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Prevalence of Antibiotic Resistance Genes among Human Gut-Derived Bifidobacteria

Sabrina Duranti,[a] Gabriele Andrea Lugli,[a] Leonardo Mancabelli,[a] Francesca Turroni,[a] Christian Milani,[a] Marta Mangifesta,[b] Chiara Ferrario,[a] Rosaria Anzalone,[a] Alice Viappiani,[b] Douwe van Sinderen,[c] Marco Ventura[a]

Laboratory of Probiogenomics, Department of Life Sciences, University of Parma, Parma, Italy[a]; GenProbio srl, Parma, Italy[b]; APC Microbiome Institute and School of Microbiology, National University of Ireland, Cork, Ireland[c]

ABSTRACT The microbiota of the human gastrointestinal tract (GIT) may regularly be exposed to antibiotics, which are used to prevent and treat infectious diseases caused by bacteria and fungi. Bacterial communities of the gut retain a reservoir of antibiotic resistance (AR) genes, and antibiotic therapy thus positively selects for those microorganisms that harbor such genetic features, causing microbiota modulation. During the first months following birth, bifidobacteria represent some of the most dominant components of the human gut microbiota, although little is known about their AR gene complement (or resistome). In the current study, we assessed the resistome of the *Bifidobacterium* genus based on phenotypic and genotypic data of members that represent all currently recognized bifidobacterial (sub)species. Moreover, a comparison between the bifidobacterial resistome and gut metagenome data sets from adults and infants shows that the bifidobacterial community present at the first week following birth possesses a reduced AR arsenal compared to that present in the infant bifidobacterial population in subsequent weeks of the first year of life. Our findings reinforce the concept that the early infant gut microbiota is more susceptible to disturbances by antibiotic treatment than the gut microbiota developed at a later life stage.

IMPORTANCE The spread of resistance to antibiotics among bacterial communities has represented a major concern since their discovery in the last century. The risk of genetic transfer of resistance genes between microorganisms has been extensively investigated due to its relevance to human health. In contrast, there is only limited information available on antibiotic resistance among human gut commensal microorganisms such as bifidobacteria, which are widely exploited by the food industry as health-promoting microorganisms or probiotic ingredients. In the current study, we explored the occurrence of antibiotic resistance genes in the genomes of bifidobacteria and evaluated their genetic mobility to other human gut commensal microorganisms.

KEYWORDS gut microbiomes, bifidobacteria, human gut, antibiotic resistance genes, resistomes

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Address correspondence to Marco Ventura, marco.ventura@unipr.it.

S.D. and G.A.L. contributed equally to this article.

The human gut microbiota plays an important role in health and disease of the host through its impact on immunology, nutrition, and pathogenesis (1, 2). The microbiota may regularly be exposed to a variety of antimicrobial agents, such as antibiotics, which are used in the treatment and prevention of bacterial infection in human and veterinary medicine (3). Antibiotic therapy may cause secondary effects such as distortion of the homeostasis of microbial gut consortia (4) and selection for antibiotic-resistant microorganisms. In fact, bacteria have counteracted the action of antibiotics

through the acquisition of a specific genetic arsenal, also known as the resistome, which is involved in inactivation and/or removal of antibiotics. A large part of the resistome is contained within chromosomal DNA, although it may also be present on extrachromosomal replicons like plasmids and phages, which are transmissible to other members of the gut microbiota through horizontal gene transfer (HGT) events (5). Furthermore, antibiotic resistance may also be provided by a mutation in a gene encoding the antibiotic target, in which case the acquired resistance is not considered to be horizontally transferable (5). Antibiotic treatment selects for antimicrobial-resistant bacteria, where this selection is positively correlated with antibiotic usage (6). Furthermore, antibiotic resistance (AR) genes in gut commensal microorganisms are considered to be undesirable as they may lead to antibiotic resistance in human pathogens. Using HGT mechanisms, AR genes not only may be exchanged among members of the indigenous gut microbiota but also may be transferred to other bacteria that are just passing through the gastrointestinal tract (GIT), including several diet-associated bacteria (7). These studies have prompted the European Food Safety Authority (EFSA) to issue guidelines on the safety assessment of microorganisms used in food and feed production.

Bifidobacteria are common human gut microbiota members and are especially abundant during the first months following birth (2). Antibiotic therapy is commonly used to treat microbial infections in both infants and pregnant mothers and for antibiotic prophylaxis in preterm infants (8). Notably, infants without any exposure to antibiotics showed a higher percentage of *Bifidobacteriaceae* (8, 9). Thus, the occurrence of AR genes in bifidobacteria may increase their ecological fitness for gut persistence and colonization (10). Recently, the pan-genome, i.e., the full complement of genes of the *Bifidobacterium* genus has been reconstructed (11, 12), thereby providing a genomic data set, which will be important to identify the bifidobacterial resistome. Furthermore, we have evaluated the presence of AR genes in different strains belonging to the bifidobacterial (sub)species that are components of the qualified presumption of safety (QPS) list in accordance with the EFSA, such as *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium adolescentis*, *Bifidobacterium longum* subsp. *longum*, and *Bifidobacterium animalis* spp. (13, 14).

In the current study, we reconstructed the resistome of the *Bifidobacterium* genus based on both phenotypic and genotypic data, which together with the prediction of its genetic mobility allowed us to assess the potential of bifidobacterial AR genes to spread to the genomes of other gut bacteria. Furthermore, comparison of the, here identified, bifidobacterial resistome with those embedded in various microbiome data sets from infants and adults provides insights into the contribution of bifidobacteria to overall antibiotic resistance in the human gut microbial ecosystem.

RESULTS AND DISCUSSION

Assessment of antimicrobial susceptibility of bifidobacteria. The susceptibility toward eight different antibiotics, i.e., ampicillin, vancomycin, gentamicin, streptomycin, erythromycin, tetracycline, chloramphenicol, and clindamycin, representing those indicated by EFSA (15, 51), was determined for 91 *Bifidobacterium* strains (Fig. 1 and Tables 1 and 2). As displayed in Fig. 1 and Table 1, we determined the breakpoint values for each antibiotic for the type strain of each of the currently known 54 bifidobacterial (sub)species. All type strains belonging to the 54 (sub)species of the *Bifidobacterium* genus showed a unimodal breakpoint value distribution for ampicillin ($<$0.03 to 2 μg/ml), vancomycin (0.25 to 1 μg/ml), gentamicin (8 to 512 μg/ml), and chloramphenicol (0.5 to 2 μg/ml) (Fig. 1a, b, e, and g). In contrast, for erythromycin, clindamycin, streptomycin, and tetracycline, a bimodal distribution with two or three different subpopulations (Fig. 1c, d, f, and h) was observed. In addition, we evaluated the susceptibility toward the same set of antibiotics at the intraspecies level (Table 2). Such analyses are crucial in order to understand the variability of antibiotic resistance within a particular taxon and to provide scientific support for the EFSA-suggested breakpoint values. Remarkably, the breakpoint values put forward by EFSA for the genus *Bifido-*

