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Differential antibiosis predisposes mice to *Campylobacter jejuni* infection: Deeper insights into the impact of the gut microbiota composition in colonization resistance

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ORIGINAL RESEARCH PAPER

ABSTRACT

Conventional laboratory mice are protected from oral *Campylobacter jejuni* infection due to colonization resistance (CR) mediated by their host-specific gut microbiota. Here, we used differential effects of distinct antibiotics on gut microbiota composition to identify microbial groups associated with CR against *C. jejuni*. Therefore, specific pathogen-free (SPF) mice were subjected to ampicillin plus sulbactam (A/S), ciprofloxacin (CIP), or vancomycin (VAN) via the drinking water for 28 days or left untreated before peroral *C. jejuni* challenge. Cultural analyses revealed that CR displayed by untreated mice was abrogated by A/S treatment, but only reduced in mice treated with CIP or VAN. Notably, differential analysis of antibiotic-induced microbiota changes and *C. jejuni* colonization dynamics identified lactobacilli and *Clostridium leptum* as key microbial groups that were associated with CR. Notably, the complete eradication of intestinal bacteria in A/S treated mice supported high intestinal *C. jejuni* colonization levels which triggered apoptosis and inflammatory responses accompanied by enhanced expression of matrix-degrading gelatinases in the colon. In conclusion, A/S treated mice represent a valuable infection model for the study of campylobacteriosis and the treatment of mice with specific antibiotics support the investigation of molecular mechanisms involved in CR against enteropathogens.

KEYWORDS

Campylobacter jejuni, enteropathogenic infection, commensal gut microbiota composition, antibiotic treatment, colonization resistance, matrix metalloproteinases, gelatinases, host-pathogen interaction, gut microbiota shifts, differential antibiotic treatment

INTRODUCTION

The Gram-negative, spirally curved *Campylobacter jejuni* are considered among the leading causes of bacterial gastroenteritis worldwide [1, 2]. Generally viewed as an accidental human pathogen, these bacteria reside as commensals in the intestines of poultry and are transmitted via the consumption of contaminated raw or undercooked meat, but even through contaminated surface water or unpasteurized milk [2–4]. Ingestion of the pathogen is accompanied by a range of symptoms that normally unfold after 1–5 days incubation period. Patients are typically presented with diarrhea, cramps, and abdominal pain, and often with fever, nausea, and vomiting [5]. Symptoms severity can vary based on the host's immune status and the strain of the pathogen, ranging from mild discomfort to bloody diarrhea and

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severe abdominal cramps [5, 6]. In some cases, infection with *C. jejuni* can even trigger post-infectious sequelae such as reactive arthritis, irritable bowel disease, and Guillain-Barré syndrome [7]. *C. jejuni* infections can be self-limiting in healthy individuals, lasting between several days and two weeks. Nevertheless, treating these infections remains challenging due to the increased resistance of *C. jejuni* to antibiotics, and the risk of disrupting the balance of the bacterial community in the intestines, leaving the host at risk of infection with other opportunistic pathogens [8, 9].

As a matter of fact, and in contrast to humans, conventional laboratory mice are highly resistant to *C. jejuni* infections due to their specific intestinal microbiota present leaving the pathogen unable to multiply and colonize in the gastrointestinal tract [10–12]. This phenomenon has been widely described and is known as colonization resistance (CR), which refers to the gut microbiota's ability to orchestrate a shield blocking out luminal growth and expression of virulence genes by incoming pathogens [13].

A plethora of pathways underly CR and can be generally divided to competition for nutrients and niches, production of antimicrobial peptides, modulation of host immune responses, and others [13]. However, despite this highly complex intestinal ecosystem, perturbations induced by several factors, most notably antibiotics, leave this system in a dysbiotic state indicated by a loss of diversity, a decrease or even loss of major bacterial groups, shifts in the metabolic activity of bacterial community members, and a reduction in CR, and thus rendering the host susceptible to infection by pathogenic bacteria [11, 12, 14–16]. Antibiotics are most commonly used as a therapeutic tool to manage bacterial infections across various domains, including the human health sector, livestock, and agriculture. A plethora of mechanisms underly antibiotics efficacy, such as inhibition of DNA replication, inhibition of protein biosynthesis, inhibition of cell wall synthesis among others [17].

Indeed, mouse models subjected to antibiotic treatments have long been recognized as valuable tools for research targeting the pathogenesis of enteric pathogens [18]. For instance, streptomycin pretreated mice have been widely employed to study *Salmonella enterica* Typhimurium (*S.* Tm) infections [19, 20]. Beyond *S.* Tm, the streptomycin model was also utilized to study other pathogens like *Clostridioides difficile*, *Listeria monocytogenes*, and both, enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC) [21–24]. Antibiotic-treated mice have also been widely applied to dissect *C. jejuni* infections [12, 18]. Ampicillin pretreatment of CBA/J mice was shown to render these animals susceptible to *C. jejuni* colonization [25]. Stahl et al. also showed that treating mice with vancomycin (VAN) paved the way for *C. jejuni* to overcome CR and establish in these mice [26]. This was also observed upon pretreating mice with therapeutic dosages of penicillin or ciprofloxacin (CIP) [27].

Shifts in the intestinal microbiota composition provides *C. jejuni* with the opportunity to invade the intestinal mucosa, residing in the intestinal epithelial cells and lamina propria, and thus activating the innate and adaptive immune responses [28–30]. This response is characterized by dendritic cells activation, neutrophils recruitment and initiation of a T-helper cell 1 (Th1) type immune response [28, 31]. Importantly, the gut microbiota contributes substantially to the development and differentiation of the host immune system [32, 33]. In fact, bacterial signals play a major role in the developmental phase of both, innate and adaptive immune cell functions [32]. For instance, treatment with fluoroquinolones such as ciprofloxacin strongly reduces aerobic Gram-negative bacteria [34], whereas vancomycin administration can decrease several Gram-positive bacterial species such as enterococci, clostridia, and bifidobacteria [35]. Notably, the eradication of bacteria in the intestinal microbiota by antibiotics causes a profound disturbation of the immune system present in the gut. For instance, VAN treatment was followed by a reduction in the numbers of colonic regulatory T cells (Tregs) in the enteric lamina propria [36] and was shown to inhibit Th17 cell differentiation [37]. Furthermore, interferon (IFN)-γ and interleukin (IL)-17 production by $CD4^+$ T lymphocytes was also reduced upon antibiotic treatment [38]. Given that dysbiosis induced by antibiotic treatments can be fast and long lasting [39, 40] it is remarkable that the immune functions suppressed by antibiotics were restored upon restoration of the gut microbiota composition by fecal microbiota transplantation [33].

