9) on BCA plates with and without meropenem, respectively, indicating that *Bacteroides* isolates with elevated meropenem MICs are not infrequent in the faecal microbiome (but about 2 orders of magnitude less than the susceptible isolates). The species distribution listed in Table S1 may reflect the real situation in the colon as non-*fragilis Bacteroides* (NFB) species were prevalent and the proportion of *B. fragilis* was only 11.9%. The *B. clarus/stercoralis, ovatus/xylanisolvens* and *vulgatus/dorei* pairs were not resolved since they had not been distinguished in the previous reference study [10]. The number of bacterial cells in faecal material is estimated to be around 10^{11}/g and the proportion of *Bacteroides* is around 30% in faecal material, hence roughly 3×10^{10}/g *Bacteroides* cells can be expected there. In this study we were able to recover an average of 1.41×10^9 *Bacteroides* per gram of sample; and about 5% of this theoretical value, which can be explained by the fact that not all *Bacteroides* isolates/species are oxygen tolerant or there were also dead cells. A study that reported live cell counts in the rabbit intestinal tract, appeared recently and it demonstrated that dead cells are also present there [18]. The ratio of *B. fragilis* isolates among the total of *Bacteroides* isolates recovered was 11.9%, which was similar to that found by Møller-Hansen et al. [19]. This once again confirmed that NFB isolates are more abundant than *B. fragilis* in the intestinal normal flora, but the actual proportion of the *B. fragilis* living cells is also significant and was higher than earlier thought (by 0.5–10%) [1].

3.2. Determination of the antibiotic susceptibilities of intestinal Bacteroides and Parabacteroides isolates

The antibiotic susceptibilities for ampicillin, amoxicillin/clavulanate, cefoxitin, imipenem, clindamycin, moxifloxacin, metronidazole, tetracycline, tigecycline and chloramphenicol were recorded for the 202 *B. fragilis* group isolates recovered from the normal microbiota by agar dilution. Tetracycline and chloramphenicol were included as they may be considered as a choice for treating infections caused by strains that are resistant to more conventional agents. Country-specific data values are displayed in Table 1 and cumulative data values are displayed in Table 2 with the MIC range, MIC_{50}, MIC_{90} and the resistance rates. The cross-correlations between pairs of antibiotics are given in the Supplementary material.

There were some isolates, 17 (8.5%) and 5 (2.5%, Table S3), that had a resistance to >3 or 5 antibiotics, respectively. In Hungary, in a recent antibiotic resistance survey, the prevalence of MDR *Bacteroides* strains was estimated to be 1.5% (6/400) [20], but we have no prevalence data on MDR *Bacteroides* from other countries that participated in this study. Also, no isolates were recovered that had

Table 1
Antibiotic susceptibility data of normal flora *B. fragilis* group isolates obtained from Europe.

