



Amoxicillin-Clavulanic Acid Resistance in the Genus *Bifidobacterium*

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ABSTRACT Amoxicillin-clavulanic acid (AMC) is one of the most frequently prescribed antibiotic formulations in the Western world. Extensive oral use of this antimicrobial combination influences the gut microbiota. One of the most abundant early colonizers of the human gut microbiota is represented by different taxa of the *Bifidobacterium* genus, which include many members that are considered to bestow beneficial effects upon their host. In the current study, we investigated the impact of AMC administration on the gut microbiota composition, comparing the gut microbiota of 23 children that had undergone AMC antibiotic therapy to that of 19 children that had not been treated with antibiotics during the preceding 6 months. Moreover, we evaluated AMC sensitivity by MIC test of 261 bifidobacterial strains, including reference strains for the currently recognized 64 bifidobacterial (sub)species, as well as 197 bifidobacterial isolates of human origin. These assessments allowed the identification of four bifidobacterial strains that exhibit a high level of AMC insensitivity, which were subjected to genomic and transcriptomic analyses to identify the putative genetic determinants responsible for this AMC insensitivity. Furthermore, we investigated the ecological role of AMC-resistant bifidobacterial strains by *in vitro* batch cultures.

IMPORTANCE Based on our results, we observed a drastic reduction in gut microbiota diversity of children treated with antibiotics, which also affected the abundance of *Bifidobacterium*, a bacterial genus commonly found in the infant gut. MIC experiments revealed that more than 98% of bifidobacterial strains tested were shown to be inhibited by the AMC antibiotic. Isolation of four insensitive strains and sequencing of their genomes revealed the identity of possible genes involved in AMC resistance mechanisms. Moreover, gut-simulating *in vitro* experiments revealed that one strain, i.e., *Bifidobacterium breve* PRL2020, is able to persist in the presence of a complex microbiota combined with AMC antibiotic.

KEYWORDS bifidobacteria, antibiotics, comparative genomics

The human gastrointestinal tract (GIT) harbors a complex and dynamic population of microorganisms, i.e., the gut microbiota, whose coordinated actions are believed to be important to support a healthy human physiology (1, 2). Microbes colonize the neonatal gut immediately following birth, and this early life event is believed to represent a crucial opportunity for microbiota modulation, which in turn may influence host health in later life (3). During a human life, the gut microbiota may frequently be exposed to various antibiotics, causing alterations in the associated microbial communities, with both immediate effects and potential indirect, long-term effects on host health (4, 5). In this

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context, early antibiotic exposure has a major impact on infant health through diversity reduction and compositional alteration of microbial communities in this ecosystem (6, 7).

Amoxicillin-clavulanic acid (AMC) is one of the most frequently prescribed antibiotics, especially in infants and adolescents (8). The combination of these two compounds is commonly used for treatment of different pediatric infectious diseases, including acute otitis media, sinusitis, pneumonia, urinary tract infections, and skin and soft tissue infections (9). Despite the extensive use of AMC, especially for pediatric patients, little is known about its impact on the gut microbiota.

The most prevalent and abundant early colonizers of the human infant gut encompass members of the *Bifidobacterium* genus (10–12). In recent years, the scientific interest in bifidobacteria has been growing because they are believed to have an impact on important immunomodulatory activities in the host at the very early stages of life and to influence the physiology of the gut ecosystem by their metabolic activities (11, 13). *In vitro* susceptibility assessments have demonstrated that *Bifidobacterium* species are generally sensitive to amoxicillin (14–16). Nonetheless, only limited knowledge is available pertaining to the effects of amoxicillin on its own or when combined with clavulanic acid on bifidobacterial communities residing in the human gut (17).

In the current study, we assessed the microbiota composition of children that had undergone AMC antibiotic treatment and compared this to that of children who had not been administered any antibiotics in the previous 6 months. Further analyses were carried out in order to investigate AMC sensitivity/resistance of a large collection of bifidobacterial isolates from humans, using the MIC assay. These analyses allowed the identification of two strains belonging to *Bifidobacterium breve* and two isolates representing *Bifidobacterium longum* subsp. *longum* strains that were shown to exhibit a relatively high level of AMC resistance. Genome sequencing coupled with transcriptomic analyses allowed the identification of genes that appear to be responsible for this high level of AMC insensitivity. Finally, we investigated the effect of AMC and AMC-insensitive bifidobacteria on the human gut microbiota through *in vitro* coculture experiments, obtaining insights into the ecological role played by such bifidobacterial strains.

RESULTS AND DISCUSSION

Microbial composition of gut microbiota of children taking AMC. In order to assess the differences between the gut microbiota composition of 23 children undergoing AMC therapy for respiratory tract infection (AMC group) compared to that of 19 healthy, age-matched individuals (control [CTRL] group) who had not been taking any antibiotic during the preceding 6 months (see Table S1 in the supplemental material), 16S rRNA gene-based microbial profiling analyses were carried out on fecal samples as described previously (18). The analyses resulted in a total of 2,274,698 reads with an average of $54,159 \pm 11,080$ reads per sample. Statistical whisker plot analyses revealed a notable difference between the two groups analyzed (Fig. 1). Specifically, the 16S rRNA gene-based analysis showed a higher diversity of the CTRL group microbiota compared to that of the AMC group (P value = 0.03), demonstrating that the microbiota composition is significantly influenced by the antibiotic therapy to which individuals were subjected (Fig. 1a).

In order to explore the absolute bacterial abundance of the analyzed fecal samples, we used a quantitative microbiome profiling approach based on flow cytometric analyses for the enumeration of microbial cells present in each fecal sample assayed. This analysis allowed the identification of the microbial load of the 42 fecal samples, which was then used to normalize the 16S rRNA sequencing data. This procedure therefore generated absolute abundance data for each profiled taxon, as previously described (19). Notably, the absolute bacterial abundances for the two groups were shown to be significantly different (average absolute bacterial abundances of $2,448,888 \pm 2,875,900$ and $5,438,290 \pm 3,345,390$ in AMC and CTRL groups, respectively; P value < 0.001), revealing that the microbial abundance of the AMC samples was significantly reduced compared to