While CR against *C. jejuni* in conventional laboratory mice is primarily mediated by species-specific commensals present within the gut microbiota, the distinct bacterial groups and their associated effector molecules responsible for inhibiting *C. jejuni* colonization remain poorly understood. In our present study we shed the light onto these major bacterial groups by depleting components of the microbial community using distinct antibiotic regimens. By analyzing the impact of antibiotic-induced dysbiosis on microbial composition and *C. jejuni* colonization dynamics, we seek to identify the key microbial groups that govern resistance or susceptibility to the pathogen.

MATERIAL AND METHODS

Mice and antibiotic pretreatment

Conventional C57BL/6j wildtype mice were bred and maintained in the Forschungsinstitute für Experimentelle Medizin, Charité – Universitätsmedizin Berlin, Germany under specific pathogen free (SPF) conditions. Mice were housed in cages equipped with filter tops within an experimental semi-barrier under standard conditions (i.e., 22–24 °C room temperature, 55 ± 15% humidity, 12 h light/12 h dark cycle) and had *ad libitum* access to autoclaved water and standard chow (food pellets: ssniff R/M-H, V1534-300, Sniff, Soest, Germany). Eight-week-old female mice were transferred to sterile cages (maximum of 3–4 animals per cage) and subjected to differential antibiotic pretreatment for 4 weeks (from day −28 until day −2; Fig. 1). Therefore, ampicillin plus sulbactam (A/S)

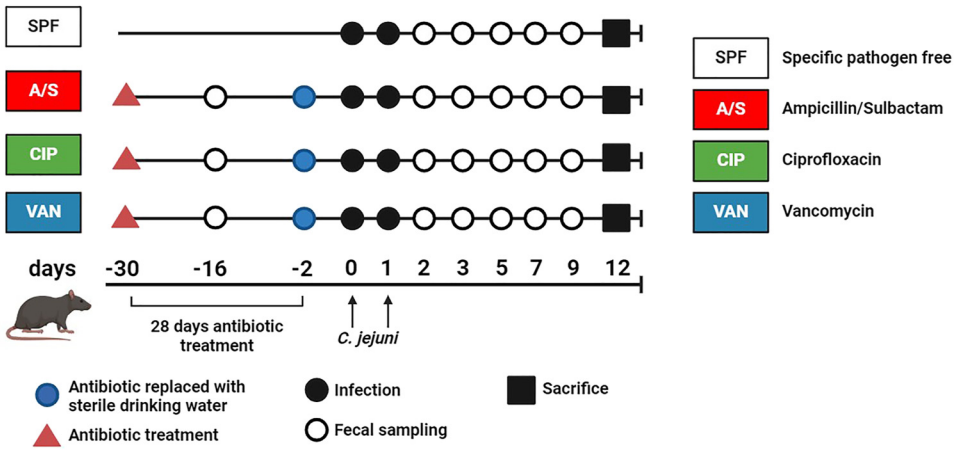

Fig. 1. **Timeline of the experimental procedures**

(2 g L^{-1}; Dr. Friedrich Eberth Arzneimittel, Ursensollen, Germany), CIP (200 mg L^{-1}; Bayer Vital, Leverkusen, Germany), or VAN (500 mg L^{-1}; Cell Pharm, Hannover, Germany) were added to the autoclaved tap water (*ad libitum*) or remained untreated (SPF controls). Two days before *C. jejuni* infection, respective antibiotic compounds were withdrawn and replaced by autoclaved tap water (*ad libitum*) to assure antibiotic washout.

C. jejuni infection and assessment of fecal shedding

C. jejuni strain 81-176 was thawed from frozen stocks and grown on selective karmali agar plates (Oxoid, Wesel, Germany). Mice were infected perorally with 10^9 colony-forming units (CFU) of the pathogen on days 0 and 1 by gavage as stated elsewhere [11]. At defined time points post-infection (p.i.), fecal and intestinal luminal *C. jejuni* bacteria were quantified by counting of CFU after growth of serial dilutions of respective samples on karmali agar plates for at least 48 h at 37 °C under microaerophilic conditions as described in detail previously [11]. The detection limit of viable pathogens was 100 CFU per g fecal matter.

Sampling procedures

On day 12 p.i., mice were sacrificed by CO_2 asphyxiation. Colonic *ex vivo* biopsies and luminal samples from the terminal ileum and colon were derived under aseptic conditions. From each mouse, colonic samples were collected in parallel for subsequent microbiological and immunohistopathological analyses.

In situ immunohistochemistry

Quantitative *in situ* immunohistochemical analyses were performed in colonic *ex vivo* biopsies following immediate fixation in 5% formalin and embedding in paraffin as reported previously [41, 42]. In brief, to detect apoptotic epithelial cells, T lymphocytes, and Tregs, colonic paraffin sections (5 µm) were stained with primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA; 1:200), CD3 (no. N1580, Dako, Glostrup, Denmark; 1:10), and FOXP3 (clone FJK-165, no. 14-5773, eBioscience, San Diego, CA, USA; 1:100), respectively. Positively stained cells were quantitated by a blinded independent investigator applying light microscopy. The average number of respective positively stained cells in each sample was determined within at least six high power fields (HPF, 0.287 mm^2; 400 × magnification).