Country	Antibiotic	MICs (mg/L)			R (%)	Country	Antibiotic	MICs (mg/L)			R (%)
		Range	MIC_{50}	MIC_{90}				Range	MIC_{50}	MIC_{90}	
All[a]	Ampicillin	1->256	128	>256	96.6	Hungary	Ampicillin	2->256	128	>256	99.0
	Amoxicillin/clavulanate	0.064–32	0.5	4	4.5		Amoxicillin/clavulanate	0.064–32	1	8	6.2
	Cefoxitin	0.5–256	16	64	14.9		Cefoxitin	0.5–128	32	128	31.2
	Imipenem	0.032–324	0.5	2	2.0		Imipenem	0.064–16	0.5	4	3.1
	Clindamycin	0.064->256	4	>256	47.3		Clindamycin	0.064->256	2	>256	35.4
	Moxifloxacin	0.064–64	1	8	11.4		Moxifloxacin	0.064–64	1	8	6.2
	Metronidazole	0.032–4	0.5	1	0		Metronidazole	0.032–4	0.5	1	0
	Tetracycline	0.064->256	32	128	66.2		Tetracycline	0.125–128	16	32	66.7
	Tigecycline	0.032–32	0.5	4	1.5		Tigecycline	0.032–8	0.25	2	0
	Chloramphenicol	0.125–8	4	8	0		Chloramphenicol	0.25–8	4	8	0
Belgium	Ampicillin	8->256	256	>256	100	Slovenia	Ampicillin	<2–>256	32	256	90.9
	Amoxicillin/clavulanate	0.125–16	1	4	3.4		Amoxicillin/clavulanate	0.064–2	0.225	1	0
	Cefoxitin	0.5–256	4	128	20.3		Cefoxitin	0.5–32	16	32	0
	Imipenem	0.064–0.5	0.5	0.5	6.8		Imipenem	0.064–16	0.5	1	4.5
	Clindamycin	0.125->256	16	>256	62.7		Clindamycin	0.064–8	4	8	0
	Moxifloxacin	0.125–64	1	16	25.4		Moxifloxacin	0.064->32	2	4	4.2
	Metronidazole	0.064–0.5	0.5	0.5	0		Metronidazole	0.064–1	0.5	1	0
	Tetracycline	0.5->256	32	64	71.2		Tetracycline	0.064–128	8	32	45.4
	Tigecycline	0.032–32	1	8	3.4		Tigecycline	0.125–4	0.5	4	0
	Chloramphenicol	0.125–8	8	8	0		Chloramphenicol	0.125–8	8	8	0
Germany	Ampicillin	4 > 256	32	>256	100	Turkey	Ampicillin	<2–>256	64	>256	87.5
	Amoxicillin/clavulanate	<0.016–0.5	0.125	0.5	0		Amoxicillin/clavulanate	0.064–16	0.5	2	2.5
	Cefoxitin	1–128	16	128	29.2		Cefoxitin	0.5–128	16	32	5.0
	Imipenem	0.064–4	0.5	2	0		Imipenem	0.032–2	0.5	1	0
	Clindamycin	0.125->256	64	>256	87.5		Clindamycin	0.064->256	4	>256	40.0
	Moxifloxacin	0.064–32	2	16	20.8		Moxifloxacin	0.064–64	2	32	20.0
	Metronidazole	0.064–4	0.5	1	0		Metronidazole	0.064–0.5	0.5	0.5	0
	Tetracycline	0.125–128	32	128	75.0		Tetracycline	0.064–128	64	128	85.0
	Tigecycline	<0.016–16	0.5	2	4.2		Tigecycline	0.032–16	0.25	4	2.5
	Chloramphenicol	0.5–4	8	8	0		Chloramphenicol	0.25–8	2	4	0

[a] From all the given countries.

Table 2
Comparison of the antibiotic susceptibility parameters of clinical and normal flora *B. fragilis* group isolates obtained in Europe.

