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ENVIRONMENTAL MICROBIOLOGY

Exploring the Ecological Effects of Naturally Antibiotic-Insensitive Bifidobacteria in the Recovery of the Resilience of the Gut Microbiota during and after Antibiotic Treatment

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ABSTRACT Amoxicillin-clavulanic acid (AMC) is the most widely used antibiotic, being frequently prescribed to infants. Particular members of the genus *Bifidobacterium* are among the first microbial colonizers of the infant gut, and it has been demonstrated that they exhibit various activities beneficial for their human host, including promotion/maintenance of the human gut microbiota homeostasis. It has been shown that natural resistance of bifidobacteria to AMC is limited to a small number of strains. In the current study, we investigated the mitigation effects of AMC-resistant bifidobacteria in diversity preservation of the gut microbiota during AMC treatment. To this end, an *in vitro* coculture experiment based on infant fecal samples and an *in vivo* study employing a rodent model were performed. The results confirmed the ability of AMC-resistant bifidobacterial strains to bolster gut microbiota resilience, while specific covariance analysis revealed strain-specific and variable impacts on the microbiota composition by individual bifidobacterial taxa.

IMPORTANCE The first microbial colonizers of the infant gut are members of the genus *Bifidobacterium*, which exhibit different activities beneficial to their host. Amoxicillin-clavulanic acid (AMC) is the most frequently prescribed antibiotic during infancy, and few strains of bifidobacteria are known to show a natural resistance to this antibiotic. In the present work, we evaluated the possible positive effects of AMC-resistant bifidobacterial strains in maintaining gut microbiota diversity during AMC exposure, performing an *in vitro* and *in vivo* experiment based on an infant gut model and a rodent model, respectively. Our results suggested the ability of AMC-resistant bifidobacterial strains to support gut microbiota restoration.

KEYWORDS *Bifidobacterium*, gut microbiota, antibiotic

The term "microbiota" refers to the complex population of microorganisms that colonize a specific ecological niche (1). In recent decades, it has become clear that the gut microbiota composition affects the health status of the (human) host (2). In fact, various studies have reported the crucial role played by the gut microbiota in maintaining physiological homeostasis of the host as well as in modulating nutritional and immunological functionalities (1, 3). At birth, microorganisms very rapidly colonize the neonatal gut, and the composition of this early-life intestinal microbiota is reported to

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be influenced by several factors, such as mode of delivery, diet, and gestational age, in addition to antibiotic treatment (4). An aberrant gut microbiota composition, sometimes referred to as dysbiosis, may influence human health directly but may also entail long-term host health consequences.

Several factors can lead to a dysbiotic state, such as diet, environment (5), and the use of antimicrobial agents, like antibiotics (6). In this context, oral antibiotics have been widely reported to exert a marked effect on the human gut microbiota composition, frequently causing dysbiosis (7, 8) and contributing to the onset of several metabolic and intestinal disorders (9–12). Amoxicillin is a beta-lactam antibiotic which is typically administered together with clavulanic acid, a beta-lactamase inhibitor (AMC), and this dual drug combination represents the most widely used antibiotic, commonly administered during infancy (13, 14). Interestingly, microbiota analysis of healthy human adult volunteers treated with AMC revealed a significant decrease in abundance of bifidobacterial species (15), which are dominant members of the human gut microbiota in the early stages of life until weaning (16). In this regard, it was recently discovered that AMC resistance is rare among bifidobacteria and appears to be a species-independent feature (17, 18). In order to evaluate possible ecological roles played by three previously identified AMC-insensitive bifidobacterial strains, i.e., *Bifidobacterium breve* 1891B, *Bifidobacterium breve* M1D, and *Bifidobacterium longum* subsp. *longum* 1898B, in maintaining and/or re-establishing homeostasis of the gut microbiota during or following AMC therapy, we assayed the mitigation effects of these strains on the gut microbiota in the presence of AMC using an *in vitro* infant gut model as well as an *in vivo* rodent study. For this purpose, quantitative PCR (qPCR) and shallow metagenomics approaches were used to evaluate the impact over time of these AMC-resistant strains on the intestinal microbiota.