Expression analysis of gelatinases and Timps

Total RNA was isolated from snap-frozen colonic *ex vivo* biopsies, reverse transcribed, and analyzed as described previously [43]. In brief, mRNAs coding for murine gelatinases A and B also referred to matrix metalloproteinase (MMP)-2 and −9, respectively, and for the tissue inhibitors of matrix metalloproteinases (Timp)-1 and −3 were detected by real-time polymerase chain reaction (PCR) with specific primers and quantified by analysis with the Light Cycler Data Analysis Software (Roche, Penzberg, Germany). The mRNA of the housekeeping gene for hypoxanthine-phosphoribosyltransferase (HPRT) was used as reference; the mRNA expression levels of the individual genes were normalized to the lowest measured value and expressed as fold expression (arbitrary units).

Gut microbiota analyses

For molecular analysis of the gut microbiota composition additionally assessing fastidious and uncultivable bacteria, DNA from fecal and colonic luminal samples was extracted as described previously [44]. In brief, DNA extracts and plasmids were quantified using Quant-iT PicoGreen reagent (Invitrogen, Paisley, UK) and adjusted to 1 ng per µl. Then, abundance of the main bacterial groups of the gut microbiota was assessed by the quantitative real time polymerase chain reaction (qRT-PCR) with group-specific 16S rRNA gene primers (Tib MolBiol, Berlin, Germany) as described previously [44–46]. The number of 16S rRNA gene

copies/ng DNA of each sample was determined and frequencies of respective bacterial groups calculated proportionally to the eubacterial (V3) amplicon.

Statistical analyses

Medians and significance levels were calculated using GraphPad Prism (version 8; San Diego, CA, USA). Normalization of data was assessed by the Anderson-Darling test. The Student's *t* test and Mann-Whitney test were used for pairwise comparisons of normally and not normally distributed data, respectively. For multiple comparisons, the one-sided ANOVA with Tukey post-correction (for normally distributed data) and the Kruskal-Wallis test with Dunn's post-correction (for not normally distributed data) were applied. Two-sided probability (*P*) values ≤0.05 were considered significant. Data sets from respective mouse groups (SPF, $n = 9$; A/S, $n = 10$; CIP, $n = 9$; VAN, $n = 10$) were pooled from three independent experiments.

RESULTS

Time course of fecal *C. jejuni* shedding following infection of mice that had been subjected to differential antibiotic pretreatment

Conventional SPF wildtype mice were pretreated with distinct antibiotics from different antibiotic classes and antimicrobial spectra that are commonly used in clinical settings. Then, we addressed the consequences of the differentially induced commensal gut microbiota shifts on murine host resistance against or susceptibility towards *C. jejuni* infection (perorally on days 0 and 1). Our cultural analyses of the enteropathogenic loads in fecal samples over time revealed that untreated control mice with a complex gut microbiota had virtually completely expelled the *C. jejuni* bacteria from the intestines within 48 h after the latest oral challenge, and in fact, the fecal samples remained *C. jejuni* culture-negative until day 12 p.i. (Fig. 2A)