Antibiotic/taxon	Clinical isolates[a]				Intestinal isolates[b]				p[d]
	MICs (mg/L)			R (%)	MICs (mg/L)			R (%)	
	Range	MIC_{50}	MIC_{90}		Range	MIC_{50}	MIC_{90}		
Ampicillin									
All isolates[c]	1->256	32	>256	98.2	1->256	128	>256	96.6	n.s.[e]
B. fragilis	1->256	32	>256	97.4	4->256	32	>256	100	n.s.
B. thetaiotaomicron	2->256	64	>256	98.8	4->256	>256	>256	100	n.s.
B. ovatus	8->256	64	>256	100	1->256	64	>256	95.8	n.s.
B. vulgatus	4->256	64	>256	100	1->256	128	>256	94.3	n.s.
B. uniformis	16->256	32	>256	100	1->256	128	>256	90.9	n.s.
P. distasonis	8->256	16	>256	100	8->256	>256	>256	100	–[f]
Other species	4->256	64	>256	100	1->256	256	>256	94.1	n.s.
Amoxicillin/clavulanic acid									
All isolates	0.016->256	1	16	10.4	0.064–32	0.5	4	4.5	**0.021**
B. fragilis	0.016->256	1	16	8.7	0.064–16	0.5	8	4.2	n.s.
B. thetaiotaomicron	0.125–32	1	16	12.0	0.064–16	1	4	5.3	n.s.
B. ovatus	0.25–32	1	16	18.4	0.064–16	0.5	4	4.2	n.s.
B. vulgatus	0.125–256	1	16	14.3	0.064–2	0.55	1	0	**0.022**
B. uniformis	0.125–64	1	64	30.0	0.064–8	2	8	0	n.s.
P. distasonis	0.5–256	2	32	21.4	0.064–32	2	32	23.1	n.s.
Other species	0.125–64	2	16	11.5	0.064–16	0.5	4	3.9	n.s.
Cefoxitin									
All isolates	1->256	16	128	17.2	0.5–256	16	64	14.9	n.s.
B. fragilis	1->256	16	256	13.7	1–32	16	32	0	**0.031**.
B. thetaiotaomicron	2->256	32	256	27.1	4–128	32	128	31.6	n.s.
B. ovatus	1–256	32	256	24.5	0.5–128	16	64	12.5	n.s.
B. vulgatus	1->256	8	64	14.3	1–128	16	64	14.3	n.s.
B. uniformis	2–256	8	256	20.0	0.5–128	16	128	18.2	n.s.
P. distasonis	8->256	32	>256	35.7	0.5–128	8	128	23.1	n.s.
Other species	2->256	16	>256	26.9	0.5–128	8	64	15.7	n.s.
Imipenem									
All isolates	0.002->32	0.5	1	0.85	0.032–324	0.5	2	2.0	n.s.
Strains isolated on BCA-B					0.032–32	0.5	1	1.4	n.s.
B. fragilis	0.002->32	0.25	0.5	1.2	0.064–16	0.5	16	13.6	**<0.001**
Strains isolated on BCA-B					0.125–16	0.5	1	10.5	**0.02**.
B. thetaiotaomicron	0.047–8	0.25	1	0	0.25–2	1	2	0	–
B. ovatus	0.016–2	0.25	1	0	0.064–16	0.5	4	2.1	n.s.
B. vulgatus	<0.125–2	0.125	0.5	0	0.064–16	0.5	2	2.9	n.s.
B. uniformis	0.125–2	0.125	1	0	0.064–1	0.5	1	0	–
P. distasonis	0.125–2	0.5	1	0	0.25–2	1	2	0	–
Other species	0.012–8	0.5	4	0	0.064–4	0.5	2	0	–
Clindamycin									
All isolates	0.016->256	2	>256	32.4	0.064->256	4	>256	47.3	**<0.001**
B. fragilis	0.016->256	1	256	28.5	0.125->256	2	>256	20.8	n.s.
B. thetaiotaomicron	0.047->256	4	>256	42.2	0.064->256	8	>256	63.2	ns.
B. ovatus	0.125->256	4	>256	44.9	0.064->256	4	>256	45.8	n.s.
B. vulgatus	0.016->256	2	>256	47.6	0.064->256	4	>256	48.6	n.s.
B. uniformis	1->256	8	256	60.0	0.25->256	128	>256	63.6	n.s.
P. distasonis	0.016->256	1	>256	28.6	0.064->256	8	>256	53.9	n.s.
Other species	0.047->256	2	256	34.6	0.064->256	8	>256	49.0	n.s.
Moxifloxacin									
All isolates	<0.125–32	1	16	13.6	0.064–64	1	8	11.4	n.s.
B. fragilis	<0.125–64	0.5	8	14.0	0.064–4	0.5	2	0	**0.032**
B. thetaiotaomicron	0.125–32	1	16	14.5	0.5–16	2	8	10.5	n.s.
B. ovatus	<0.125–32	1	4	8.2	0.064–64	1	8	8.3	n.s.
B. vulgatus	<0.125–64	1	32	21.4	0.064–64	2	32	11.4	n.s.
B. uniformis	<0.125–4	1	4	0	0.064–32	4	32	45.5	**0.023**
P. distasonis	<0.125–2	0.5	1	0	0.25–16	0.5	16	30.8	**0.048**
Other species	<0.125–32	0.25	4	11.5	0.064–64	1	4	7.8	n.s.
Metronidazole									
All isolates	0.016–256	0.5	1	0.5	0.5–1	0.5	1	0	n.s.
B. fragilis	0.016–32	0.5	1	0.5	0.064–4	0.5	1	0	n.s.
B. thetaiotaomicron	<0.125->256	0.5	2	1.2	0.064–2	0.5	1	0	n.s.
B. ovatus	0.032–2	0.5	2	0	0.064–4	0.5	1	0	–
B. vulgatus	<0.125–4	0.5	1	0	0.064–4	0.5	1	0	–
B. uniformis	0.016–2	0.5	1	0	0.032–1	0.5	1	0	–
P. distasonis	0.125–2	0.5	1	0	0.064–0.5	0.5	0.5	0	–
Other species	<0.125–4	0.5	2	0	0.064–2	0.5	1	0	–
Tetracycline									
All isolates					0.064->256	32	128	66.2	
B. fragilis					0.125–128	32	128	58.3	
B. thetaiotaomicron					0.064–256	32	128	89.5	
B. ovatus					0.125–128	16	64	52.1	
B. vulgatus					0.125–128	32	128	88.6	