RESULTS AND DISCUSSION

Impact of AMC-resistant bifidobacterial strains on the infant gut microbiota in the presence of AMC. In order to evaluate the possible effects of AMC treatment on a gut microbiota encompassing AMC-resistant *Bifidobacterium* strains, coculture experiments involving infant fecal samples inoculated with *B. breve* 1891B, *B. breve* M1D, or *B. longum* subsp. *longum* 1898B (18) were carried out using the MiPro model (19). Specifically, 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, (iv) fecal sample with AMC and each AMC-insensitive strain (see Materials and Methods; also, see Fig. S1a in the supplemental material). The cocultures were monitored at two different time points, i.e., 12 h and 24 h after inoculation (T0). For each time point and for each cultivation condition tested, the changes in the microbiota composition were assessed by shallow-shotgun metagenomics analysis, while the absolute abundance of bacterial cells was evaluated by flow cytometry. Principal-coordinate analysis (PCoA) based on absolute bacterial abundance at species level revealed a clear difference between each T0 and the corresponding 12-h and 24-h time points, revealing a general selection of certain bacteria during cultivation (Fig. S1b). Notably, cultures of fecal samples from infant 1 in all cases seemed to select bacteria belonging to *Clostridium paraputrificum*, *Enterococcus faecalis*, *E. faecium*, *Escherichia coli*, and *Klebsiella michiganensis* (prevalence $>$ 80%) (see Table S1 in the supplemental material). Similarly, cultures of fecal samples from infant 2 and infant 3 revealed a selection of species belonging to the genus *Escherichia* (prevalence $>$ 80%) and *Bacteroides fragilis* (prevalence $>$ 80% in infant 3) (Table S1). Furthermore, analysis of cell number variations between 12 h and 24 h indicated that 83% of AMC-supplemented cocultures showed a decrease in cell number over time, while 75% of cocultures without AMC treatment revealed an opposite trend. These results confirm the previously reported impact of antibiotic treatment on the microbiota by decreasing microbial abundance and complexity (7, 8). In order to confirm the ability of the three AMC-insensitive *Bifidobacterium* strains to resist antibiotic treatment, we applied strain-specific PCR to all fecal samples, which revealed, as expected, the absence of the AMC-resistant bacterial strains in the original

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fecal samples used for the experiments yet highlighted the presence of these strains in the cultures of fecal samples at 12 h and 24 h (Fig. S1c).

Assessment of bifidobacterial colonization following antibiotic treatment. In order to validate the capability of AMC-insensitive *Bifidobacterium* strains in preserving the gut microbiota as indicated by the *in vitro* analyses, an *in vivo* study based on a rodent model was performed. Specifically, to assess the level of intestinal microbial colonization by AMC-resistant strains of bifidobacteria, we applied a qPCR approach to quantify bacterial DNA extracted from fecal samples from each rat included in our study. qPCR analysis revealed the presence of these bifidobacterial strains in each fecal sample from time point 1 (T1). The two applied *B. breve* strains, i.e., M1D and 1891B, showed a relatively linear trend of microbial load across the different experimental times (Fig. 1d); these persistence data confirmed their insensitivity to AMC (18). Furthermore, *B. breve* 1891B exhibited a higher microbial load than *B. breve* M1D, in accordance with the previously published *in vitro* experiments that reported greater MICs for *B. breve* 1891B than other *B. breve* strains (18) (Fig. 1d). Conversely, evaluation of the microbial load of *B. longum* subsp. *longum* 1898B revealed a nonlinear variation (Fig. 1d). In particular, the qPCR analysis demonstrated that the microbial load for this strain increases at T3 and decreases at T4, followed by a second considerable increase at T5 (analysis of variance [ANOVA] P value < 0.05 at T3 and T5) (Fig. 1d). Notably, previously reported MIC analyses involving the AMC-insensitive strains used here revealed that *B. longum* subsp. *longum* 1898B is associated with a lower MIC than strains belonging to the species *B. breve* (18), while under the conditions used here, this strain showed the highest microbial load compared to *B. breve* strains 1891B and M1D (Fig. 1d), suggesting a superior *in vivo* colonization ability of this strain despite antibiotic treatment.

Impact of AMC-based antibiotic therapy on the gut microbiota of rats. The gut microbiota is reported to be strongly influenced by antibiotic therapy (20, 21). In order to evaluate the impact of AMC-based antibiotic therapy in association with AMC-resistant bifidobacterial strains on rodent physiology, the parameters body weight (BW) (Fig. 1b) and food intake (FI) (Fig. 1c) were measured at different time points during the experiment for each rat. However, no statistically significant differences were found between the investigated groups (see the supplemental material). In order to evaluate the impact of AMC-based antibiotic therapy on the gut microbiota of rats in association with the administration of AMC-insensitive bifidobacterial strains, shallow shotgun metagenomics analysis of fecal samples from each rat was performed. A total of 64 fecal samples were analyzed, resulting in a total of 5,626,619 reads, with an average of 87,916 $\pm$ 52,213 reads per sample (Table S2).

Analysis of bacterial species richness indicated a significantly higher number of species in untreated control samples at T0 than samples from treated rats at subsequent time points (ANOVA P value < 0.01) (Fig. 2a). In contrast, only rats treated with AMC in association with *B. breve* 1891B strain (group 2 [G2]) showed at T1 a nonsignificant difference from the control (Tukey's honestly significant difference [HSD] test *post hoc* P value > 0.05). These results indicate that the presence of this strain impacts the complexity of the microbiota. Furthermore, analysis of beta-diversity based on the Bray-Curtis dissimilarity, represented through a PCoA representation, revealed three different clusters associated with each time point (permutational multivariate analysis of variance [PERMANOVA] P value < 0.05) (Fig. 2b). The three clusters appear to be independent of the bifidobacterial strain administered along with AMC treatment and strongly correlated with time. In particular, while T0, T3, and T5 samples grouped as three condensed independent clusters, samples at T1 constitute a heterogeneous group, indicating a severe initial impact of antibiotic treatment on the gut microbiota composition of the rats (22).