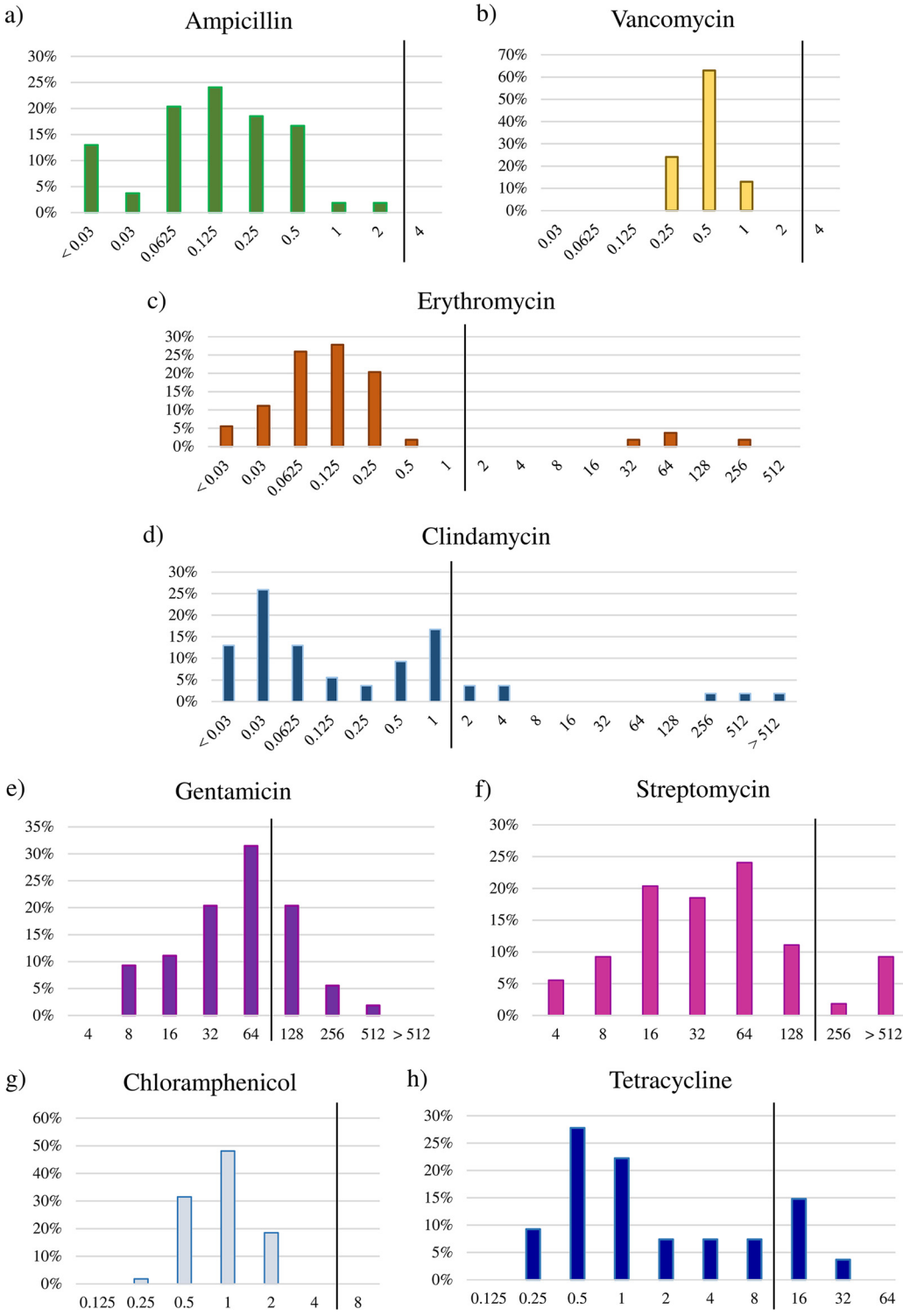

FIG 1 Microbiological breakpoint values. Distribution of breakpoint values for ampicillin (a), vancomycin (b), erythromycin (c), clindamycin (d), gentamicin (e) streptomycin (f), chloramphenicol (g), and tetracycline (h) in all type strains of the 54 (sub)species belonging to the *Bifidobacterium* genus. All bar plots report the antibiotic concentration values on the *x* axis (micrograms per milliliter) with the *y* axis showing the percentage of strains. The vertical black line represents the EFSA breakpoint value.

bacterium are based on values that were determined for a very small number of strains or for just a single strain (although proposed to apply to the whole genus) (15). Our analyses highlight a unimodal distribution of antibiotic resistance toward ampicillin, vancomycin, gentamicin, erythromycin, clindamycin, and chloramphenicol for all bifi-

TABLE 1 Antibiotic sensitivity of all type strains belonging to the *Bifidobacterium* genus