that of the CTRL group (Fig. 1b). Interestingly, these analyses revealed differences in the composition at the genus level of the AMC group compared to the CTRL group microbiota, probably due to the intensive selective pressure imposed by the antibiotic therapy to which the AMC group children had been subjected. Specifically, comparison of the gut microbiota compositions of AMC and CTRL groups based on Student's *t* test and linear discriminant analysis effect size (LEfSe) statistical analysis revealed significantly higher absolute abundance of 25 genera, such as *Dialister* (P value = 0.0001; false-discovery rate [FDR] = 0.0001), *Roseburia* (P value = 0.001; FDR = 0.001), *Ruminococcus 1* (P value = 0.013; FDR = 0.025), *Agathobacter* (P value = 0.008; FDR = 0.019), and *Odoribacter* (P value = 0.009; FDR = 0.02), in CTRL samples (Fig. 1b; see also Tables S2 and S3 in the supplemental material). This finding is consistent with those of other studies that had previously assessed the microbiota of individuals treated with antibiotics (20–23). Interestingly, the comparison of the gut microbiota between the AMC and the CTRL groups also showed a 1.8-fold decrease in absolute abundance of the genus *Bifidobacterium*, revealing a possible correlation between the use of the antibiotic and the decrease of this bacterial genus. Bifidobacteria are widely considered to represent a positive biomarker for a healthy gut status and are characteristic of the infant's microbiota (13, 24–26). Although no statistically significant differences were observed, the decrease in members of this genus is consistent with previous data that also showed a decline in bifidobacterial abundance following antibiotic exposure (5, 6).

In order to evaluate the interindividual differences between AMC and CTRL samples, we analyzed the beta diversity based on the weighted UniFrac distance metric and represented the obtained results through principal coordinate analysis (PCoA) (Fig. 1c). Interestingly, the PCoA analysis showed that most of the samples were grouped as two different clusters corresponding to AMC or CTRL samples, respectively, thus underlining the distinct microbiota composition of individuals that had or had not been treated with AMC (Fig. 1c). Moreover, a permutational analysis of variance (PERMANOVA) statistical analysis on the PCoA data revealed a significant division between the two analyzed groups (P value = 0.001, pseudo- F = 4.91, adonis P value = 0.001, R^2 = 0.11). Furthermore, we performed a PCoA based on the host age (see Fig. S1 in the supplemental material), which revealed that there was no correlation between host age and gut microbiota profile (PERMANOVA P value = 0.541, pseudo- F = 0.97, adonis P value = 0.06, R^2 = 0.04). These findings corroborate the fact that microbiota profiles differ considerably between samples, yet in the case of children that had been treated with antibiotics it was clear that such samples had generally suffered from bacterial depletion compared to control samples, underlining the expected detrimental effect of AMC on the microbiota composition.

Isolation of novel bifidobacterial strains and assessment of AMC susceptibility.

As previously reported, bifidobacterial species are commonly present in the infant microbiota and are generally considered to represent a positive microbial biomarker for gut health (13, 24–26). The possible correlation between the use of the antibiotic and the decrease in abundance of the *Bifidobacterium* genus (see above) prompted us to investigate the AMC sensitivity of the species belonging to this genus. For the purpose of evaluating the AMC sensitivity of different members of the *Bifidobacterium* genus, we performed MIC assays involving 237 different strains, including 63 type strains of currently recognized bifidobacterial (sub)species and 174 different strains belonging to four *Bifidobacterium* species, i.e., *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, and *Bifidobacterium longum* (see Table S4 in the supplemental material), representing common colonizers of the human gut (27–31). Moreover, in order to isolate bifidobacterial strains with AMC insensitivity, we applied a bifidobacterial isolation protocol on fecal samples belonging to individuals subjected to AMC therapy (Table S4). The analysis (32, 33) described in Materials and Methods allowed the isolation of 24 novel strains (Table 1), of which 18 were shown to belong to the *B. longum* species, three to the *B. breve* species, and three to the *B. pseudocatenulatum* species. All tested strains showed a unimodal distribution of AMC MIC (MIC_{AMC}) breakpoint values, ranging from 0.125 $\mu\text{g/ml}$ to 32 $\mu\text{g/ml}$ (see Fig. S2 and Table S4 in the supplemental material). Of the strains, 96.5% exhibited a MIC of $\leq 1 \mu\text{g/ml}$, five strains displayed a breakpoint value equal to 2 $\mu\text{g/ml}$, and, notably, four bifidobacterial

TABLE 1 *Bifidobacterium* strains isolated from AMC samples and corresponding MIC_{AMC} values

| Species | Strain | MIC _{AMC} (μg/ml) |
|--|---------|----------------------------|
| <i>Bifidobacterium breve</i> | PRL2020 | 32 |
| <i>Bifidobacterium breve</i> | M1D | 16 |
| <i>Bifidobacterium breve</i> | M5B | 1 |
| <i>Bifidobacterium longum</i> subsp. <i>longum</i> | 1898B | 8 |
| <i>Bifidobacterium longum</i> | 2195B | 1 |
| <i>Bifidobacterium longum</i> | 2196B | 1 |
| <i>Bifidobacterium longum</i> | 2197B | 1 |
| <i>Bifidobacterium longum</i> | 2198B | 0.5 |
| <i>Bifidobacterium longum</i> | 2199B | 1 |
| <i>Bifidobacterium longum</i> | 2202B | 0.125 |
| <i>Bifidobacterium longum</i> subsp. <i>longum</i> | 39B | 4 |
| <i>Bifidobacterium longum</i> | AD12C | 1 |
| <i>Bifidobacterium longum</i> | E2C | 1 |
| <i>Bifidobacterium longum</i> | E4F | 2 |
| <i>Bifidobacterium longum</i> | E6H | 1 |
| <i>Bifidobacterium longum</i> | F2A | 0.25 |
| <i>Bifidobacterium longum</i> | G7G | 0.25 |
| <i>Bifidobacterium longum</i> | G8F | 0.5 |
| <i>Bifidobacterium longum</i> | L5G | 2 |
| <i>Bifidobacterium longum</i> | MISS1F | 0.25 |
| <i>Bifidobacterium longum</i> | T3 | 2 |
| <i>Bifidobacterium pseudocatenulatum</i> | AN3D | 2 |
| <i>Bifidobacterium pseudocatenulatum</i> | L3G | 0.5 |
| <i>Bifidobacterium pseudocatenulatum</i> | M8H | 2 |