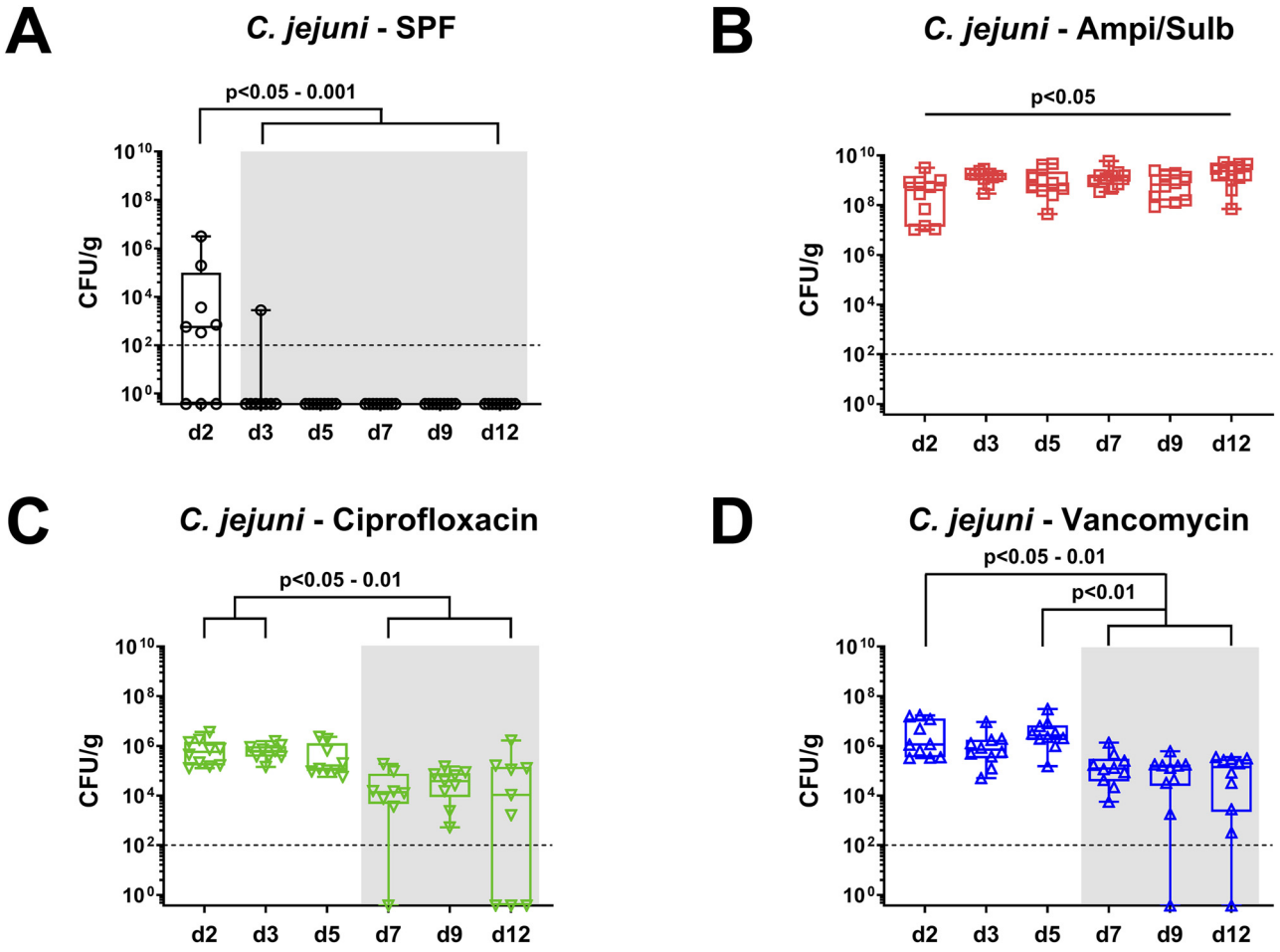

Fig. 2. **Time course of fecal *C. jejuni* shedding following infection of mice that had been subjected to differential antibiotic pretreatment** Conventional C57BL/6j mice harboring a specific pathogen free (SPF) gut microbiota remained **(A)** untreated (SPF; black circles) or were pretreated with different antibiotics such as **(B)** ampicillin plus sulbactam (Ampi/Sulb; red squares), **(C)** ciprofloxacin (CIP; green inverted triangles) or **(D)** vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 until d-2). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain and the fecal pathogen loads were surveyed over time post-infection as indicated by culture and expressed as colony-forming units per gram feces (CFU/g). The box plots representing the 25th and 75th percentiles of the median (bar within box), the total range, and the significance levels (*P* values) determined by the Kruskal-Wallis test with Dunn's post-hoc test are given. Shown data were pooled from 3 independent experiments

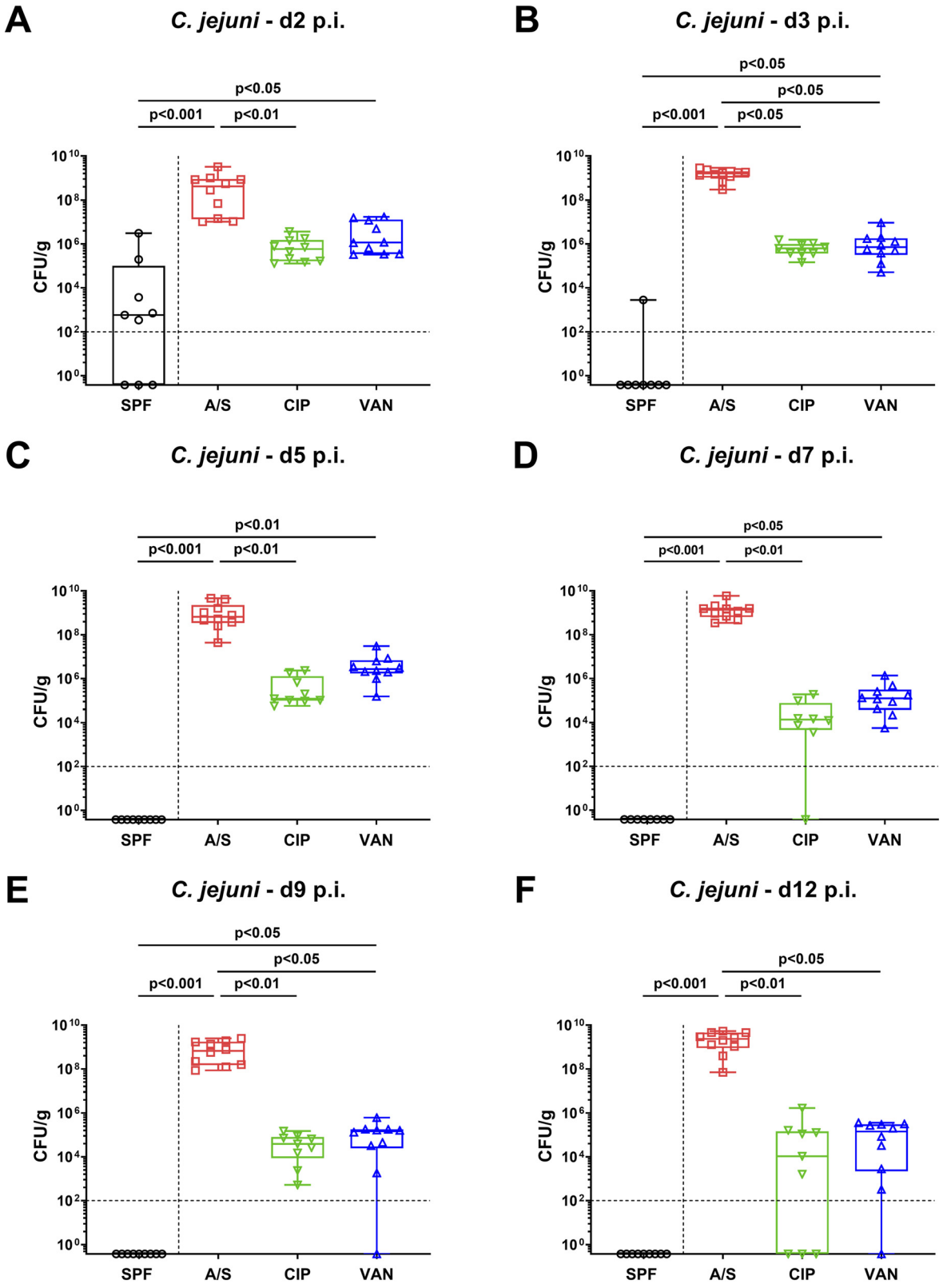

Fig. 3. **Fecal pathogen loads at defined time points post *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment**

Conventional C57BL/6j mice harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S; red squares), ciprofloxacin (CIP; green inverted triangles) or vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 until d-2) or remained untreated (SPF; black circles). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain. The intestinal pathogen loads were surveyed in fecal samples over time post-infection (p.i.) as indicated (**A–F**) by culture and expressed as colony-forming units per gram feces (CFU/g). The box plots representing the 25th and 75th percentiles of the median (bar within box), the total range, and the significance levels (*P* values) determined by the Kruskal-Wallis test with Dunn's post-hoc test are given. Shown data were pooled from 3 independent experiments

indicative for a strong CR against the enteropathogens. Conversely, A/S pretreated mice were stably colonized by *C. jejuni* as indicated by high median fecal pathogen loads of approximately 10^9 CFU per g feces (Fig. 2B). In mice that had been pretreated with CIP or VAN, however, approximately 4 orders of magnitude lower fecal *C. jejuni* numbers (i.e., medians of approximately 10^6 CFU/g) were detected on days 2 and 3 p.i., whereas between days 7 and 12 p.i., median bacteria numbers of between 10^4 CFU/g and 10^5 CFU/g were observed in the feces of respectively pretreated mice (Fig. 2C and D). Notably, feces of single mice from the CIP (33.3%) and the VAN (10%) cohorts were completely *C. jejuni* culture-negative at the end of the experiment (Fig. 2C and D).