Species	Resistance data (μg/ml) for:							
	Ampicillin	Vancomycin	Gentamicin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
B. actinocoloniiforme DSM 22766	0.0625	0.5	8	4	0.03	0.03	2	0.5
B. adolescentis ATCC 15703	0.0625	0.5	128	32	0.0625	0.03	1	1
B. aesculapii MRM 3/1	0.0625	0.5	64	16	0.0625	<0.03	8	0.5
B. angulatum LMG 11039	0.125	0.5	128	64	0.03	0.03	0.25	0.5
B. animalis subsp. *animalis* LMG 10508	0.125	0.5	64	32	0.25	4	1	2
B. animalis subsp. *lactis* DSM 10140	0.125	0.5	128	64	0.25	4	8	2
B. asteroides LMG 10735 (PRL2011)	0.0625	0.5	16	32	0.0625	0.0625	1	2
B. biavatii DSM 23969	0.125	0.5	32	8	0.0625	0.03	0.25	0.5
B. bifidum LMG 11041	<0.06	0.25	8	8	0.125	<0.06	0.5	0.5
B. bohemicum DSM 22767	0.25	0.5	8	4	0.03	<0.03	2	0.25
B. bombi DSM 19703	<0.03	1	8	4	0.0625	0.5	0.5	1
B. boum LMG 10736	<0.03	0.25	512	256	0.0625	0.125	32	0.5
B. breve LMG 13208	0.0625	0.5	64	16	0.125	0.03	0.5	0.5
B. callitrichos DSM 23973	0.0625	0.5	256	64	0.25	0.03	8	0.5
B. catenulatum LMG 11043	<0.03	0.5	128	>512	0.0625	1	1	1
B. choerinum LMG 10510	0.25	0.25	128	64	0.125	2	0.5	1
B. coryneforme LMG 18911	0.25	0.5	32	16	0.0625	0.0625	4	2
B. crudilactis LMG 23609	0.125	1	16	32	0.25	1	4	2
B. cuniculi LMG 10738	0.5	0.5	32	32	0.125	1	2	1
B. dentium LMG 11045 (Bd1)	0.25	0.5	32	32	0.125	0.03	0.5	1
B. eulemuris LMM E3	0.5	1	32	64	0.125	0.5	0.5	1
B. gallicum LMG 11596	0.25	0.5	64	16	<0.03	0.0625	0.5	0.5
B. gallinarum LMG 11586	0.03	0.5	64	>512	<0.03	0.5	1	1
B. hapali MRM 8.14	0.25	0.5	64	16	0.0625	<0.03	0.5	1
B. indicum LMG 11587	0.25	1	32	128	64	512	16	2
B. kashiwanohense DSM 21854	0.5	0.5	128	64	32	256	0.5	1
B. lemurum LMC13	0.5	1	64	32	0.03	<0.03	0.5	1
B. longum subsp. *infantis* ATCC 15697	0.125	0.5	8	>512	0.125	0.0625	4	0.5
B. longum subsp. *longum* LMG 13197	0.5	0.5	32	64	0.125	<0.03	1	1
B. longum subsp. *suis* LMG 21814	<0.03	1	32	>512	0.0625	0.03	16	1
B. magnum LMG 11591	<0.03	0.25	8	8	<0.03	0.03	16	1
B. merycicum LMG 11341	0.0625	0.5	64	64	0.125	0.03	16	1
B. minimum LMG 11592	0.5	0.5	64	16	0.25	0.25	0.5	2
B. mongoliense DSM 21395	0.25	1	16	8	0.25	0.0625	0.5	1
B. moukalabense DSM 27321	0.125	0.5	128	128	0.125	1	0.5	0.5
B. myosotis MRM 5.9	0.5	0.25	32	32	0.03	<0.03	0.25	1
B. pseudocatenulatum LMG 10505	0.0625	0.25	128	64	0.25	0.03	16	1
B. pseudolongum subsp. *globosum* LMG 11569	0.125	0.5	128	128	0.0625	1	8	1
B. pseudolongum subsp. *pseudolongum* LMG 11571	0.0625	0.25	64	128	0.125	0.125	2	1
B. psychraerophilum LMG 21775	0.125	0.5	64	32	0.0625	1	4	2
B. pullorum LMG 21816	1	0.25	32	16	64	>1,024	32	2
B. reuteri DSM 23975	0.125	0.5	32	16	0.25	0.5	0.25	0.5
B. ruminantium LMG 21811	0.25	0.25	16	8	0.125	1	0.25	0.5
B. saeculare LMG 14934	0.125	0.5	64	>512	256	1	16	0.5
B. saguini DSM 23967	0.5	0.5	64	16	0.25	1	1	1
B. scardovii LMG 21589	0.5	0.5	64	16	0.25	0.25	1	1
B. stellenboschense DSM 23968	<0.03	0.5	16	16	0.0625	0.03	0.5	0.5
B. stercoris DSM 24849	0.0625	0.5	64	64	0.25	0.03	16	1
B. subtile LMG 11597	0.25	0.5	64	32	0.0625	0.5	1	1
B. thermacidophilum subsp. *porcinum* LMG 21689	0.03	0.25	256	128	0.125	0.0625	1	0.5
B. thermacidophilum subsp. *thermacidophilum* LMG 21395	0.125	0.25	16	64	0.125	0.125	0.5	2
B. thermophilum JCM 1207	0.0625	0.25	256	128	0.125	0.0625	1	0.5
B. tissieri MRM 5.18	2	0.25	128	64	0.03	0.03	1	1
B. tsurumiense JCM 13495	0.125	0.5	128	64	0.5	2	1	1

dobacterial species belonging to the QPS list. In contrast, we observed susceptibility variations to streptomycin and tetracycline at the intraspecific level for *B. bifidum* as well as *B. breve* and for *B. bifidum*, *B. animalis*, and *B. adolescentis*, respectively (Table 2). This information indicates that antibiotic resistance in bifidobacteria does not appear to follow a vertical route of evolution but may have been acquired through horizontal gene transfer, in a fashion similar to that previously observed for other gut commensal microorganisms (7).

TABLE 2 Antibiotic sensitivity of different strains belonging to the QPS list

Species	Strain	Resistance data (μg/ml) for:							
		Ampicillin	Vancomycin	Gentamicin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
B. bifidum	PRL2010	0.0625	1	64	32	0.125	<0.03	1	1
	LMG13195	<0.03	0.5	64	>1,024	0.0625	0.03	0.5	0.5
	LMG11041	<0.06	0.25	8	8	0.125	<0.06	0.5	0.5
	IPLA20017	0.03	0.5	32	>1,024	0.125	0.0625	0.5	0.5
	IPLA20015	0.0625	0.5	32	>1,024	0.0625	0.0625	8	0.5
	A8	0.03	1	64	8	0.0625	0.03	0.5	0.5
	156B	0.0625	0.5	64	16	0.03	0.0625	1	1
	85B	0.0625	0.5	32	16	0.03	0.0625	1	0.5
	324B	0.0625	1	64	16	0.0625	0.0625	1	0.5
	LMG13200	0.5	0.5	16	256	0.0625	0.25	8	0.5
	LMG11583	0.5	0.5	32	16	0.03	0.25	16	1
	LMG11582	0.5	0.5	32	>1,024	0.125	0.25	16	0.5
B. breve	689B	0.5	0.5	32	>1,024	0.125	0.25	0.5	0.5
	DSM20213	0.0625	0.5	64	16	0.125	0.03	0.5	0.5
	12L	0.25	1	32	32	0.125	0.25	0.5	0.5
	2L	0.25	1	16	32	0.125	0.25	0.25	0.5
	D1-16	0.25	0.5	8	>1,024	0.125	<0.03	0.5	1
	31L	0.5	1	32	>1,024	0.125	<0.03	0.5	0.5
	UCC2003	0.125	1	64	32	0.25	0.5	0.125	1
B. animalis subsp. *lactis*	BI12	0.125	0.5	128	64	0.25	4	8	2
	BLC1	0.125	0.5	128	64	0.25	4	8	2
	DSM10140	0.125	0.5	128	64	0.25	4	8	2
	646	0.125	0.5	128	64	0.25	4	8	2
	BB12	0.125	0.5	128	64	0.25	4	8	2
	ADO11	0.125	0.5	128	64	0.25	4	8	2
B. animalis subsp. *animalis*	ATCC25527	0.125	0.5	64	32	0.25	4	1	2
B. adolescentis	22L	<0.03	0.25	32	32	0.5	<0.03	0.5	2
	ATCC15703	0.0625	0.5	128	32	0.0625	0.03	1	1
	LMG18897	<0.03	0.25	16	32	0.25	<0.03	0.25	2
	LMG11579	0.25	0.25	32	32	1	1	16	1
	LMG10733	0.0625	0.25	64	32	0.25	<0.03	1	0.5
	LMG10734	0.125	0.25	64	32	0.5	<0.03	0.5	2
	42B	0.0625	0.25	32	16	0.25	<0.03	0.5	2
	70B	0.0625	0.25	64	64	0.25	0.03	0.5	2
	487B	<0.03	0.25	32	32	0.5	0.125	0.5	2
	703B	0.25	0.25	64	32	0.5	<0.03	1	2
	AD2-8	0.03	0.25	16	32	0.25	<0.03	0.5	2
	AL12-4	0.0625	0.25	32	16	0.5	<0.03	1	2
	AL46-2	1	<0.03	32	32	<0.03	0.125	16	0.5
	AL46-7	0.0625	0.25	32	16	0.25	0.25	16	2
	JCM15918	0.0625	0.5	64	64	0.25	0.03	16	1
B. longum subsp. *longum*	296B	0.125	0.5	32	64	<0.03	<0.03	1	2
	LMG13197	0.5	0.5	32	64	0.125	<0.03	1	1
	B2A2	0.125	0.25	32	64	0.0625	<0.03	1	1