strains showed a MIC equal to or higher than 4 μg/ml (Fig. S2 and Table S4). Interestingly, the newly isolated strain *B. breve* PRL2020 displayed the highest MIC_{AMC} value, i.e., 32 μg/ml (Table 1), which was 11-fold higher than the average bifidobacterial MIC_{AMC}. Moreover, *B. breve* strain M1D showed a MIC value of 16 μg/ml, which was 5-fold higher than the *Bifidobacterium* MIC average (Table 1). This antibiotic insensitivity is unusual for *B. breve* species, which in general exhibit a high sensitivity to various antibiotics, including AMC (34). Furthermore, two isolates belonging to the *B. longum* species, i.e., *B. longum* subsp. *longum* 1898B and *B. longum* subsp. *longum* 39B, were shown to exhibit MIC values of 8 μg/ml and 4 μg/ml (Table 1), 8- and 4-fold higher, respectively, than the average MIC value of other *B. longum* strains. These findings suggest that different levels of resistance/insensitivity to AMC reflect strain-specific characteristics rather than being a species-specific feature. Notably, the MIC_{AMC} breakpoints identified in 98.5% of the bifidobacterial strains tested were very low compared to MIC_{AMC} values previously identified for other members of the human gut microbiota, such as *Escherichia coli*, *Citrobacter* spp., *Bacteroides* spp., and *Parabacteroides* spp., which showed MIC_{AMC} breakpoint values higher than 8 μg/ml (35, 36). Interestingly, these data indicate that AMC resistance/insensitivity in bifidobacteria does not appear to follow a vertical route of evolution but may have been acquired through horizontal gene transfer (HGT), in a similar way to that described for other gut commensal microorganisms (37). Finally, we evaluated the susceptibility to the antibiotic amoxicillin alone for the four strains of *Bifidobacterium* insensitive to AMC. Interestingly, each strain showed higher MIC values, i.e., *B. breve* PRL2020 showed a MIC value of 64 μg/ml, *B. breve* strain M1D exhibited a MIC breakpoint of 32 μg/ml, and *B. longum* subsp. *longum* 1898B and *B. longum* subsp. *longum* 39B presented MIC values of 8 μg/ml. These data confirm the resistance of these strains to amoxicillin, both as the sole antibiotic and in combination with clavulanic acid.

Comparative genomics and identification of putative resistance genes in AMC-insensitive *Bifidobacterium* strains. In order to identify the genetic features that are involved in AMC insensitivity in the four identified bifidobacterial strains, we sequenced, annotated and *in silico* analyzed the genomes of *B. breve* PRL2020, *B. breve* M1D, *B. longum*

TABLE 2 Genetic features of AMC-resistant *Bifidobacterium* strains

| Strain | Genome length (bp) | No. of contigs | No. of ORFs | No. of tRNAs | No. of rRNA loci | Accession no. |
|---|--------------------|----------------|-------------|--------------|------------------|-----------------|
| <i>B. breve</i> PRL2020 | 2,427,222 | 6 | 2,102 | 54 | 3 | JACZEM000000000 |
| <i>B. breve</i> M1D | 2,421,612 | 7 | 2,053 | 54 | 3 | JACZEL000000000 |
| <i>B. longum</i> subsp. <i>longum</i> 1898B | 2,464,874 | 9 | 2,065 | 56 | 4 | JACZEK000000000 |
| <i>B. longum</i> subsp. <i>longum</i> 39B | 2,287,000 | 25 | 1,842 | 56 | 1 | JACZEJ000000000 |

subsp. *longum* 39B, and *B. longum* subsp. *longum* 1898B, which were shown to elicit the highest MIC_{AMC} values (Table 1). The coverage depth of these four newly isolated *Bifidobacterium* chromosomes ranged from 72- to 388-fold, which upon assembly generated 1 to 25 contigs (Table 2). The number of predicted open reading frames (ORFs) ranged from 1,842 for *B. longum* subsp. *longum* 39B to 2,102 for *B. breve* PRL2020 (Table 2). We then evaluated the genetic similarities of *B. breve* PRL2020, *B. breve* M1D, *B. longum* subsp. *longum* 39B, and *B. longum* subsp. *longum* 1898B to other chromosome sequences that are publicly available for *B. breve* and *B. longum* subsp. *longum* through a comparative genomics analysis. Specifically, the genome sequences of *B. breve* PRL2020, *B. breve* M1D, *B. longum* subsp. *longum* 39B, and *B. longum* subsp. *longum* 1898B were compared with the chromosome sequences of six *B. breve* and five *B. longum* subsp. *longum* strains, including AMC-sensitive strains (see Table S5 in the supplemental material). *In silico* analysis revealed that 1,272 genes are commonly shared among the analyzed *B. breve* strains, representing the core genome of this taxon (Fig. 2a), whereas the core genome of the assessed *B. longum* subsp. *longum* strains was shown to be composed of 1,106 genes. Moreover, there are variable numbers of truly unique genes (TUGs) in each of the two investigated species, ranging between 175 for *B. breve* UCC2003 and 41 for *B. breve* 7E, and ranging from 585 for *B. longum* subsp. *longum* 1897B and 113 for *B. longum* subsp. *longum* 39B (Fig. 2a and Table S5). Interestingly, this analysis revealed the presence of 323 accessory genes shared between *B. breve* AMC-insensitive strains, i.e., *B. breve* PRL2020 and *B. breve* M1D, and absent in AMC-sensitive *B. breve* strains (Fig. 2a). Furthermore, the genomes of the AMC-resistant strains *B. longum* subsp. *longum* 39B and *B. longum* subsp. *longum* 1898B were shown to contain 25 genes that are shared between them but are absent in other analyzed *B. longum* strains (Fig. 2a). In addition, the genomes of the four identified AMC-insensitive *Bifidobacterium* strains were screened to identify putative antibiotic resistance (AR) genes, using the MEGARes database (38). This *in silico* analysis showed that the predicted resistome of *B. breve* PRL2020, *B. breve* M1D, *B. longum* subsp. *longum* 39B, and *B. longum* subsp. *longum* 1898B ranged from 167 genes in the case of *B. breve* M1D to 175 genes for *B. longum* subsp. *longum* 1898B (Fig. 2b). We included in our analyses both sequences coding for antibiotic-removing transporters and sequences specifying antibiotic-inactivating enzymes. The number of predicted antibiotic-removing transporters ranged from 109 for *B. longum* subsp. *longum* 39B to 127 genes for *B. breve* PRL2020 and *B. breve* M1D (Fig. 2b). Interestingly, the analyzed strains were shown to harbor genes putatively encoding beta-lactamases as part of their resistome; *B. longum* strains were predicted to encode five distinct beta-lactamases, whereas the assessed *B. breve* strains were predicted to specify four beta-lactamases. However, since these putative beta-lactamases are conserved among different strains of the same species, it appears that they are not involved in the observed AMC insensitivity phenotype for some strains.