In order to evaluate the metagenomics-based microbial composition divergence between samples and the T0 group, a specific analysis based on a modified dysbiosis score (23) was performed (Fig. 2c). This analysis assessed the difference of the median Bray-Curtis dissimilarity of the T0 reference group and each treated subgroup, i.e., G1,

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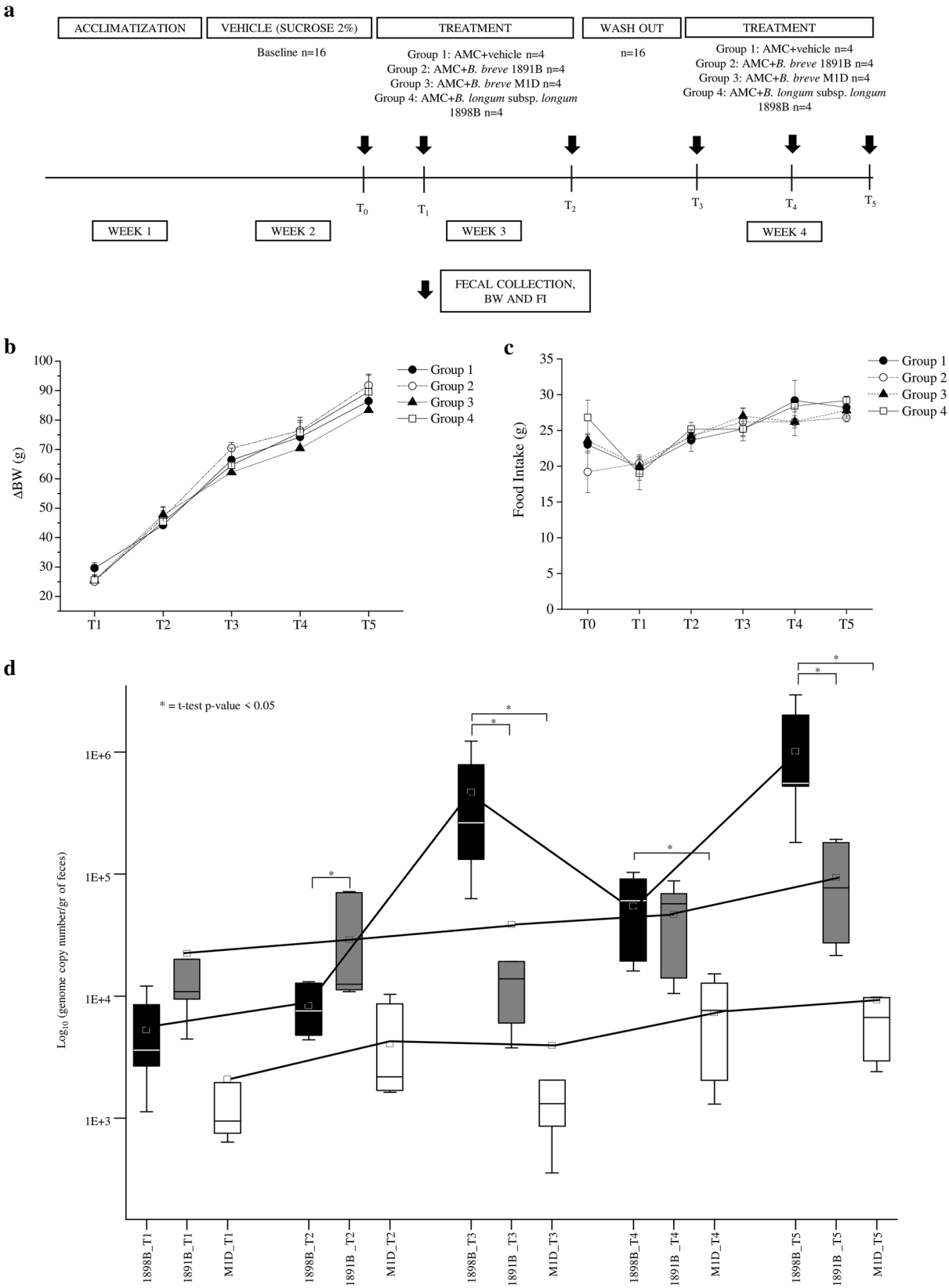


FIG 1 Timeline and experimental results of the *in vivo* study. (a) Timeline of the experimental procedures in rats. (b and c) Body weight and food intake measurements at different time points (T0 to T5). Values are means ± standard errors of the means (SEM). Statistical results are reported in the supplemental material. (d) qPCR evaluation of the numerical load of bifidobacterial strains in stool samples from rats. The graph reports the average abundances of *B. breve* 1891B, *B. longum* subsp. *longum* 1898B, and *B. breve* M1D as calculated by qPCR at T0, T1, T2, T3, T4, and T5.