Table 2 (*continued*)

Antibiotic/taxon	Clinical isolates[a]				Intestinal isolates[b]				p[d]
	MICs (mg/L)			R (%)	MICs (mg/L)			R (%)	
	Range	MIC_{50}	MIC_{90}		Range	MIC_{50}	MIC_{90}		
B. uniformis					0.064–128	32	128	63.6	
P. distasonis					0.5–32	16	64	69.2	
Other species					0.125–64	32	32	58.8	
Tigecycline									
All isolates	0.016–32	0.25	2	1.7	0.032–32	0.5	4	1.5	n.s.
B. fragilis	0.016–32	0.5	2	1.8	0.064–8	2	4	0	n.s.
B. thetaiotaomicron	0.064–8	0.25	0.5	0	0.064–32	1	4	5.3	n.s.
B. ovatus	0.032–16	0.25	2	2.0	0.064–16	0.25	8	2.1	n.s.
B. vulgatus	<0.125–16	2	4	4.8	0.064–16	0.25	2	2.9	n.s.
B. uniformis	0.016–16	0.125	1	10.0	0.032–2	0.064	8	0	n.s.
P. distasonis	0.125–4	0.5	2	0	0.125–4	0.5	2	0	–
Other species	0.047–8	0.125	1	0	0.032–8	0.5	2	0	–
Chloramphenicol									
All isolates					0.125–8	4	8	0	
B. fragilis					4–8	4	8	0	
B. thetaiotaomicron					1–8	8	8	0	
B. ovatus					0.125–8	4	8	0	
B. vulgatus					0.125–8	4	8	0	
B. uniformis					0.5–8	4	8	0	
P. distasonis					0.25–8	2	8	0	
Other species					0.125–8	4	8	0	

[a] From Ref. [10].
[b] Data from this study.
[c] All *Bacteroides* and *Parabacteroides* species.
[d] The significance values of differences obtained by χ^2-tests (significances are given in bold).
[e] Here, n. s. means non-significant.
[f] Equal values.

a resistance to both imipenem and metronidazole or both imipenem and tigecycline.

3.3. A comparison of our new data with the antibiotic susceptibility data in the previous European study on clinical Bacteroides isolates

The findings of this study were also compared with those of the clinical isolates in the latest European *Bacteroides* susceptibility survey [10] (see Table 2), using regular epidemiological parameters such as MIC range, MIC_{50}, MIC_{90} and the resistance rate. Our comparison of resistance data for the normal microbiota and clinical isolates tells us that the general trends are similar for both intestinal and clinical isolates: almost 100% resistance for ampicillin, intermediate rates of resistance (13–44%) for moxifloxacin, cefoxitin and clindamycin and very low resistance rates (0–4%) for amoxicillin/clavulanate, imipenem, metronidazole and tigecycline [10,21]. The resistance rate of the *B. fragilis* group isolates for tetracycline was 69.7%, which is close to that (75.9%) for clinical isolates obtained recently in Romania [22]. No chloramphenicol-resistant strain was found and this is consistent with the findings of other studies. However, at present there is a scarcity of data for these two last antibiotics.