Moreover, further analysis of the *in silico* resistome data and comparison between genes found in the insensitive strains but absent from the sensitive strains allowed the identification of 11 predicted AR genes that are present only in the *B. breve* AMC-insensitive strains (see Table S6 in the supplemental material). Eight of these identified genes were predicted to encode transporters, whereas two genes seemed to encode enzymes that provide protection against glycopeptide antibiotics (39). Finally, one gene was predicted to encode a glycosyl hydrolase involved in the modification of the lipopolysaccharide (LPS) core and lipid A region with ethanolamine and addition of aminoarabinose to the 4' phosphate of lipid A (40). Furthermore, carrying out the same analysis

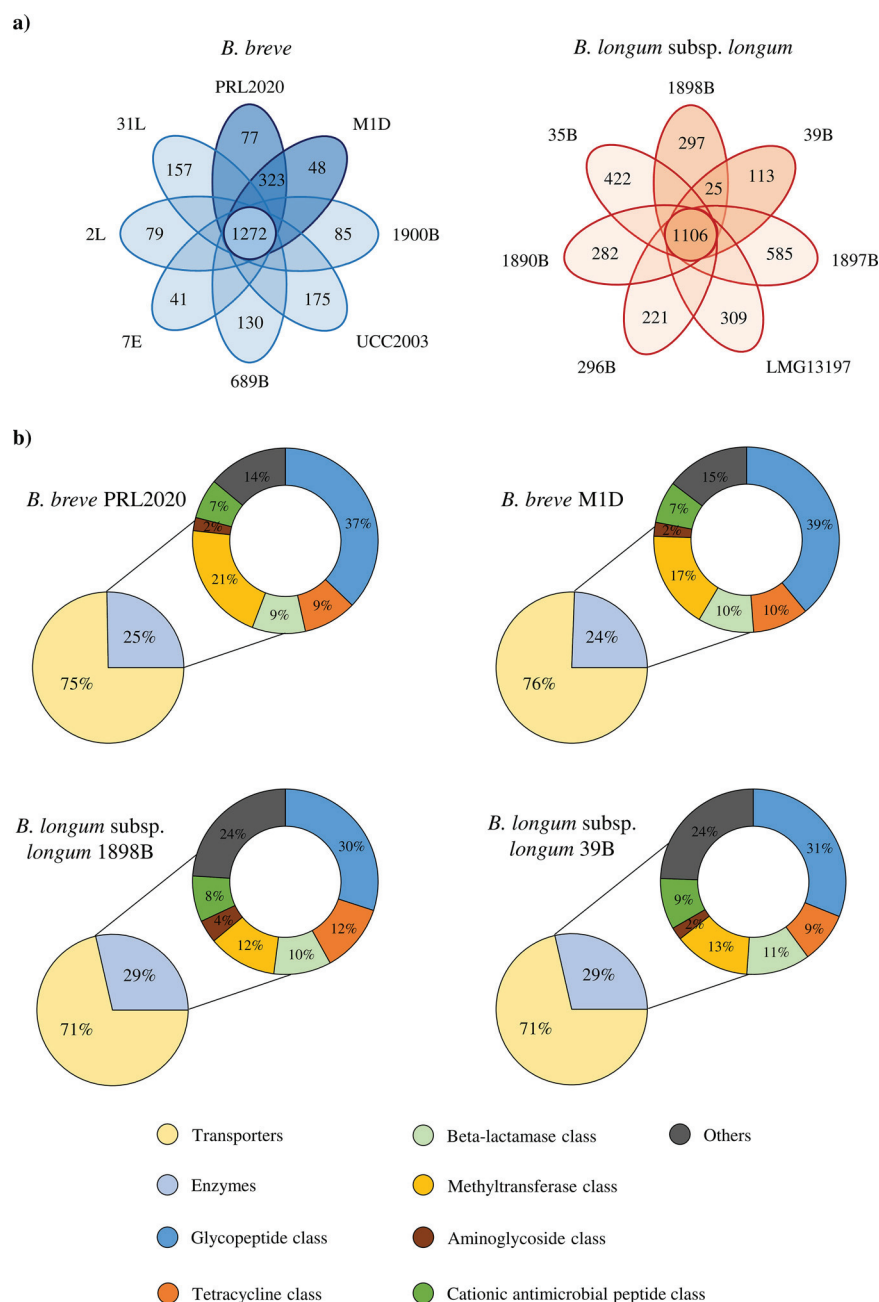


FIG 2 Comparative genomic and resistome analyses of AMC-resistant *Bifidobacterium* strains. (a) Venn diagrams. The numbers in the central circles represent the numbers of genes in the core genomes of the *B. breve* and *B. longum* subsp. *longum* strains analyzed. The numbers in the overlapping sections represent the numbers of shared genes between AMC-resistant strains, while the numbers in the ovals depict the number of TUGs for each strain. (b) Predicted resistomes of the AMC-resistant *Bifidobacterium* strains.

for members of the *B. longum* subsp. *longum* taxon, we identified just one gene uniquely shared between AMC-resistant *B. longum* subsp. *longum* strains, predicted to encode a putative AR transporter (Table S6). These identified genes are indeed credible genetic candidates responsible for the high MIC_{AMC} breakpoint values identified in *B. breve* PRL2020, *B. breve* M1D, *B. longum* subsp. *longum* 39B, and *B. longum* subsp. *longum* 1898B.

Transcriptomic analysis of *Bifidobacterium breve* PRL2020. In order to investigate if and how the presence of AMC in the growth medium modulates the

transcription of genes putatively involved in the AR described above, we explored the transcriptome of *B. breve* PRL2020 when cultivated in the presence or absence of AMC in De Man, Rogosa, and Sharpe (MRS) medium. This analysis showed significant modulation of 163 genes (>2-fold induction; P value ≤ 0.05), 109 of which were upregulated in the MRS_{AMC} medium, while 52 genes were downregulated compared to the reference condition (Fig. 3). Functional assignment of the upregulated and downregulated transcribed genes based on the eggNOG database (41) revealed that the overexpressed transcripts corresponded to genes involved in defensive mechanisms and in the metabolism and transport of inorganic ions (20.2% and 9.2%, respectively) (Fig. 3). Conversely, downregulated transcripts corresponded to genes involved in the transport and metabolism of carbohydrates and nucleotides (13.7% and 25.5%, respectively). Interestingly, among the upregulated genes, one particular ORF, associated with locus tag PRL2020_1181 and encoding a predicted ABC transporter, exhibited 3.6-fold-increased transcription (P value = 0.0001; FDR = 0.002) (Fig. 3). This gene had been identified in the resistome of strain *B. breve* PRL2020, being shared with other *B. breve* AMC-resistant strains but not present in *B. breve* AMC-sensitive strains (Table S6). These findings suggest that this ABC transporter-encoding gene is involved in the observed insensitivity of *B. breve* PRL2020 to the AMC antibiotic. In addition, two TUGs observed in the genome of strain *B. breve* PRL2020, i.e., ORFs PRL2020_1167 and PRL2020_1282, were shown to be transcriptionally induced in the presence of AMC, exhibiting an upregulation of 3.4-fold (P value = 0.039; FDR = 0.04) and 2.7-fold (P value = 0.049; FDR = 0.044), respectively (Fig. 3), and in both cases encoding hypothetical proteins. These data indicate that these unique genes are involved in the high resistance of *B. breve* PRL2020 to AMC antibiotic.