Prediction of the bifidobacterial resistome. The genomes of 91 bifidobacterial strains that had been assessed for their susceptibility to antibiotics (see above) were screened for putative AR genes. This analysis was performed using the CARD AR gene database (16), to which two genes, predicted to encode aminoglycoside phosphotransferases (APH), had been added. Notably, the presence of these two genes in *B. breve* has previously been shown to be associated with resistance to gentamicin, streptomycin, and kanamycin (17). These *in silico* analyses revealed that the resistome of the genus *Bifidobacterium* is predicted to consist of 783 genes (see Table S1 in the supplemental material). Of the AR genes identified, 47% were shown to be conserved in the 54 (sub)species of the genus *Bifidobacterium*, thus representing bifidobacterial core genome sequences (12, 18). While 74% of these genes encode β-lactamases, 26% are involved in antimicrobial peptide resistance (Table S1). Interestingly, analysis of chromosomal sequences revealed the presence of a single copy of a predicted APH-

encoding gene in all *Bifidobacterium* strains, Furthermore, in 20 bifidobacterial genomes, we identified two copies of such a putative APH-encoding gene, which may account for higher resistance toward streptomycin and gentamicin in these bifidobacterial strains (see Table S2 in the supplemental material). In contrast, *tet*(W) homologs, previously identified in *Bifidobacterium animalis* subsp. *lactis* (19, 20), are present in the genomes of 11 bifidobacterial species (Fig. 2), based on *in silico tet*(W) domain prediction, thus highlighting the fact that this AR gene represents a relatively broad species-specific genetic signature within the bifidobacterial pan-genome. Notably, *in vitro*-based MIC assays involving these strains revealed high resistance toward tetracycline (from 8 μg/ml to 32 μg/ml), consistent with the presence of the *tet*(W) gene in their genomes (Fig. 2). In addition, tetracycline resistance may be conferred by additional genetic features, since *Bifidobacterium indicum* LMG 11587 and *Bifidobacterium callitrichos* DSM 23973 encompass no less than three *tet*(W) domains (Fig. 2).

When the antibiotic susceptibility data were coupled to the predicted resistome information, we were able to detect good correspondence between phenotype and genotype. For instance, in *B. indicum* LMG 11587 and *Bifidobacterium coryneforme* LMG 18911, different susceptibilities to streptomycin, erythromycin, clindamycin and tetracycline were identified, which appeared to be supported by the genotypic data. Recently, Lugli et al. (12) highlighted that a very close genetic relatedness exists between *B. indicum* LMG11587 and *B. coryneforme* LMG18911 (average nucleotide identity [ANI] value of 98.13). Comparative genomic analysis involving the chromosomal sequences of these strains allowed the identification of genes that are absent in the genome of *B. coryneforme* LMG 18911 (Fig. 3). Interestingly, an in-depth functional analysis of the *B. indicum* LMG 11587 unique genes highlighted the presence of an open reading frame (ORF) predicted to encode a major facilitator superfamily (MFS) transporter that is classified as a member of the drug:H^+-antiporter-3 (12 spanner) (DHA3) family, which may be responsible for the extrusion of erythromycin, streptomycin, and clindamycin from the (bifido)bacterial cell (Fig. 3). Similarly, *Bifidobacterium pullorum* LMG 21816 was shown to exhibit a high level of resistance against clindamycin (>1,024 μg/ml) compared to that identified for other type strains of the 54 (sub)species of the genus *Bifidobacterium*. Comparative genomics involving all type strains of the genus showed 138 protein-encoding genes that appeared to be unique to *B. pullorum* LMG21816. Notably, one of these genes (BPULL_1285) was predicted to encode an MFS transporter classified as a DHA3 family transporter. This putative multidrug transporter may thus be responsible for the noted resistance to clindamycin as also observed for *B. coryneforme* LMG 18911. Nonetheless, in five cases, *Bifidobacterium catenulatum* LMG 11043, *Bifidobacterium gallinarum* LMG 11586, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, *Bifidobacterium longum* subsp. *suis* LMG 21814, and *Bifidobacterium saeculare* LMG 14934 (Table S1), the observed high resistance to streptomycin (>512 μg/ml) does not appear to be associated with the presence of a specific AR gene. This resistance may therefore either be due to a point mutation in a particular chromosomal gene causing innate resistance against streptomycin or be caused by high expression of a particular AR gene. Furthermore, based on the reconstruction of the genetic evolution of species of the genus *Bifidobacterium* based on their pan-genomes (11), we predicted the genetic origins of AR identified in bifidobacteria. Notably, 6% of the predicted bifidobacterial resistome appears to be acquired from other microbial genera such as *Gardnerella* and *Lactobacillus*, representing bacterial taxa that share the same environmental niche as bifidobacteria (Table S1). Nonetheless, we cannot exclude the possibility that these genes might be received independently from another common source. These findings therefore suggest that a sizeable portion of the genetic AR arsenal of bifidobacteria has been acquired by means of HGT events.