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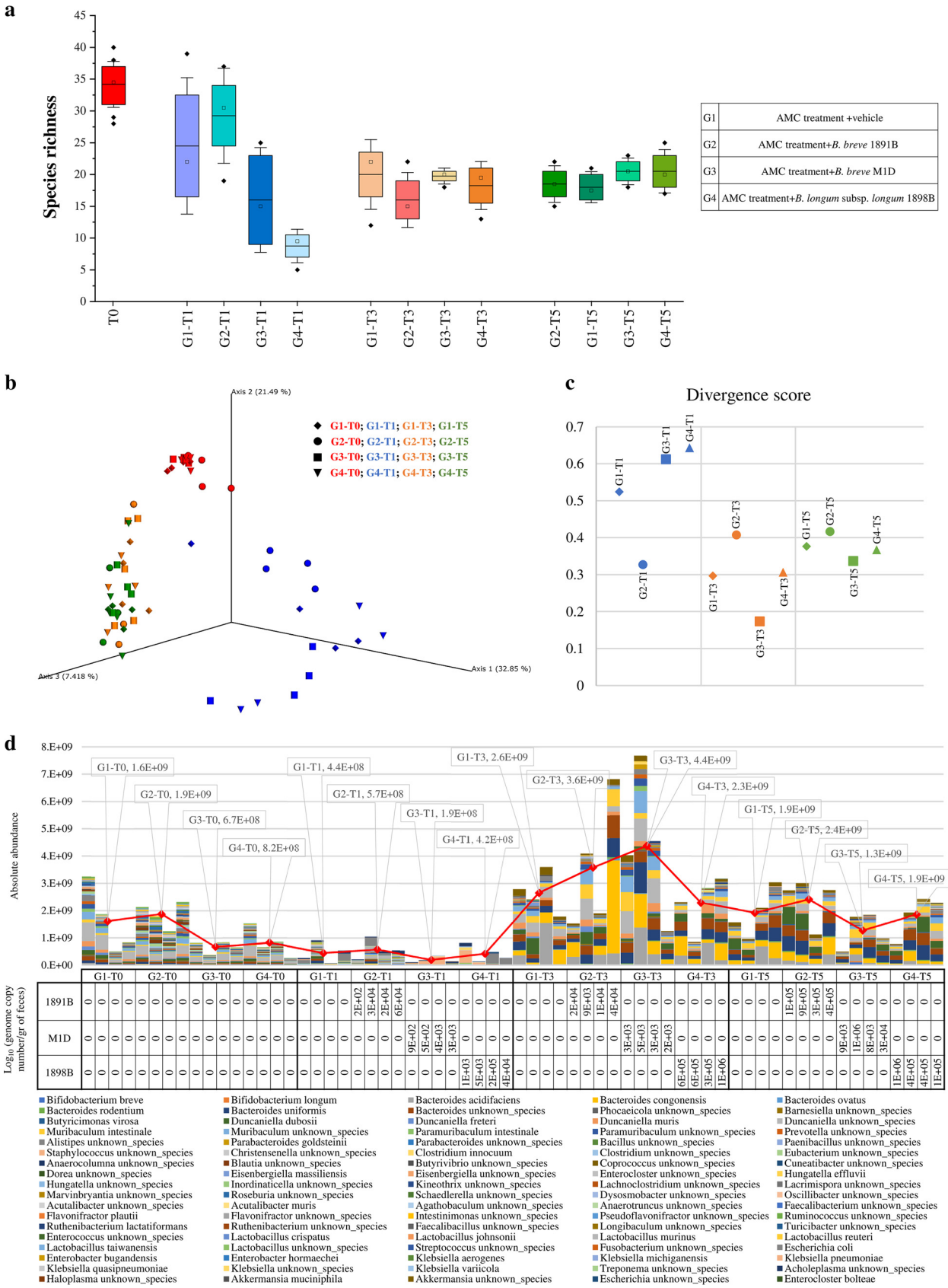


FIG 2 Evaluation of fecal microbiotas of rats at different time points. (a) Whisker plot reporting the species richness value for each experimental group at different time points. The *x* axis represents the different time points for each group, while the *y* axis indicates the number of species.

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G2, G3, and G4, in order to detect any divergence in microbial composition. Rats treated with AMC in association with *B. breve* 1891B (G2) at T1 showed a less divergent microbiota than the reference T0 group (Fig. 2c), indicating that this strain mitigates the disruptive effect of AMC treatment on the stability of the microbiota. Intriguingly, for subsequent time points, i.e., T3 and T5, analysis indicated a lower divergence of the samples belonging to G3 and G4 than the T0 reference (Fig. 2c), apparently reflecting the stabilizing influence of *B. breve* M1D and *B. longum* subsp. *longum* 1898B on the microbial composition over the course of the experiment.