Effect of resistant *Bifidobacterium* strains on the human gut microbiota in the presence of AMC. The GIT microbiota composition is known to be influenced by antibiotic compounds (4, 5). In particular, antibiotic exposure will alter the microbial composition in the infant gut community (6, 7). In order to evaluate the effects of AMC on the human gut microbiota in the presence of AMC-insensitive *Bifidobacterium* strains retrieved in this study, three different fresh infant fecal samples were used separately to perform coculture experiments. In detail, each fecal sample was used to test four different coculture conditions for each AMC-insensitive strain, i.e., (i) fecal sample, (ii) fecal sample with AMC-insensitive strain, (iii) fecal sample with AMC, and (iv) fecal sample with AMC and AMC-insensitive strain (see Materials and Methods) (4). The cocultures were monitored at four different time points, i.e., 12 h, 18 h, 24 h, and 36 h after the addition of inoculum. For each time point and for each cultivation condition tested, the changes in the microbiota composition were assessed by shallow shotgun metagenomics analysis. The analyses resulted in a total of 4,659,664 reads, with an average of $38,831 \pm 21,806$ reads per sample (see Table S7 in the supplemental material). In order to obtain a comprehensive biological interpretation of the analyzed batch culture microbiome complexity, we performed a quantitative microbiome profiling experiment based on flow cytometric analyses for the enumeration of microbial cells present in each coculture condition at each time point (19). Interestingly, comparison of each coculture experiment with its own control revealed a decrease of the number of microbial cells in 95% of the samples to which AMC was added (Fig. 4a; see also Table S8 in the supplemental material). Moreover, statistical analysis revealed a significant decrease (P value = 0.0001) of 1.94-fold in the number of cells in the AMC-treated group (average of $6.85 \times 10^7 \pm 4.32 \times 10^7$) compared to the control sample group (average of $1.33 \times 10^8 \pm 8.21 \times 10^7$).

Focusing on the absolute abundance of the species corresponding to the identified AMC-resistant *Bifidobacterium* strains, the strains that seemed to increase in abundance following the AMC treatment were *B. breve* PRL2020 and *B. breve* M1D (Fig. 4b and Table S8). In fact, the fecal samples treated with AMC and cocultivated with PRL2020 or M1D showed an increase of abundance of *B. breve* species in 42% and 33% of the samples, respectively, compared to each untreated condition (Fig. 4b). Moreover, the *B. breve* PRL2020 strain displayed the greatest AMC resistance, with a decrease in

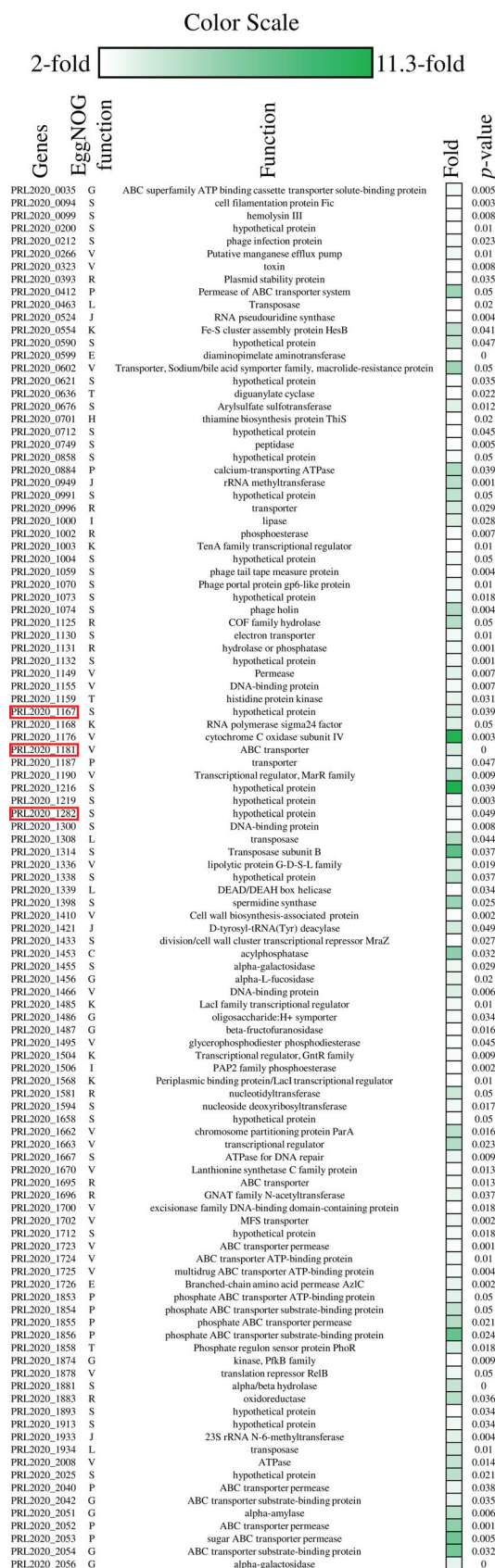


FIG 3 Transcriptional modulation of *B. breve* PRL2020 genes in the presence of AMC. The heatmap displays the subset of significantly upregulated encoding genes. Red boxes highlight the ORFs (Continued on next page)

abundance of <50% in only 33% of the samples, while *B. breve* M1D revealed a decrease in abundance of <50% in 50% of the samples (Fig. 4b). In contrast, *B. longum* subsp. *longum* 39B and *B. longum* subsp. *longum* 1898B showed a decrease in absolute abundance always greater than 50% (Fig. 4b). Furthermore, alignment analysis based on Bowtie 2 software revealed that all these reads classified as *B. breve* or *B. longum* align with each AMC-resistant *Bifidobacterium* strain's reference genome (alignment identity of 99%). Therefore, these results may support the apparent improved ability of *B. breve* PRL2020 to grow in the presence of AMC antibiotic, making this strain an interesting candidate for the development of *Bifidobacterium*-containing probiotic products. Certainly, further *in vitro* experiments aimed at evaluating the ability of the strain to survive gastrointestinal passage and to achieve a high level of viability during industrial production and storage could promote the *B. breve* PRL 2020 strain as a putative novel health-promoting bacterium.