In addition, a systematic analysis using the χ^2-test to compare the resistance rates of clinical isolates and the normal microbiota revealed statistically significant differences for amoxicillin/clavulanate (decreased resistance) and clindamycin (increased resistance); see Table 1. For *B. fragilis* isolates. Higher resistance rates were found for imipenem irrespective of whether they were isolated on BCA-A or BCA-B plates (Table 1). Statistically different resistance rates were found with some species for some antibiotics, but high significance values (<0.001) were only found for clindamycin for all *Bacteroides/Parabacteroides* isolates and for imipenem in the case of *B. fragilis*, regardless of which BCA plate type was used for the isolation (Table 1). Some earlier studies also assessed the antibiotic susceptibility of *Bacteroides* isolates obtained from normal microbiota and reported similar results to those for clinical isolates. However, it should also be mentioned that these studies were different in various respects, such as the taxa involved, anatomical sites of isolation and the age and health of the subjects [19,23–29]. They also did not include comparisons with isolates from infections and examined fewer strains than ours; hence a clear conclusion could not be drawn. But Hansen et al. reported that carbapenem therapy significantly increased the number of carbapenem-resistant strains recovered from patient's faecal samples [19].

The differences found in resistance in our study might be accounted by the following factors: (i) the resistance data simply changed over the time that had elapsed between the two studies (2008–10 and 2014–16); (ii) because of differences between countries and different species composition; and (iii) there were real differences in antibiotic resistance between the clinical and normal microbiota isolates.

As there were country- and species-specific differences in the resistance data (Table S2) we think that temporal changes were probably chiefly responsible for the observed differences. This is supported by the fact that if we plot the fairly constant clindamycin consumption against the resistance percentage in Hungary an increase in resistance can be seen (Fig. S1). This may be explained by models showing that above a certain threshold of antibiotic use, an increase in the resistance rate can be expected regardless of whether the usage increases further [30].

However, if we also compare the data from Hungary from this study with the antibiotic susceptibilities of a parallel study on clinical *Bacteroides* isolates also obtained from Hungary in the same period of time [31], we observe differences for cefoxitin, moxifloxacin and tetracycline (Table S4). In our opinion this means that the difference depends on the isolation sites, e.g. fecal microbiota versus clinical isolates. Recently differences were found in the microbial compositions of the mucosa and lumen of the small bowel, large bowel and feces of humans and this may indicate that the antimicrobial resistance and resistance mechanisms also change depending on the anatomical site [32–34]. It is also known

that the resistance elements for cefoxitin and tetracycline are mobile so the mobility of the resistance elements for these antibiotics may also differ in the mucosal microbiota and feces. Transfer regulations were described for the tetracycline resistance conjugative transposons of *Bacteroides* by tetracycline itself [35] and for Tn*916* of Firmicutes by ribosome targeting antibiotics [36], and hence a similar differential regulation of horizontal spread is anticipated in the intestine.

4. Conclusions

Overall, we can state the following points: (i) the antibiotic susceptibilities of strains obtained from five European countries were found to be similar to the previous European susceptibility study on clinical *Bacteroides* group strains; (ii) exceptions were noted for amoxicillin/clavulanate and clindamycin; and (iii) these exceptions were most probably caused by temporal, spatial and taxonomical differences, but differences in the anatomical origin and thus between normal microbiota and clinical strains can also be expected. We think that a more extensive, comparative investigation on the frequency of antibiotic resistance genes harboured by these species will help us clarify this issue.

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Transparency declarations

None declared.

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Appendix A. Supplementary data

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