Identification of putative mobile bifidobacterial AR genes. In order to evaluate the occurrence of bifidobacterial AR genes located on or in the proximity of mobile elements such as episomes, conjugative transposons, and prophages, we analyzed the

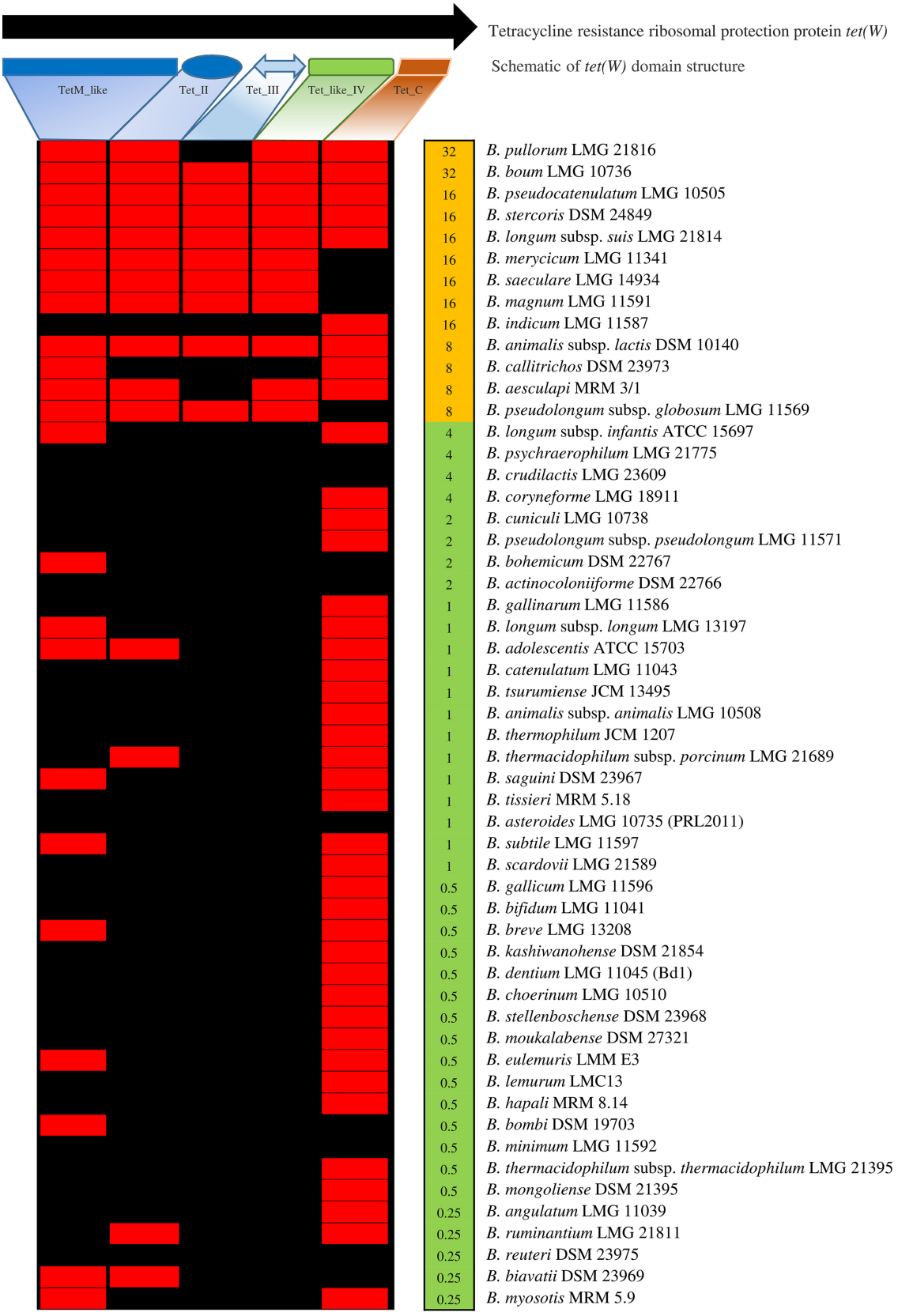

FIG 2 Comparison of *tet*(W) gene identified in *B. animalis* subsp. *lactis* with the corresponding domains from all type strains of bifidobacteria. The top part of the figure depicts a schematic representation of the different domains identified in *tet*(W). The heat map displays an *in silico* prediction of domains encoded by *tet*(W) and the breakpoint value for tetracycline in all type strains belonging to the *Bifidobacterium* genus. The red color indicates gene presence, and the black color represents their absence.