Conclusions. Many pharmaceuticals are well known to influence the human gut microbiota. Since the combination of amoxicillin and clavulanic acid is one of the most frequently prescribed antibiotics in the Western world, especially during infancy and adolescence, we decided to explore the impact of this antibiotic formulation on the gut microbiota of 23 children and compare this to that of a control group who had not been treated with AMC. Interestingly, results indicated a drastic reduction of bacteria in AMC-treated children compared to the CTRL group, including a reduction in absolute abundance of the *Bifidobacterium* genus. We decided to perform MIC experiments with several strains belonging to our collection and novel strains that had been isolated from children treated with AMC. The determination of sensitivity/resistance of these intestinal *Bifidobacterium* strains showed that 98.5% of them are sensitive to this antibiotic. We isolated four strains that showed higher resistance, of which *B. breve* strain PRL2020 displayed the highest MIC_{AMC} value. We identified gene candidates responsible for the higher resistance of certain bifidobacterial strains to AMC compared to that of the majority of bifidobacteria, which appear to be highly sensitive to these antimicrobial molecules. In a follow-up study, we will further investigate the genetic determinants and molecular mechanism of the observed AMC resistance by applying gene inactivation protocols and/or the heterologous expression of the predicted AMC resistance genes in sensitive bifidobacterial strains. Finally, by simulating gut microbiota experiments it emerged that the PRL2020 strain is able to survive in the presence of a complex microbiota combined with AMC antibiotic, opening up the possibility, after verifying that horizontal gene transfer of the AR genes is unlikely to take place, of using this strain in a probiotic product when AMC therapy is prescribed.

MATERIALS AND METHODS

Sample collection. For the purpose of this study, a total of 42 human fecal samples were collected and divided into two groups; the first one was represented by fecal samples obtained from 23 children who were undergoing AMC (amoxicillin:clavulanic acid ratio of 7:1) treatment from 7 to 10 days (average of 8.9 ± 1.3 days) due to respiratory tract infections, while the second group was represented by fecal samples from 19 healthy children (see Table S1 in the supplemental material). Collected samples, which consisted of approximately 10 g of fresh fecal material, were kept on ice, shipped under subzero conditions to the laboratory, and stored at -80°C until further processing.

Bacterial DNA extraction and 16S rRNA gene PCR amplification and sequencing. Stool samples were subjected to DNA extraction using the QIAamp DNA stool minikit following the manufacturer's instructions (Qiagen). Partial 16S rRNA gene sequences were amplified from extracted DNA using the primer pair Probio_Uni/Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence (18).

FIG 3 Legend (Continued)

PRL2020_1181, PRL2020_1167, and PRL2020_1282, which are putatively involved in the AMC resistance mechanism. The color scale (green) at the top of the figure indicates increased transcription levels compared to those of the reference samples. The EggNOG letter for each significant upregulated gene is reported. Each letter stands for a function, as follows: S, function unknown; L, replication, recombination, and repair; R, general function prediction only; G, carbohydrate transport and metabolism; J, translation, ribosomal structure, and biogenesis; E, amino acid transport and metabolism; K, transcription; P, inorganic ion transport and metabolism; C, energy production and conversion; V, defense mechanisms; T, signal transduction mechanisms; H, coenzyme transport and metabolism; and I, lipid transport and metabolism.