Fecal pathogen loads at defined time points post *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment

When comparing *C. jejuni* loads between respective pretreatment groups at defined time points (Fig. 3) it turned out that between days 2 and 9 p.i., pathogen numbers were higher in fecal samples taken from A/S and VAN ($P < 0.05$–0.001), but not CIP pretreated mice (not significant (n.s.)) if compared to untreated but infected SPF controls (Fig. 3A–E). At the end of the experiment, fecal *C. jejuni* numbers did not differ between mice from the SPF, CIP, and VAN groups in the multivariate analysis (n.s.; Fig. 3F). Notably, whereas all SPF mice were *C. jejuni* culture-negative, the enteropathogens could be isolated from the feces of individual mice from the CIP and VAN cohorts at day 12 p.i.

Furthermore, both, CIP and VAN pretreated mice harbored lower *C. jejuni* numbers in their feces if compared to mice from the A/S cohort on days 3, 9, and 12 p.i. ($P < 0.05$–0.01; Fig. 3B, E and F), which also held true for mice from the CIP ($P < 0.01$), but not VAN cohorts (n.s. due to high standard deviations) on days 2, 5, and 7 p.i. (Fig. 3A, C and D).

Upon necropsy we isolated *C. jejuni* from the terminal ileum and colon lumen and found higher *C. jejuni* counts in respective intestinal samples derived from the A/S as compared to the CIP ($P < 0.01$) and VAN ($P < 0.05$) pretreated mice which also held true upon comparison with the untreated, but infected SPF controls ($P < 0.001$; Fig. 4). Notably, not only in the feces, but also in the terminal ileum and colon, the *C. jejuni* counts did not differ between mice from the CIP, VAN, and SPF groups on day 12 p.i. (n.s.; Fig. 4), whereas all SPF mice remained *C. jejuni* culture-negative. Collectively, mice without antibiotic pretreatment were protected from oral *C. jejuni* infection, whereas pretreatment with A/S compromised the physiological CR most prominently as indicated by highest fecal enteropathogen loads until the end of the observation period.

Apoptotic and immune cell responses in the colon upon *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment

Then, we addressed the impact of *C. jejuni* infection in antibiotics pretreated mice on pro-inflammatory immune

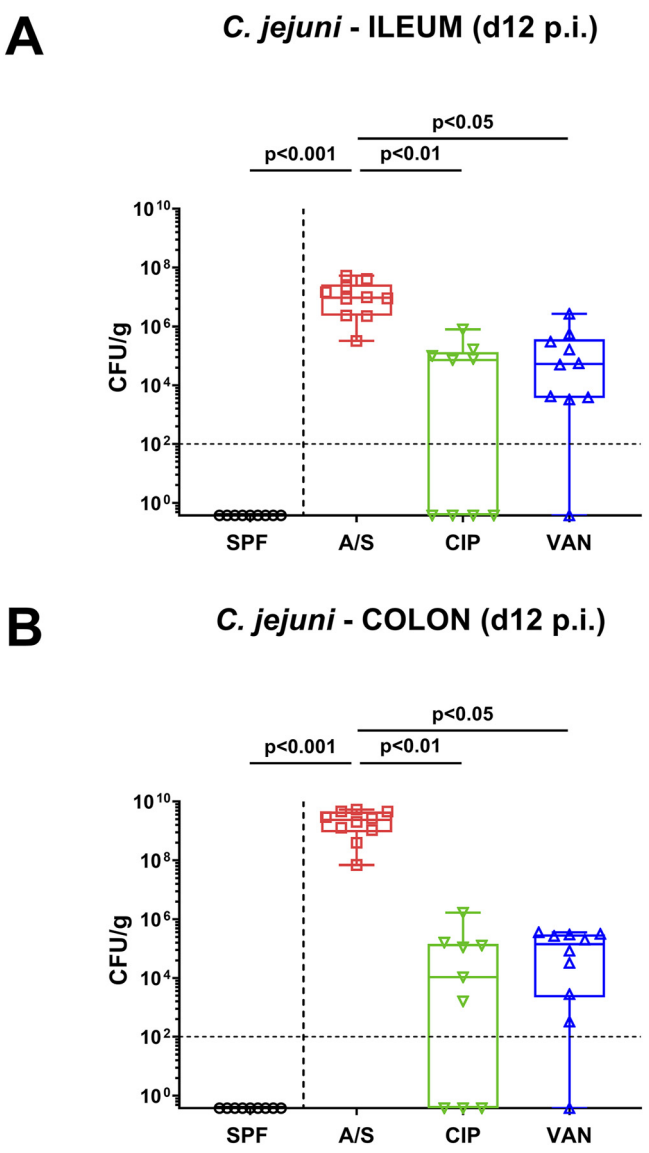

Fig. 4. **Ileal and colonic pathogen loads post *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment**
Conventional C57BL/6j mice harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S; red squares), ciprofloxacin (CIP; green inverted triangles) or vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 until d-2) or remained untreated (SPF; black circles). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain. Following necropsy on day 12 post-infection (p.i.), the pathogen loads were analyzed in luminal samples derived from the **(A)** terminal ileum and **(B)** colon by culture and expressed as colony-forming units per gram feces (CFU/g). The box plots indicating the 25th and 75th percentiles of the median (bar within the box), the total range, and the significance levels (*P* values) determined by the Kruskal-Wallis test with Dunn's post-hoc test are given. Shown data were pooled from 3 independent experiments