In order to obtain a comprehensive biological interpretation of the analyzed fecal microbiome complexity, we performed a quantitative microbiome profiling experiment based on flow-cytometric analyses for the enumeration of microbial cells present in each sample at T0, T1, T3, and T5. Interestingly, comparison of absolute abundance in all fecal samples at different time points revealed a 3.1-fold decrease of microbial cells at T1 compared to T0 (ANOVA P value < 0.01), highlighting a depletion of the bacterial community that is most likely due to AMC treatment (Fig. 2d). Conversely, samples at T3 and T5 revealed increases of 0.4-fold (ANOVA P value < 0.01) and 0.7-fold (ANOVA P value < 0.05), respectively, in microbial cells compared to T0 samples (Fig. 2d), probably due to microbial resilience and proliferation of AMC-insensitive strains. Focusing our interest on the most abundant species representative of each fecal sample, the metagenomic analysis revealed that the fecal microbiota composition of rats at T0 was relatively stable and was mainly characterized by species belonging to the genus *Duncaniella*, such as *Duncaniella muris* (absolute average of 6.93E+07 ± 5.59E+07, prevalence of 56%), *Duncaniella dubosii* (absolute average of 5.72E+07 ± 3.67E+07, prevalence of 69%) and an as-yet-unclassified species of *Duncaniella* (absolute average of 2.47E+08 ± 1.72E+08, prevalence of 100%) (Table S3). Remarkably, T1 samples showed a substantial change in microbiota composition compared to T0 (Table S3), indicating an interspecific diversity between samples that is presumed to be due to the effect of antibiotic treatment on the microbiota composition (24, 25). Conversely, analysis of fecal samples at T3 and T5 highlighted a partial restoration of bacterial taxa characteristic of those present in T0 samples, mainly represented by uncharacterized species of *Duncaniella* (Table S3). Moreover, T3 and T5 samples highlighted a homogeneous microbiota composition characterized by species belonging to the genus *Bacteroides*, such as *Bacteroides congonensis* (absolute average of 5.11E+08 ± 6.85E+08, prevalence of 72%) and *Bacteroides uniformis* (absolute average of 3.30E+08 ± 2.16E+08, prevalence of 97%) (Table S3), suggesting a possible bacterial adaptation to AMC treatment.

Covariances between AMC-insensitive bifidobacterial strains and the gut microbiotas of AMC-treated rats. In order to determine if administration of AMC-insensitive bifidobacterial strains is involved in delineating the overall taxonomic composition of the rat fecal microbiota, we performed a covariance analysis through Spearman's rho coefficient. To do this, we evaluated the presence of AMC-insensitive bifidobacterial strains in the rodent fecal samples through a qPCR approach to overcome our failure to detect these strains by shallow shotgun metagenomic analysis (presumed to be due to their abundance being below the detection limit of this metagenomic technique). Thus, we correlated the absolute abundance observed for all taxa and qPCR results, revealing a variable impact of AMC-insensitive bifidobacterial strains on the gut microbiota composition of the experimental rats. Specifically, *B. breve* 1891B revealed five positive and one negative correlation with other bacteria, suggesting a rather modest interaction of this strain with other gut microbiota members. Conversely, correlation analysis showed that *B. breve* M1D and *B. longum* subsp. *longum* 1898B exhibit the highest ability to negatively impact the presence of other

FIG 2 Legend (Continued)
The boxes represent the 25th and 75th percentiles, and the whiskers represent the standard deviations. The lines in the boxes represent the averages, while the squares represent the medians. The rhombi indicate outliers. (b) PCoA of rat samples, subdivided by treatment group and time point. (c) Divergence in microbial composition (divergence score) calculated through the difference of the median Bray-Curtis dissimilarity between the T0 reference group and each treated subgroup. (d) Bar plots of the absolute abundance of each rat fecal sample based on the treatment group and the results of the qPCR analysis based on the bifidobacterial strains.

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TABLE 1 Covariance analysis between AMC-insensitive bifidobacterial strains and fecal samples microbiota calculated through Spearman's rho coefficient[a]