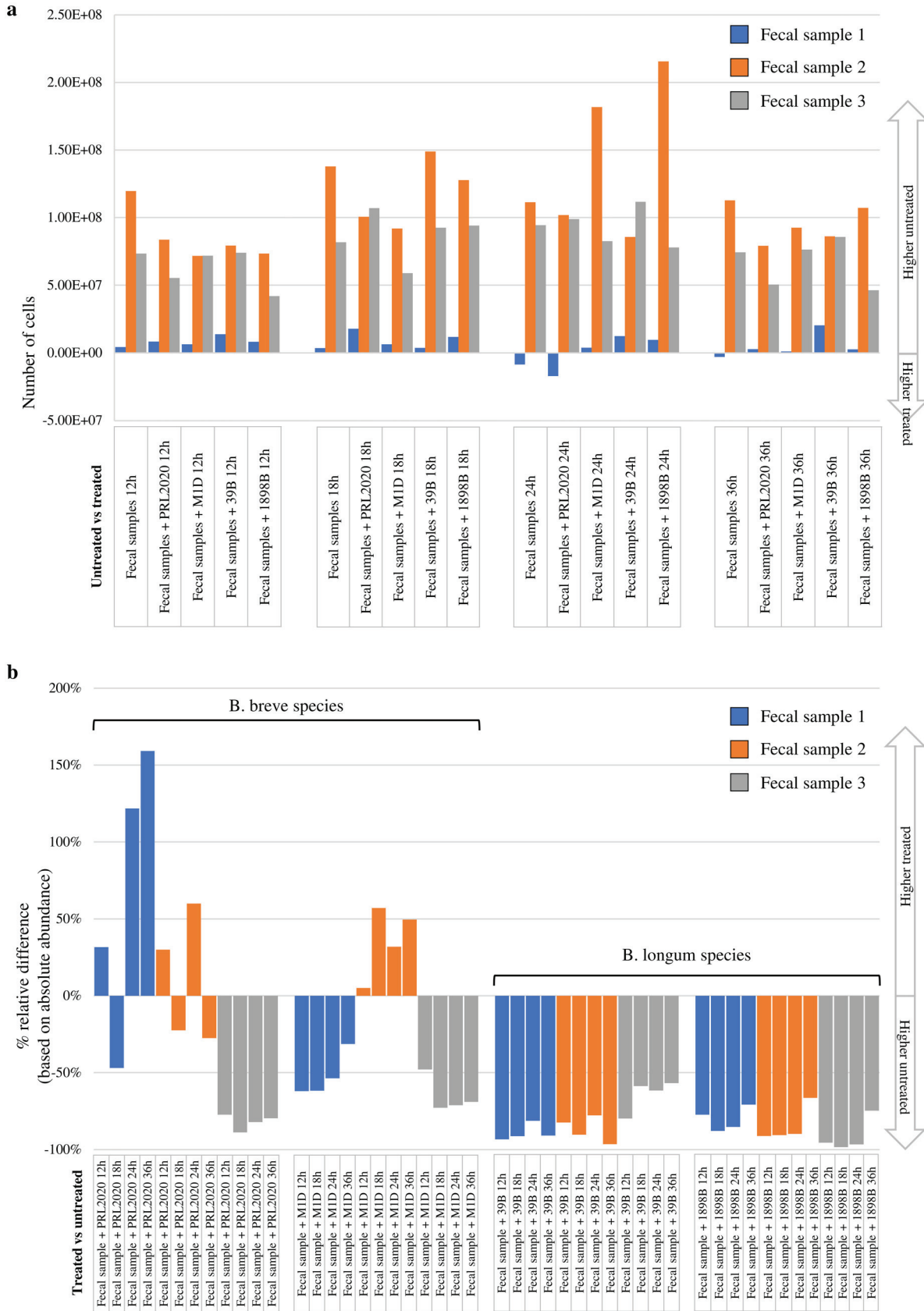


FIG 4 Growth experiments in fecal medium. (a) Comparison of the absolute abundances of the coculture experiments in MRS_{AMC} and the reference condition. (b) Relative percentage difference between AMC-treated and untreated samples based on the absolute abundance of the bacterial species corresponding to the strain analyzed (*B. breve* for PRL2020 and M1D strains and *B. longum* for 39B and 1898B strains).

Illumina adapter overhang nucleotide sequences were added to these partial 16S rRNA gene-specific amplicons, which were further processed employing the 16S metagenomic sequencing library preparation protocol (part no. 15044223 rev. B; Illumina). Amplifications were carried out using a Verity thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on a 2200 TapeStation instrument (Agilent Technologies, USA). DNA products obtained following PCR-mediated amplification of the 16S rRNA gene sequences were purified by a magnetic purification step involving Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. The DNA concentration of the amplified sequence library was determined by a fluorimetric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a concentration of 4 nM, and 5- μ l quantities of each diluted DNA amplicon sample were mixed to prepare the pooled final library. Sequencing was performed using an Illumina MiSeq sequencer with MiSeq reagent kit v3 chemicals. Following sequencing, the FASTQ files were processed using a custom script based on the QIIME software suite (42). Paired-end read pairs were assembled to reconstruct the complete Probio_Uni/Probio_Rev amplicons. Quality control retained sequences with a length between 140 and 400 bp and a mean sequence quality score of >20 , while sequences with homopolymers of >7 bp and mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices and UniFrac analysis), 16S rRNA operational taxonomic units (OTUs) were defined at 100% sequence homology using DADA2 (43); OTUs not encompassing at least 2 sequences derived from the same sample were removed. Notably, this approach allows highly distinctive taxonomic classification at single-nucleotide accuracy (43). All reads were classified to the lowest possible taxonomic rank using QIIME2 (42, 44) and a reference data set from the SILVA database. Biodiversity within a given sample (alpha diversity) was calculated based on the observed OTU index. Similarities between samples (beta diversity) were calculated by weighted UniFrac (45). The range of similarities is calculated between values 0 and 1. PCoA representations of beta diversity were performed using QIIME2 (42, 44).

Evaluation of cell density by flow cytometry assay. For bacterial cell counting, 0.2 g of fecal sample was diluted in a physiological solution (phosphate-buffered saline [PBS]). Subsequently, bacterial cells were stained with 1 μ l SYBR green I and incubated in the dark for at least 15 min before measurement. All count experiments were performed in triplicate using an Attune NxT flow cytometer (Invitrogen, Thermo Fisher Scientific) equipped with a blue laser set at 50 mW and tuned to an excitation wavelength of 488 nm. Multiparametric analyses were performed on both scattering signals (forward scatter [FSC] and side scatter [SSC]) and SYBR green I fluorescence was detected on the FL1 channel. Cell debris and eukaryotic cells were excluded from acquisition analysis by a sample-specific FL1 threshold. All data sets were statistically analyzed with Attune NxT flow cytometer software. Utilizing these cell counts to normalize the sequencing data into absolute abundance of each profiled taxon, we were able to perform quantitative microbiome profiling using a previously described method (19).

Isolation of novel *Bifidobacterium* strains. In order to explore the AMC resistance of the *Bifidobacterium* genus, 24 novel strains were isolated from fecal samples of individuals that had been treated with AMC for various numbers of days (Table 1). One gram of feces from each collected fecal sample was mixed with 9 ml of PBS (pH 6.5). Serial dilutions and subsequent plating were performed using MRS agar supplemented with 50 μ g/ml mupirocin (Delchimica, Italy), 0.05% (wt/vol) L-cysteine hydrochloride, and 8 μ g/ml of AMC (Merck, Germany). Morphologically distinct colonies that developed on MRS plates were randomly picked and restreaked in order to isolate purified bacterial strains. All novel isolates were subjected to DNA isolation and characterized as previously described (46). The *Bifidobacterium* strains isolated in this study are listed in Table 1.

Amoxicillin-clavulanic acid susceptibility tests. The MIC breakpoints (in μ g/ml) of AMC were determined using the broth microdilution method (MDIL) (47). 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). Furthermore, the same MIC analysis was performed for the antibiotic amoxicillin alone (European Standard, Merck, Germany). The MIC breakpoint represents the highest concentration of a given antibiotic to which a particular bacterial strain was shown to be resistant.

Genome sequencing and assemblies. DNA samples extracted from AMC-resistant bifidobacterial isolates were subjected to genome sequencing using the MiSeq platform (Illumina, UK) at GenProbio srl (Parma, Italy) according to the supplier's protocol (Illumina, UK). FASTQ files of the paired-end reads obtained from targeted genome sequencing of isolated strains were utilized as input for genome assemblies through the MEGAnnotator pipeline (48). SPAdes software was used for *de novo* assembly of each *Bifidobacterium* genome sequence (49, 50), while protein-encoding ORFs were predicted using Prodigal (51).

Comparative genomics. In order to identify unique protein families encoded by newly isolated AMC-resistant *Bifidobacterium* strains, a PGAP analysis was performed (52). *B. breve* PRL2020 and *B. breve* M1D genomes were analyzed with six other *B. breve* genomes tested for AMC resistance (Table S5). Simultaneously, *B. longum* subsp. *longum* 39B and *B. longum* subsp. *longum* 1898B genomes were compared with five *B. longum* subsp. *longum* genomes used in the MIC_{AMC} analyses (Table S5). Each predicted proteome of a given *Bifidobacterium* genome was screened for orthologues against the proteome of every *Bifidobacterium* strain belonging to the same species by means of BLAST analyses (53) (E value cutoff of $<1 \times 10^{-4}$ and 50% identity across at least 80% of both protein sequences). Protein families shared between analyzed genomes allowed us to identify the core genomes of *B. breve* and *B. longum* subsp. *longum*.