responses in the colon. Whereas no overt clinical including macroscopic intestinal sequelae of oral pathogenic challenge of mice could be observed (data not shown), A/S pretreated

mice displayed higher numbers of apoptotic colonic epithelial cells on day 12 p.i. when compared to mice from the CIP, VAN, and SPF cohorts ($P < 0.01$–0.001; Fig. 5A). Interestingly, apoptotic cell numbers were higher in the colonic epithelia of CIP ($P < 0.05$), but not VAN versus SPF mice (n.s.; Fig. 5A). In addition, we determined adaptive immune cell responses in the intestinal tract upon oral enteropathogen application applying quantitative *in situ* immunohistochemistry. In all antibiotic pretreatment groups, we found *C. jejuni* induced increases in $CD3^+$ T lymphocyte numbers in the colonic mucosa and lamina propria on day 12 p.i. if compared to untreated, but infected SPF counterparts ($P < 0.05$–0.001; Fig. 5B). These increases were, however, less pronounced in CIP and VAN versus A/S pretreated mice ($P < 0.001$; Fig. 5B). Moreover, mice from the A/S cohort displayed higher $FOXP3^+$ Treg numbers in their colonic mucosa and lamina propria as compared to CIP, VAN, and SPF mice ($P < 0.05$–0.001), whereas Treg counts did not differ between the latter three groups (n.s.; Fig. 5C). Hence, A/S pretreatment did not only result in highest intestinal *C. jejuni* burdens, but also in most pronounced apoptotic and distinct adaptive immune cell responses in the colon.

Colonic gelatinase and TIMP expression upon *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment

Given that intestinal inflammation is accompanied by cellular matrix turnover, we measured the expression of distinct matrix-degrading gelatinases and counter-regulatory Timp molecules. On day 12 p.i., both, MMP2 and MMP9 mRNA expression levels were higher in *ex vivo* colonic biopsies taken from A/S as compared to VAN pretreated mice ($P < 0.001$) and to untreated SPF counterparts ($P < 0.01$–0.001; Fig. 6A and B). Furthermore, colonic Timp1 mRNA levels were higher in A/S as compared to the other three cohorts on day 12 p.i. ($P < 0.05$–0.001; Fig. 6C). In case of colonic Timp3 expression, higher mRNA values were obtained for A/S versus VAN pretreated mice only ($P < 0.001$; Fig. 6D). Hence, A/S pretreated mice displayed the most prominently enhanced gelatinase and Timp mRNA expression in the infected colon.

Fecal microbiota composition in mice following differential antibiotic pretreatment and immediately before *C. jejuni* infection

Furthermore, we addressed the impact of the antibiotics induced gut microbiota shifts rendering the mice susceptible for *C. jejuni* infection. Therefore, we performed a comprehensive survey of the fecal microbiota composition both, immediately before *C. jejuni* infection on day 0 (Fig. 7) and at the end of the experiment on day 12 p.i. (Fig. 8). Our quantitative molecular analyses revealed that if compared to untreated SPF controls with a preserved physiological CR, A/S mice harbored lower total bacterial gene numbers in their feces ($P < 0.01$; Fig. 7A) due to decreased enterobacteria ($P < 0.05$; Fig. 7B), lactobacilli ($P < 0.05$; Fig. 7D), *Bacteroides/Prevotella* spp. ($P < 0.001$; Fig. 7F), *Clostridium coccoides* ($P < 0.01$; Fig. 7G), *Clostridium leptum* ($P < 0.01$; Fig. 7H), and Mouse Intestinal Bacteroides ($P < 0.001$; Fig. 7I) as analyzed on day 0. In case of VAN pretreatment, fecal microbiota shifts were less pronounced given that particularly gene numbers of obligate anaerobic bacteria such as *C. coccoides* ($P < 0.001$; Fig. 7G), *C. leptum* ($P < 0.01$;

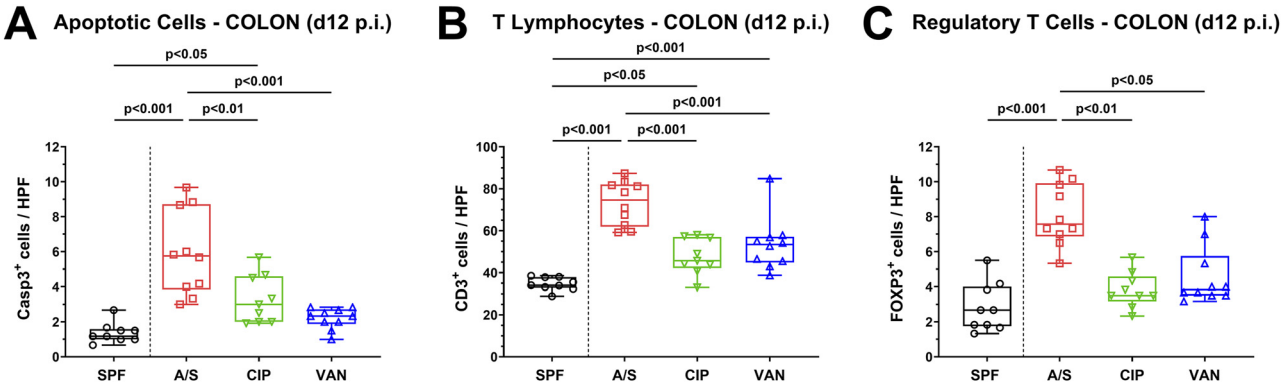

Fig. 5. **Apoptotic and immune cell responses in the colon upon *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment**

Conventional C57BL/6j mice harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S; red squares), ciprofloxacin (CIP; green inverted triangles) or vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 until d-2) or remained untreated (SPF; black circles). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain. Following necropsy on day 12 post-infection (p.i.), colonic paraffin sections were stained with antibodies against **(A)** cleaved caspase-3 (Casp3), **(B)** CD3, and **(C)** FOXP3 in order to determine the average numbers of apoptotic colonic epithelial cells, T lymphocytes, and regulatory T cells, respectively, out of six high power fields (HPF, 400× magnification, light microscopy) per mouse. The box plots indicating the 25th and 75th percentiles of the median (bar within box), the total range, and the significance levels (P values) determined by the one-sided ANOVA with Tukey post-hoc test (A,B) or the Kruskal-Wallis test with Dunn's post-hoc test (C) are indicated