		Correlation (Spearman's rho coefficient value) for:		
Phylum	**Species**	**1898B**	**M1D**	**1891B**
Actinobacteria	*Bifidobacterium longum*	−0.086	0.436	−0.086
Actinobacteria	*Bifidobacterium breve*	−0.123	0.078	0.371
Bacteroidetes	*Bacteroides ovatus*	0.251	−0.078	0.145
Bacteroidetes	*Bacteroides rodentium*	0.142	0.060	0.272
Bacteroidetes	*Duncaniella freteri*	−0.316	−0.089	−0.120
Bacteroidetes	*Paramuribaculum intestinale*	−0.356	−0.001	−0.312
Bacteroidetes	*Prevotella*, unknown species	−0.248	0.052	−0.137
Firmicutes	*Clostridium innocuum*	0.382	0.289	−0.204
Firmicutes	*Eubacterium*, unknown species	−0.286	−0.281	−0.173
Firmicutes	*Blautia*, unknown species	−0.217	−0.297	−0.210
Firmicutes	*Eisenbergiella*, unknown species	−0.287	−0.356	−0.113
Firmicutes	*Enterocloster bolteae*	0.315	−0.060	−0.060
Firmicutes	*Enterocloster*, unknown species	−0.269	−0.226	−0.158
Firmicutes	*Hungatella*, unknown species	−0.293	−0.293	−0.076
Firmicutes	*Inordinaticella*, unknown species	−0.060	0.255	−0.060
Firmicutes	*Kineothrix*, unknown species	−0.293	−0.293	−0.157
Firmicutes	*Marvinbryantia*, unknown species	−0.25	−0.25	−0.083
Firmicutes	*Roseburia*, unknown species	−0.346	−0.292	−0.161
Firmicutes	*Schaedlerella*, unknown species	−0.265	−0.346	−0.170
Firmicutes	*Dysosmobacter*, unknown species	−0.226	−0.314	−0.099
Firmicutes	*Acutalibacter*, unknown species	−0.282	−0.282	−0.059
Firmicutes	*Flavonifractor*, unknown species	−0.253	−0.346	−0.070
Firmicutes	*Intestinimonas*, unknown species	−0.253	−0.346	−0.069
Firmicutes	*Pseudoflavonifractor*, unknown species	−0.193	−0.282	−0.116
Firmicutes	*Ruminococcus*, unknown species	−0.215	−0.318	−0.206
Firmicutes	*Ruthenibacterium*, unknown species	−0.060	−0.060	0.275
Firmicutes	*Longibaculum*, unknown species	−0.060	0.255	−0.060
Firmicutes	*Lactobacillus crispatus*	−0.060	−0.060	0.275
Firmicutes	*Lactobacillus taiwanensis*	−0.106	−0.106	0.253
Proteobacteria	*Escherichia*, unknown species	0.336	−0.086	−0.086
Proteobacteria	*Enterobacter bugandensis*	−0.060	0.265	−0.060
Proteobacteria	*Enterobacter hormaechei*	−0.153	0.373	0.000
Proteobacteria	*Klebsiella aerogenes*	−0.060	0.265	−0.060
Proteobacteria	*Klebsiella pneumoniae*	0.072	0.269	−0.148
Proteobacteria	*Klebsiella quasipneumoniae*	0.115	0.258	−0.139
Proteobacteria	*Klebsiella variicola*	0.115	0.258	−0.139
Tenericutes	*Haloplasma*, unknown species	−0.060	0.255	−0.060

[a]Dark gray shading indicates significant negative correlations, while light gray shading indicates significant positive correlations.

bacteria, as indicated by negative correlations (P value < 0.05) with 16% of the taxa identified by the analysis (Table 1). Moreover, *B. breve* M1D positively correlates with 13% of the species included in the analysis and seems to promote the presence of species belonging to the genera *Enterobacter*, *Klebsiella*, and *Clostridium* (Table 1), which may contribute to intestinal dysbiosis (14, 26).

Conclusions. Antimicrobial agents, including antibiotics, are known to influence the composition and the complexity of the human gut microbiota. One of the antibiotics most frequently recommended during infancy and adolescence is the combination of amoxicillin and clavulanic acid (AMC), with consequent major effects in reducing the complexity of the gut microbiota composition and thus promoting the development of gut dysbiosis. Our hypothesis is that the reinforcement of the gut microbiota during AMC treatment with naturally AMC-insensitive strains prevents or reverses dysbiosis and/or re-establishes the natural resilience of the gut microbiota. This notion was verified here with three AMC-insensitive bifidobacterial strains, i.e., *Bifidobacterium breve* 1891B, *B. breve* M1D, and *B. longum* subsp. *longum* 1898B, which were assessed by employing *in vitro* and *in vivo* models. In this context, interesting effects pertaining to the recovery of the original microbial diversity were observed for all these strains, even if with different and strain-specific consequences. The biological explanation of these

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important ecological effects may be linked to the abilities of these bifidobacterial strains to support growth of other members of the gut microbiota by establishing mutualistic trophic interactions, such as those previously observed for various bifidobacterial strains (27–32). Several metagenomic-based studies have reported the establishment of positive correlations between bifidobacteria and the other members of the gut microbiota, which are ultimately important to promote homeostasis of the microbial communities (3, 33–35). Recent studies have reported the positive effect of the occurrence of members of some bifidobacterial species, such as *B. breve* and *B. longum*, in modulating the transcriptome of other members of the gut microbiota (36). Therefore, correlations between different bacterial species may explain the positive ecological effects of bifidobacteria in driving the establishment of the microbiota in the early life and in recovering the natural resilience of the gut microbiota during and following antibiotic treatment.