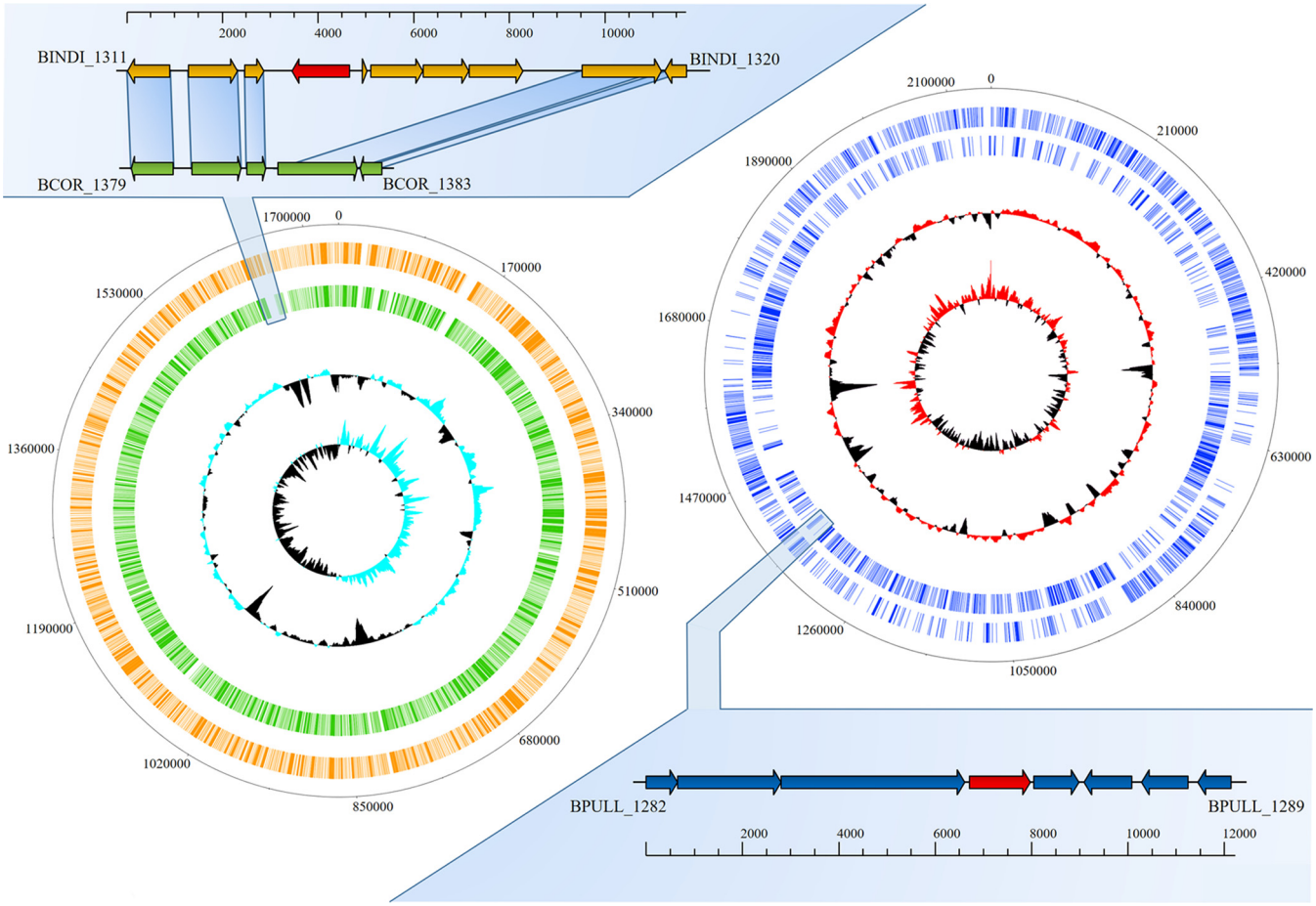

FIG 3 Unique genetic loci identified in the genome of *B. indicum* LMG 11587 and *B. pullorum* LMG 21816. On the left, a circular genome atlas of *B. indicum* LMG 11587 (orange circle) and *B. coryneforme* LMG 18911 (green circle) are shown, while on the right, the same representation is proposed for the *B. pullorum* LMG 21816 genome (blue circles). Internal circles represent the G+C percent deviation followed by the GC skew (G−C/G+C). In the reported genetic maps, each arrow indicates an ORF where the red ones correspond to the major facilitator superfamily (MFS) identified in *B. indicum* LMG 11587 (orange arrows) and *B. pullorum* LMG 21816 (blue arrows).

flanking DNA sequences of the predicted AR genes (see Tables S1 and S3 in the supplemental material). Such genetic elements may be responsible for AR gene mobilization from and/or to other microorganisms. Homology-based analyses of the surrounding DNA regions of the predicted bifidobacterial AR genes were performed and combined with outputs of the software package COLOMBO (21). These analyses identified 208 AR genes placed within genomic regions that have putatively been acquired by HGT, thus representing the predicted horizontally acquired bifidobacterial resistome (HABR) (Fig. 4). Notably, HABR represents about 27% of the predicted bifidobacterial resistome, disregarding the proximity of the genes to transposable elements. Taken together, these results suggest that a substantial proportion of the bifidobacterial AR genes identified were acquired by HGT. Furthermore, *in silico* analysis disclosed that only seven of the AR genes identified are placed near a predicted prophage, while two are adjacent to putative plasmid replication genes.

Remarkably, we noticed that the largest part of the predicted bifidobacterial HABR is directed toward glycopeptides (12%), followed by β-lactamase-encoding genes (10%) (Fig. 4a). Furthermore, the AR genes flanking prophage and plasmid DNA regions are represented by genes predicted to encode bleomycin, kanamycin, and bacitracin resistance (Table S1). Another intriguing bifidobacterial mobile AR gene predicted to be carried on a conjugative transposon is represented by the *tet*(W) gene, which is found flanking a mobile element in the genomes of all of the *B. animalis* subsp. *lactis* strains analyzed (Fig. 4b). Such findings confirm previous genomic data about this bifidobac-