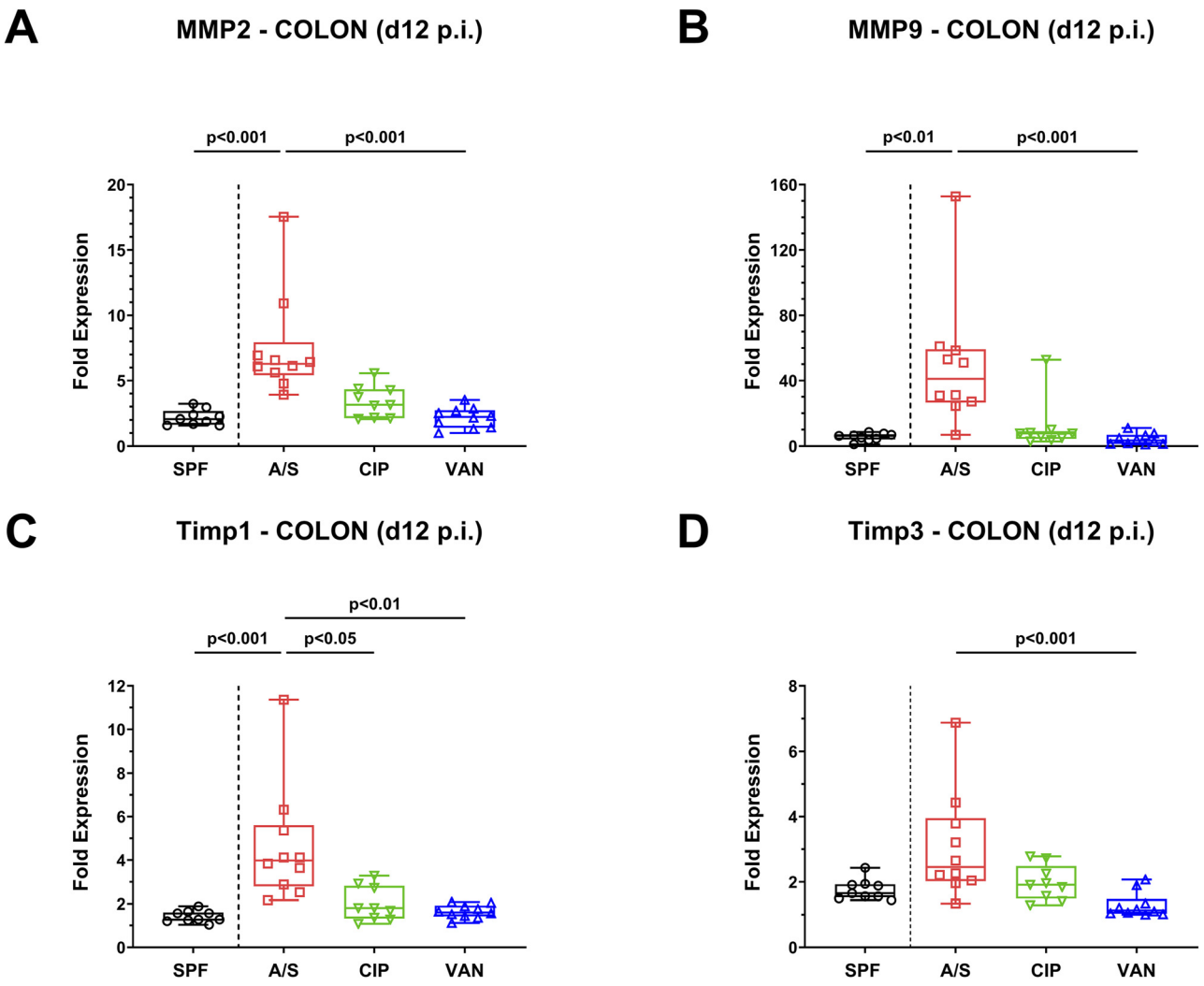

Fig. 6. **Colonic MMP and TIMP expression upon *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment** Conventional C57BL/6j mice harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S; red squares), ciprofloxacin (CIP; green inverted triangles) or vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 until d-2) or remained untreated (SPF; black circles). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain. Following necropsy on day 12 post-infection (p.i.), expression levels of **(A)** MMP2, **(B)** MMP9, **(C)** Timp1, and **(D)** Timp3 were measured in colonic *ex vivo* biopsies and expressed as arbitrary units (fold expression). Box plots indicating the 25th and 75th percentiles of the median (bar within the box), the total range, and the significance levels (*P* values) determined by the Kruskal-Wallis test with Dunn's post-hoc test are given. Shown data were pooled from 3 independent experiments

Fig. 7H), and Mouse Intestinal Bacteroides ($P < 0.001$; Fig. 7I) were lower in VAN as compared to untreated mice before *C. jejuni* infection, whereas at least a trend towards lower fecal *Bacteroides/Prevotella* spp. loads could be assessed (n.s. in the multi-variate analysis; Fig. 7F). Notably, VAN pretreated mice exhibited approximately 4 orders of magnitude higher median enterobacterial gene numbers in their feces in comparison to SPF counterparts, but the difference did not reach statistical significance in the multi-variate analysis (n.s; Fig. 7B). Remarkably, following CIP pretreatment only higher enterococci gene copies were measured in the feces on day 0 as compared to SPF counterparts ($P < 0.01$; Fig. 7C). Hence, mice that had been subjected to A/S pretreatment displayed the most prominent gut microbiota changes immediately before *C. jejuni* infection, whereas VAN and particularly CIP only resulted in moderate to rather subtle fecal microbiota changes if compared to untreated SPF controls as summarized in Fig. 9.