MATERIALS AND METHODS

Ethics statement. All experimental procedures and protocols involving animals were approved by the Veterinarian Animal Care and Use Committee of Parma University (approved protocol 370/2018-PR) and conducted in observance with the European Community Council Directives dated 22 September 2010 (2010/63/UE). The study protocol for fecal samples was approved by the ethics committee, number 2016/0028558. Signed informed consent was obtained from the legally authorized representative(s) of each infant enrolled in this study.

Strains and cultivation conditions. *Bifidobacterium* strains used in this study were *Bifidobacterium breve* 1891B, *Bifidobacterium breve* M1D, and *Bifidobacterium longum* subsp. *longum* 1898B (18). Strains were grown anaerobically in De Man, Rogosa, Sharpe (MRS) medium (Scharlau) supplemented with 0.05% L-cysteine–HCl and incubated at 37°C for 24 h. Anaerobic conditions were achieved using an anaerobic cabinet (Ruskin), in which the atmosphere consisted of 17% CO_2, 80% N_2, and 2.99% H_2.

***In vitro* gut microbiota cultivation.** For the purpose of this study, fecal samples from three infants (aged from 0 to 6 months), which consisted of approximately 10 g of fresh fecal material, were collected, kept on ice, shipped under subzero conditions to the laboratory, and stored at −20°C until further processing. These collected fecal samples were used for *in vitro* cultivation of the gut microbiota employing a medium essentially as described by Macfarlane et al. (37). A 96-deep-well plate was prepared as previously described (19). Briefly, 1.5 g of a freshly thawed infant fecal sample was placed in a 50-mL tube containing 7 mL of sterile phosphate-buffered saline (PBS) solution. Subsequently, the sample was homogenized with a vortex mixer. Sample homogenates were pipetted into each well in the medium for batch cultivation at final inoculum of 10^7 CFU/mL (cell density was determined by means of flow cytometry [38]). The final volume of the medium in each well was 1.5 mL. We tested four different growth conditions. The first condition involved inoculation of only the fecal sample in the medium. The second condition consisted of the fecal sample together with the antibiotic AMC at a final concentration of 20 μM (7, 18). The third condition consisted of the fecal sample inoculum with each single AMC-insensitive bifidobacterial strain, i.e., *B. breve* M1D, *B. breve* 1891B, and *B. longum* subsp. *longum* 1898B, at a 10^5 CFU/mL final inoculum concentration. The fourth condition consisted of the fecal sample with each of the three AMC-insensitive bifidobacterial strains in the medium supplemented with 20 μM AMC. For each growth condition, six replicates were collected at three different time points, i.e., T0, 12 h, and 24 h (Fig. S1a). Each aliquot was subjected to DNA extraction using the QIAamp DNA stool mini-kit following the manufacturer's instructions (Qiagen, UK) for sequence library preparation.

Evaluation of *Bifidobacterium* cell numbers by qPCR and PCR analysis. In order to evaluate *Bifidobacterium* cell numbers in rodent (rat) fecal samples and the presence of *Bifidobacterium* strains in co-culture experiments, we used the strain-specific primers 1891B _FW (5′-GGTTGAGCTTACCGAAGACC-3′) and 1891B _RV (5′-TAAGGCTCCTTCTGGTGTGG-3′) for *B. breve* 1891B. For *B. breve* M1D, we used the primers M1D_FW (5′-CGCTATCGACACCGACTACA-3′) and M1D_RV (5′-GATATCGGCCTTGGAACAGA-3′). For *B. longum* subsp. *longum* 1898B, we employed the primers B1898_0685_Fw (5′-GACGCGCAAGGTTCAATAAC-3′) and B1898_0685_Rev (5′-ACTATACAATGCGCCGTTGG-3′). qPCR was performed using qPCR green master mix (Biotechrabbit, Germany) on a CFX96 system (Bio-Rad, CA, USA) following previously described protocols (39). PCR products were detected with SYBR green fluorescent dye and amplified according to the following protocol: one cycle of 95°C for 2 to 3 min, followed by 40 cycles of 95°C for 15 s and 60 to 65°C for 30 s. The melting curve was 65°C to 95°C with increments of 0.5°C/s. In each run, negative controls (no DNA) were included. A standard curve was generated using the CFX96 software (Bio-Rad).

Each 12.5-μL PCR mixture contained 30 to 40 ng of genomic DNA, 2× PCRBIO HS *Taq* mix (PCR Biosystems, USA), and a 100 μM concentration of each oligonucleotide. Each PCR consisted of an initial denaturation step of 5 min at 94°C, followed by 30 amplification cycles as follows: denaturation at 94°C for 20 s, annealing at a temperature of 58°C for *B. longum* subsp. *longum* 1898B and *B. breve* M1D and 60°C for *B. breve* 1891B for 30 s, and 72°C for 30 s and finalized by an elongation step at 72°C for 5 min. PCRs were performed on a Verity thermocycler (Applied Biosystems, USA). PCR products were analyzed by electrophoresis on a 1.5% agarose gel and visualized by SYBR Safe DNA gel stain (Invitrogen).