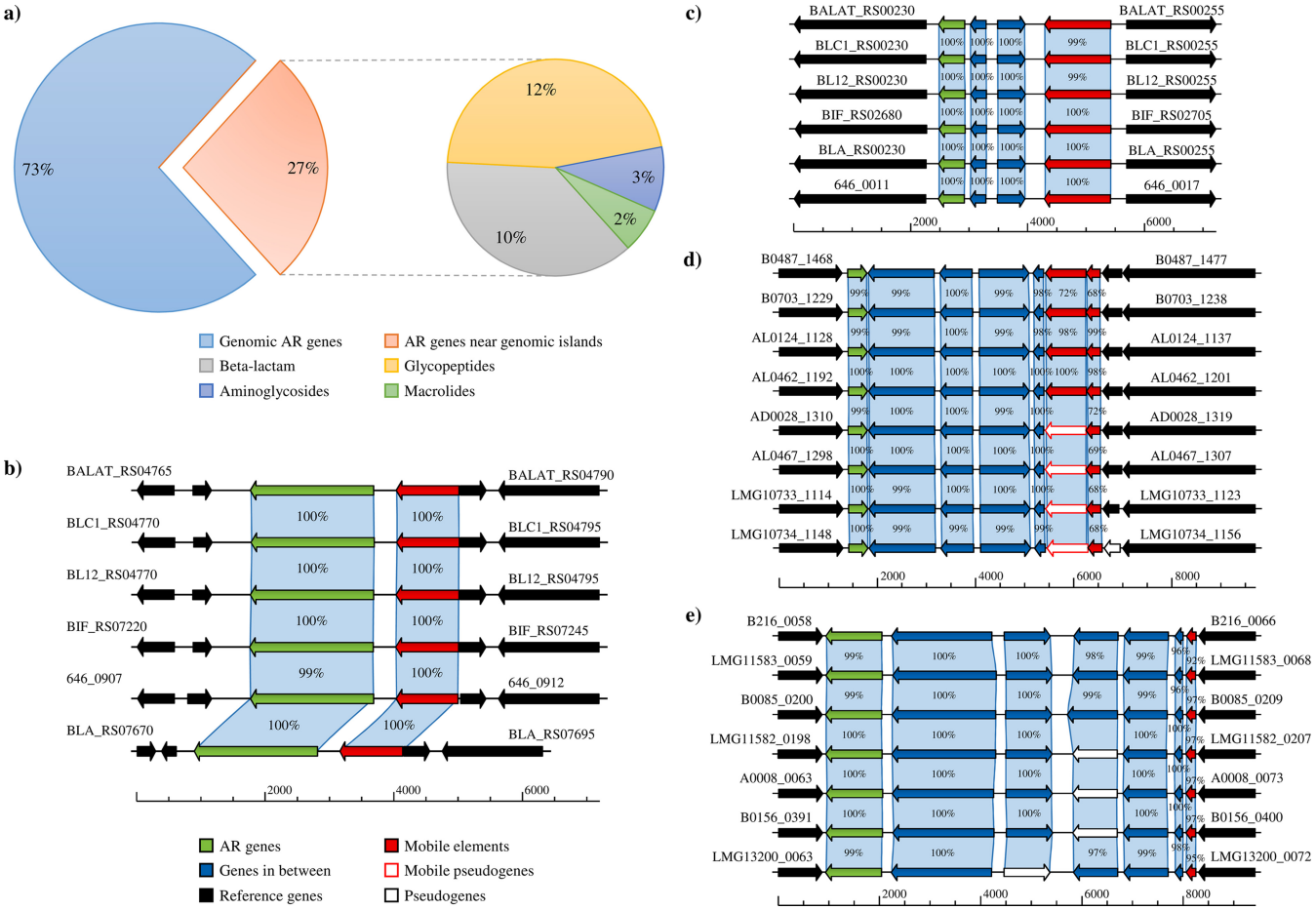

FIG 4 Distribution of mobile bifidobacterial AR genes. (a)The left pie chart represents chromosomal bifidobacterial AR genes that are (red) or are not (blue) predicted to be acquired by HGT, and the pie chart on the right displays the classification of such predicted HGT-acquired AR genes. (b to e) Bifidobacterial genomic regions containing putative AR genes (green arrows) located near mobile elements (red arrows) belonging to *B. animalis* subsp. *lactis*, *B. adolescentis*, and *B. bifidum*, respectively.

terial taxon (19) and support the hypothesis that this resistance is due to a mobile *tet*(W) gene representing a common genetic feature of *B. animalis* subsp. *lactis*. Furthermore, we identified a putative mobile AR gene that encodes a predicted aminoglycoside protein with an APH domain for kanamycin resistance in all publicly available genomes of *B. animalis* subsp. *lactis* (Fig. 4c; see also Table S3 in the supplemental material). Yet another intriguing example is represented by analysis of the genomes of *B. adolescentis*, of which eight encompass an AR gene predicted to exert resistance against bleomycin, located within 4 kb from a putative mobile element (Fig. 4d and Table S3). Remarkably, seven strains of *B. bifidum* exhibit β-lactamase-encoding genes near truncated transposases (Fig. 4e and Table S3). Nevertheless, none of these transposase-encoding genes can be classified as a conjugal transposon, thus reducing the possibility of AR gene mobilization by HGT. These findings indicate that only a small fraction (1%) of the predicted resistome found in the genus *Bifidobacterium* resides in nearby mobile elements, which may facilitate AR gene transfer to other bacteria. The distribution of AR genes in bifidobacteria may thus be due to selective pressure imposed by extensive antibiotic use in their animal and human hosts in a fashion that is similar to what has previously been observed for other lactic acid bacteria (LAB) such as *Lactobacillus* (22), a phenomenon that is considered to represent microbe-host coevolution.

Metagenome analysis targeting bifidobacterial AR. Bifidobacteria have predominantly been isolated from the mammalian GIT (23), where their functional contribution

FIG 5 Metagenomic abundance of bifidobacterial AR genes. (a) Average abundance of bifidobacterial reads retrieved in the metagenome samples of infants (healthy and preterm), children treated with antibiotics, and adults. (b) Abundance of different classes of bifidobacterial AR genes with respect to the total BGS identified in the samples. (c) Average abundance of bifidobacterial AR reads with respect to the total BGS retrieved in the metagenomes, where each number corresponds to the days following birth of healthy infants (H) or preterm infants (P).

in terms of metabolism of various dietary carbohydrates and host glycans has been investigated (11, 18, 24–26). However, their contribution to the resistome of the mammalian gut microbiome has not been studied in any great detail. We therefore assessed the presence of AR-encoding bifidobacterial DNA sequences within two gut metagenome data sets from healthy human beings, including adult gut microbiomes (27) and infant gut microbiomes (https://www.broadinstitute.org/scientific-community/science/projects/microbiome-projects), which in both cases had been collected from U.S. citizens (see Table S4 in the supplemental material). In addition, we explored the bifidobacterial AR contribution to the resistomes of preterm infant gut microbiomes (https://www.broadinstitute.org/scientific-community/science/projects/microbiome-projects) and of 2- to 7-year-old children who had received intensive antibiotic treatment, i.e., a combination of β-lactams and macrolides (28). These metagenomic data sets were assayed for the presence of bifidobacteria by searching for bifidobacterial gene sequences (BGS), i.e., the presence of any of the combined genes collected from the 91 taxa analyzed. The minimum coverage of each gene included in the BGS collection was computed based on the metagenomic reads with 98% full-length identity. As displayed in Fig. 5, healthy infants showed a higher average percentage of metagenomic reads that correspond to BGS (6.1%, $P < 0.001$), ranging from 0.01% to 52%, than adults (0.1%), while preterm infants and children treated with antibiotics exhibited an average of 1.5%. This is consistent with previous findings supporting the ecological behavior of bifidobacteria as core gut microorganisms of the healthy human gut during the suckling stage (2, 23, 29–36). Furthermore, our analysis revealed reduced bifidobacterial populations in preterm infants and children extensively treated with antibiotics, which is consistent with the existing literature (37).

The bifidobacterial AR genes collected from the genomic analysis allowed us to evaluate the contribution of the bifidobacterial resistome with respect to the total BGS identified in each sample. Interestingly, among the most frequently represented bifidobacterial AR genes in the metagenomic data sets from adult samples, an extensive repertoire of specific AR-encoding genes, such as those specifying β-lactamases, which confer resistance against penicillin, cephalosporin, carbapenem, and monobactam, is noteworthy, followed by genes that specify glycopeptide and aminoglycoside resistance (Fig. 5b). Such findings reinforce the notion that despite the lower abundance of bifidobacteria detected in the adult human gut, their functional contribution to the human gut microbiome may be important in terms of expanding the overall resistome of the human gut and thus affecting the maintenance of a gut climax following antibiotic therapies. Of note, the bifidobacterial AR-encoding gene distribution within the infant's microbiome was shown to be similar to that of adults with a preponderance of β-lactamase-encoding genes (Fig. 5b).

The availability of microbiomes from the Metagenome from Infant Gut project collected at different time points, i.e., 7, 14, 21, 60, 150, and 360 days, allowed us to evaluate the dynamics of bifidobacterial AR-encoding genes during the first year of life of healthy and preterm infants. Looking at the overall representation of bifidobacterial AR reads in the metagenomic data sets, a lower value is obtained for healthy and preterm infants in the first week of life, followed by an increase, which then stabilizes during a 60- to 360-day period, thus being comparable with an adult's profile (Fig. 5c). In contrast, in the metagenome data set of children extensively treated with antibiotics, we observed a higher abundance of bifidobacterial AR reads (1.34%, $P < 0.006$), suggesting the increased presence of bifidobacterial species that tolerate the administered dosage of antibiotics assumed during treatment (Fig. 5c). Furthermore, we observed a higher abundance in β-lactamase reads (0.99% of the bifidobacterial reads, $P < 0.001$) in the antibiotic-treated children with respect to those in the other three groups (ranging from 0.29% to 0.43%) (Fig. 5b), reflecting the antibiotic treatment provided. Accordingly, during the first days following birth, the infants' microbiota may be more sensitive to antibiotic treatment due to a very simple and fragile bifidobacterial community, which appears to be unable to cope with a high level of antibiotic administration. Thus, the particular antibiotic administered to the newborn may bring about an intestinal microbiota disturbance that can prevent or delay subsequent development of a normal microbiota (38).