Functional annotation of each protein of *B. breve* PRL2020 was performed employing the eggNOG database (41).

Prediction of antibiotic resistance genes. The *in silico* proteomes of four *Bifidobacterium* genomes isolated in this study were screened for proteins with similarity to antibiotic resistance proteins acting through inactivation and/or removal of antibiotic molecules. The screening was carried out using the MEGARes database through BLASTP analysis (E value cutoff of 1×10^{-5}) (38, 53, 54). The core database was obtained by nonredundant compilation of sequences contained in ResFinder, ARG-ANNOT, the Comprehensive Antibiotic Resistance Database (CARD), and the NCBI Lahey Clinic beta-lactamase archive (55–58). Following this, a manual examination of sequences with an E value below 1×10^{-5} was performed in order to detect distant homologs.

RNA extraction. Aliquots of *B. breve* PRL2020 cells were grown to an optical density at 600 nm ranging from 0.6 to 0.8. The experiment was conducted in three biological replicates. Total RNA was isolated from bifidobacterial cultures grown in MRS or MRS with $32 \mu\text{g/ml}$ of AMC. RNA extraction was performed as previously described (59). Briefly, cultures were centrifuged at 4,000 rpm for 10 min at 4°C. Cells pellets were treated with TES buffer (Tris-EDTA-sodium dodecyl sulfate buffer) and lysozyme, followed by resuspension in 1 ml of QIAzol lysis reagent (Qiagen, UK), and placed in a sterile tube containing glass beads (Merck, Germany). The cells were lysed by shaking the mix on a Mini-BeadBeater-24 (BioSpec Products, USA) for 2 min followed by 2 min of static cooling; this step was repeated three times. The lysate was centrifuged at $12,000 \times g$ for 15 min, and the upper phase was recovered. RNA samples were washed from proteins by means of chloroform, and, finally, samples were purified by means of the RNeasy minikit (Qiagen, UK), following the manufacturer's instructions. Quality and integrity of the RNA were checked by 2200 TapeStation (Agilent Technologies, USA) analysis. RNA concentration was then determined by a fluorimetric Qubit quantification system (Life Technologies).

Transcriptome sequencing (RNAseq) analysis performed by the MiSeq Illumina platform. Total RNA (1 μg) was treated by the TruSeq stranded total RNA protocol (Illumina, Inc., San Diego, CA), following the manufacturer's instructions. Ribosomal depletion is included in the protocol. Quality and quantity of libraries were checked by 2200 TapeStation (Agilent Technologies) analysis and a fluorimetric Qubit quantification system (Life Technologies), respectively. Samples were loaded into a flow cell from the MiSeq v3 kit (600 cycles; Illumina) as instructed by the technical support guide. The reads were depleted of adapters, quality filtered (with overall quality, quality window, and length filters), and aligned to the *Bifidobacterium* reference genomes through Bowtie 2 software (60). Reads per kilobase per million (RPKM) values were evaluated by means of Artemis software (61).

***In vitro* simulation of the effect of AMC and Bifidobacterium strains on the human gut microbiota.** The effect of AMC antibiotic and four selected AMC-insensitive *Bifidobacterium* strains on the human gut microbiota was evaluated *in vitro* through anaerobic, pH- and temperature-controlled batch cultures. We evaluated four different conditions, and the growth medium used was based on the fecal medium described by Macfarlane et al. (62). Each batch culture condition was performed using three different fresh fecal samples from children (aged 4 years) who had not undergone antibiotic treatment for at least 3 months prior to sample collection and who had not consumed probiotic bacteria. The fresh fecal sample was previously analyzed in order to confirm the absence of *B. breve* and *B. longum* species DNA. We tested four different growth conditions for each strain. Briefly, the first condition consisted of 40 ml of growth medium supplemented with AMC (20 μM) in which an overnight culture of a particular *Bifidobacterium* strain and the fresh fecal sample were inoculated, each at 1% (vol/vol and wt/vol, respectively). The concentration of AMC used was based on another study in which different antibiotic and nonantibiotic compounds were tested for their impact on the gut microbiota (4). The second condition consisted of 40 ml of fecal medium inoculated with 1% (vol/vol) of an overnight culture of a *Bifidobacterium* strain and 1% (wt/vol) of the fresh fecal sample. The third batch culture was made of 40 ml of fecal medium supplemented with AMC at 20 μM and in which the fecal sample was inoculated at 1% (wt/vol). Finally, the fourth condition was composed of 40 ml of growth medium in which only the fecal sample was inoculated at 1% (wt/vol). For each experiment, an aliquot of culture was taken at four different time points, namely, 12 h, 18 h, 24 h, and 36 h after inoculum addition. Each aliquot was subjected to DNA extraction using the QIAamp DNA stool minikit following the manufacturer's instructions (Qiagen, UK) for sequencing library preparation.

Shallow shotgun metagenomics and evaluation of cell density by flow cytometry assay of coculture experiments. Extracted DNA was prepared for sequencing purposes following the Illumina Nextera XT protocol. Briefly, DNA samples were enzymatically fragmented, barcoded, and purified using magnetic beads. Then, samples were quantified using the fluorometric Qubit quantification system (Life Technologies), loaded on a 2200 TapeStation instrument (Agilent Technologies, USA), and normalized to 4 nM. Single-end sequencing was performed using an Illumina MiSeq sequencer with a flow cell from the MiSeq v3 kit (600 cycles; Illumina). For bacterial cell counting, the batch cultures were diluted in PBS physiological solution. Subsequently, bacterial cells were stained with 1 μl SYBR green I and incubated in the dark for at least 15 min before measurement. All count experiments were performed in triplicate as described previously (see above). All data sets were statistically analyzed with Attune NxT flow cytometer software. Utilizing these cell counts to normalize the sequencing data into absolute abundance of each profiled taxa, we were able to perform quantitative microbiome profiling using a previously described method (19).

Statistical analysis. SPSS software (IBM, Italy) was used to perform statistical analysis between the AMC group and the healthy group, as well as to apply Student's *t* test to the RNAseq. PCoA statistical analysis was performed using PERMANOVA and adonis tests.

Data availability. Raw sequences of the 16S profiling experiments, shallow shotgun metagenomics experiments, and RNAseq experiments are accessible under BioProject accession number [PRJNA663786](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA663786).

Newly isolated *Bifidobacterium* genomes were sequenced and deposited in DDBJ/ENA/GenBank under the accession numbers reported in Table 2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.2 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 6, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 7, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 8, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 9, PDF file, 0.05 MB.

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