Animal housing and design of the *in vivo* experiment. The *in vivo* rodent experiments involved 16 male, 5-week-old Wistar rats. After weaning, rats were housed in same-sex sibling groups in rooms with controlled humidity (50% ± 10%) and temperature (22 ± 2°C) conditions, with a 12-h light-dark cycle

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(lights on at 7 a.m.), and with food and water available *ad libitum*. Experiments were conducted on 5-week-old male Wistar rats ($n = 16$). In rats, this age corresponds to the beginning of the human periadolescent phase (40) After weaning on postnatal day 28, rats were housed in sibling groups in rooms under controlled humidity (50% ± 10%) and temperature (22 ± 2°C) conditions, with a 12-h light-dark cycle (lights on at 7 a.m.) and with food and water available *ad libitum*, and did not undergo any type of treatment (Fig. 1a).

On day 35, experimental rats were housed individually in polymethyl methacrylate (Plexiglas) cages (39 cm by 23 cm by 15 cm), continued to consume a standard chow and were orally given a 500-μL sucrose solution (2%) with a syringe to adapt them to this form of administration. This time point represents the reference control of the experiment, also considered the baseline for microbiota analyses (36) (Fig. 1a). For the next 12 days, the rats were randomly assigned to four different treatment groups, i.e., G1, G2, G3, and G4. All four groups were orally administered amoxicillin-clavulanic acid (Augmentin) at a dosage of 35 mg kg of body weight^{-1} dissolved in water, two times per day using a syringe (41) (Fig. 1a). Furthermore, groups G2, G3, and G4 were also orally administered *B. breve* 1891B, *B. breve* M1D, and *B. longum* subsp. *longum* 1898B, respectively, while group G1 received only the antibiotic and 500 μL sucrose solution (2%) without any *Bifidobacterium* strain. *Bifidobacterium* strains were cultivated as previously described (42). The resulting cell cultures were subsequently centrifuged, washed, and resuspended in 500 μL of sucrose solution (2%). Body weight (BW) and food intake (FI) were recorded and fresh fecal samples were collected at five time points. The first fecal sample collection was performed before the oral administration of AMC and bifidobacteria (T0); then, fecal samples were collected on days 2, 5, 8, 10, and 12 (T1, T2, T3, T4, and T5, respectively) (Fig. 1a). The sawdust was changed 1 h before each fecal collection. Afterward, fresh fecal samples were collected in the morning and stored at −20°C until analysis. BW was measured as previously described (43) (Fig. 1b and c). FI was calculated as the amount (in grams) of food consumed over 24 h. BW and FI data were collected every 2 days at five time points (T0, T1, T2, T3, T4, and T5).

Fecal bacterial DNA extraction and shallow shotgun metagenomics. Rodent fecal samples were subjected to DNA extraction using the QIAamp DNA stool minikit following the manufacturer's instructions (Qiagen). Extracted DNA was then processed according to the Illumina Nextera XT protocol. DNA samples were enzymatically fragmented, barcoded, and purified by using magnetic beads. Then, samples were quantified using a fluorometric Qubit quantification system (Life Technologies, USA), loaded on a 2200 Tape Station instrument (Agilent Technologies, USA) and normalized to 4 nM. Paired-end sequencing was performed using an Illumina MiSeq sequencer with flow cell v3 for 600 cycles (Illumina Inc., San Diego, USA). The obtained fastq files were filtered for quality (>20) and length (>80 bp) of the reads. Filtered data were then used to reconstruct the taxonomic profile of the analyzed samples (44) using the bioinformatic software platform METAnnotatorX2 (45). The taxonomic classification of each read was obtained by MegaBLAST analysis (46) using as a reference the database of nonredundant genome sequences retrieved from the database at the National Center for Biotechnology Information (NCBI). In addition, the beta diversity among the analyzed samples was calculated by means of the Bray-Curtis dissimilarity and based on species abundance. The results of this analysis were represented by three-dimensional (3D) PCoA using the QIIME2 software (47, 48).

Evaluation of cell density by flow cytometry assay. For bacterial cell counting, 0.1 g of a rat fecal sample was diluted in a physiological solution (PBS). 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 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 (38).

Statistical analysis. Two-way ANOVA for repeated measures with "group" as the between-subject factor (4 groups) was performed for both BW and FI data, with "time" as the within-subject factor (six levels: T0, T1, T2, T3, T4, and T5). All statistical analyses were performed with SPSS v. 25 software (www.ibm.com/software/it/analytics/spss/). In particular, ANOVA was performed to evaluate the relative differences in abundance of bacterial species.

Data availability. Raw sequences of the shallow-shotgun metagenomics profiling experiments are accessible through SRA under study accession number PRJNA803045.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.04 MB.

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