In conclusion, bifidobacteria are dominant members of the infant gut microbiota, and their contribution to host health is well documented (24). Despite many reports investigating the susceptibility of human bifidobacterial species to various antibiotics, very little is known about their resistome. In this study, we performed a detailed assessment of the genetic traits that support antibiotic resistance in bifidobacteria. Notably, varying susceptibilities to antibiotics at the intraspecific level were identified for certain bifidobacterial species, an observation of relevance with respect to the scientific rationale for breakpoint values proposed by EFSA (15), as currently employed breakpoint values are based on MIC values determined for a single bifidobacterial strain. In addition, based on our analyses, the identification of higher MIC values with respect to breakpoint values proposed by EFSA does not always correspond with the occurrence of predicted mobile AR genes and thus does not necessarily pose any risk of genetic transferability. Interestingly, the resistome of bifidobacteria represents a substantial proportion of the predicted mobilome of the genus *Bifidobacterium*, thus supporting the hypothesis that the antibiotic era has somehow shaped the pangenome of this group of commensal microorganisms. Notably, a similar trend has already been observed for other gut commensal microorganisms such as *Lactobacillus* (22). Acquisition of resistance to antibiotics represents a way used by bacteria to survive and thus to increase their ecological fitness (39). Characterization of the bifidobacterial resistome allowed us to obtain insights into the manner by which the bifidobacterial community contributes to the overall gut microbiota resistome. Our data show that the bifidobacterial communities in the infant gut possess a reduced AR arsenal compared

to that present in the bifidobacterial gut microbiota of an older child. These data reinforce the notion that the infant gut microbiota, particular that present during the first weeks following birth, is more prone to perturbations following antibiotic therapy and may thus be highly susceptible to long-term disturbances, compared to the stable and more robust (adult) gut microbiota that develops subsequently.

MATERIALS AND METHODS

Bacterial strains. All type strains belonging to the *Bifidobacterium* genus and several previously characterized strains belonging to species present on the qualified presumption of safety (QPS) list (11, 13, 19, 40–44) were used in this study (Tables 1 and 2). Cultures were grown under anaerobic conditions (2.99% H_2, 17.01% CO_2, and 80% N_2) in a chamber (Concept 400; Ruskin) on De Man-Rogosa-Sharpe (MRS) broth (Scharlau Chemie, Barcelona, Spain) supplemented with 0.05% (wt/vol) L-cysteine hydrochloride and were incubated at 37°C. Prior to performance of the antibiotic susceptibility test, strains were precultivated (to allow adaptation) in the same medium used for the susceptibility test, based on the use of Iso-Sensitest (IST) broth (Oxoid).

Antibiotic susceptibility tests. For selected *Bifidobacterium* strains, the MIC breakpoints (micrograms per milliliter) of eight antibiotics (ampicillin, vancomycin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol) were determined using the broth microdilution method (MDIL) according to the ISO standard guidelines (15). All antibiotics were purchased from Sigma-Aldrich (Italy). Microplates were incubated under anaerobic conditions for 48 h at 37°C. Cell density was monitored by optical density measurements at 600 nm (OD_{600}) using a plate reader (BioTek, VT, USA). The MIC breakpoint represents the highest concentration of a given antibiotic to which a particular bacterial strain is resistant.

Antibiotic resistance gene prediction. The *in silico* proteome of 93 *Bifidobacterium* strains (Tables 1 and 2) was screened for enzymes that act as antibiotic inactivators using a custom script based on RapSearch2 software (45) and the database CARD (16). We decided not to include transporters in our analyses due to the low accuracy with which antibiotic transporters can be predicted.

Prediction of the mobile bifidobacterial AR genes. The bifidobacterial strains used in this study were screened for genomic islands, evaluating the genes flanking the predicted AR genes in the range of 10 kb, using homology searches (46) and the software COLOMBO (21). Putative mobile elements such as episomes, conjugative transposons, and prophages were predicted through homology searches against in-house-generated databases (http://probiogenomics.unipr.it/sw/MobElemDB.zip), including genes retrieved from the National Center for Biotechnology Information (NCBI) database.

***In silico* analysis for resistome reconstruction.** All identified bifidobacterial AR genes were aligned with whole-genome sequencing (WGS) reads previously deposited at the NCBI (Sequence Read Archive [SRA] BioProject). This information was obtained from shotgun sequencing microbiome data sets of (fecal samples of) healthy adults from the Human Microbiome Project (PRJNA48479), healthy and preterm infants from the Metagenome from Infant Gut project (PRJNA63661), and children with high-frequency antibiotic treatment from the Child Gut Microbiome under Antibiotics project (PRJEB11685). Metagenomic data sets were filtered using the fastq-mcf script (https://expressionanalysis.github.io/ea-utils/) (minimum mean quality score, 20; window size, 5; quality threshold, 25; and minimum length, 80) to achieve high-quality reads exclusively. The resulting reads were aligned against the human genome using the Burrows-Wheeler Aligner program (47) (BWAMEM algorithm with trigger reseeding, 1.5; minimum seed length, 19; matching score, 1; mismatch penalty, 4; gap open penalty, 6; and gap extension penalty, 1) and further processed with the SAMtools software package (48) in order to remove human reads. The final mapping against putative bifidobacterial AR genes was performed using Bowtie 2 (49) through multiple-hit mapping and "very-sensitive" policy. The mapping was performed using a minimum score threshold function (–score-min C,−13,0) in order to limit reads of arbitrary length to two mismatches and retain those matches with at least 98% full-length identity. The software employed to calculate read counts corresponding to either bifidobacterial genes or bifidobacterium-specific AR genes was HTSeq (50) (running in union mode). The percentages of bifidobacterial genes for each sample were based on the total amount of filtered reads, while the percentages of bifidobacterial AR genes reported were based on the counts of reads mapped to bifidobacterial genes in each sample.

Statistical analysis. SPSS software (IBM, Italy) was used to perform statistical analysis between groups by an analysis of variance (ANOVA) test.

Accession number(s). The bifidobacterial sequences reported in this article have been deposited in the GenBank database under accession numbers MLZK00000000 and MLZL00000000. The versions described in this paper are MLZK01000000 and MLZL01000000.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.02894-16.

DATASET S1, XLSX file, 0.1 MB.

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We declare that we have no competing interests.

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