Fecal microbiota composition on day 12 post *C. jejuni* infection of mice that had been subjected to differential antibiotic pretreatment

Furthermore, we surveyed the fecal microbiota in *C. jejuni* infected mice from all cohorts at the end of the experiment (Fig. 8). Again, in the A/S pretreatment group, the most distinct differences in fecal bacterial gene numbers were

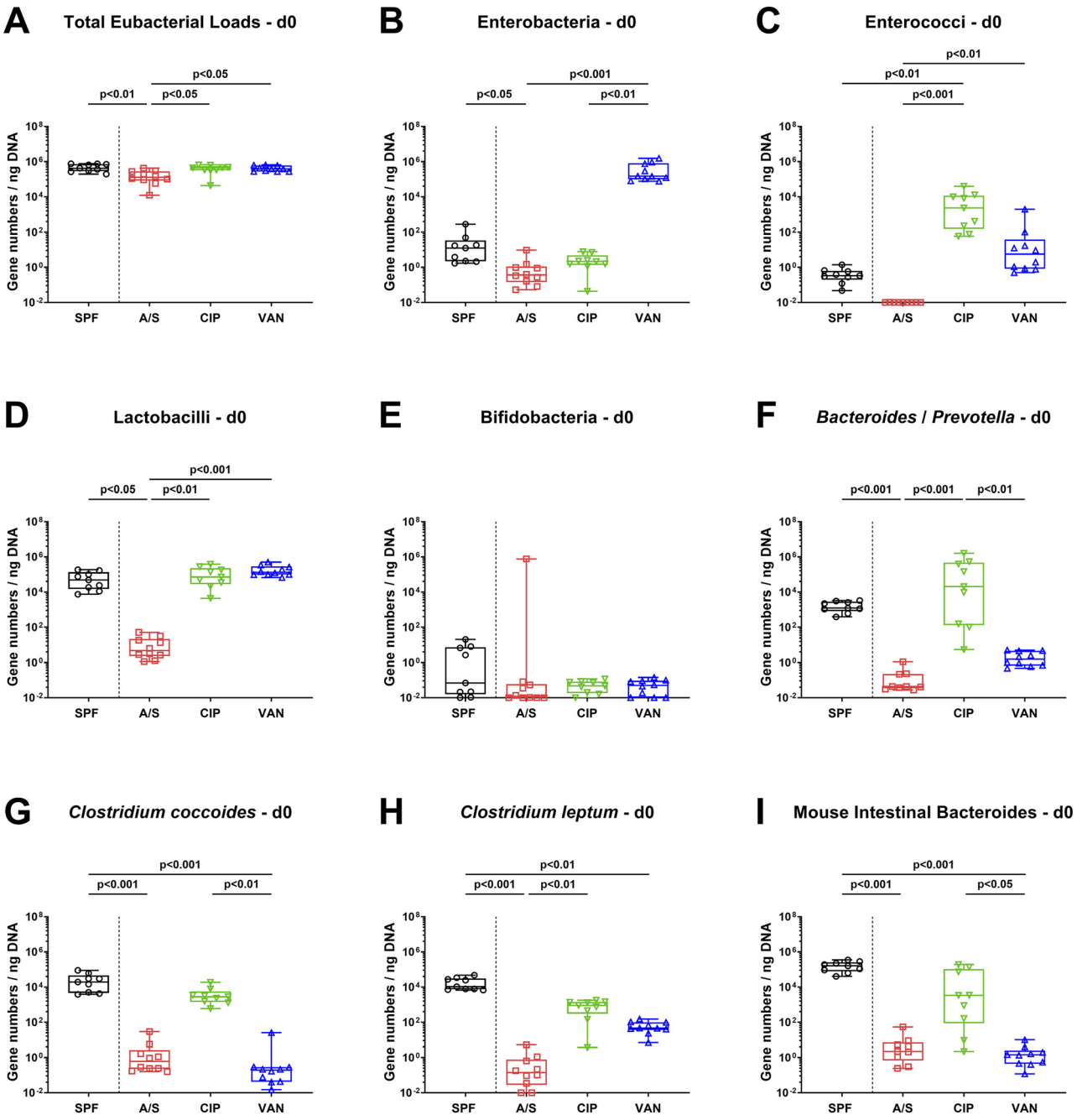

Fig. 7. **Fecal microbiota composition in mice following differential antibiotic pretreatment and immediately before *C. jejuni* infection** Conventional C57BL/6j mice (naive) harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S; red squares), ciprofloxacin (CIP; green inverted triangels) or vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 until d-2) or remained untreated (SPF; black circles). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). Immediately before *C. jejuni* 81-176 strain infection on d0, the gut microbiota composition was surveyed in fecal samples applying culture-independent (16S rRNA-based molecular) methodology (see methods). Individual gene copy numbers per ng DNA are given for **(A)** total eubacterial loads, **(B)** enterobacteria, **(C)** enterococci, **(D)** lactobacilli, **(E)** bifidobacteria, **(F)** *Bacteroides/Prevotella* species, **(G)** *Clostridium coccoides* group, **(H)** *Clostridium leptum* group, and **(I)** Mouse Intestinal Bacteroides. Box plots indicating the 25th and 75th percentiles of the median (bar within the box), the total range, and the significance levels (*P* values) determined by the one-sided ANOVA with Tukey post-hoc test (A) or the Kruskal-Wallis test with Dunn's post-hoc test (B–I) are indicated. Shown data were pooled from 3 independent experiments

detected on day 12 p.i. if compared to infected SPF counterparts given fewer gene copies for total eubacterial loads ($P < 0.001$; Fig. 8A), enterobacteria ($P < 0.01$; Fig. 8B), enterococci ($P < 0.05$; Fig. 8C), lactobacilli ($P < 0.001$; Fig. 8D), *Bacteroides/Prevotella* spp. ($P < 0.01$; Fig. 8F), *C. leptum* ($P < 0.01$; Fig. 8H), and Mouse Intestinal Bacteroides (MIB; $P < 0.01$; Fig. 8I), whereas *C. coccoides* gene numbers were higher versus SPF controls ($P < 0.01$; Fig. 8G).

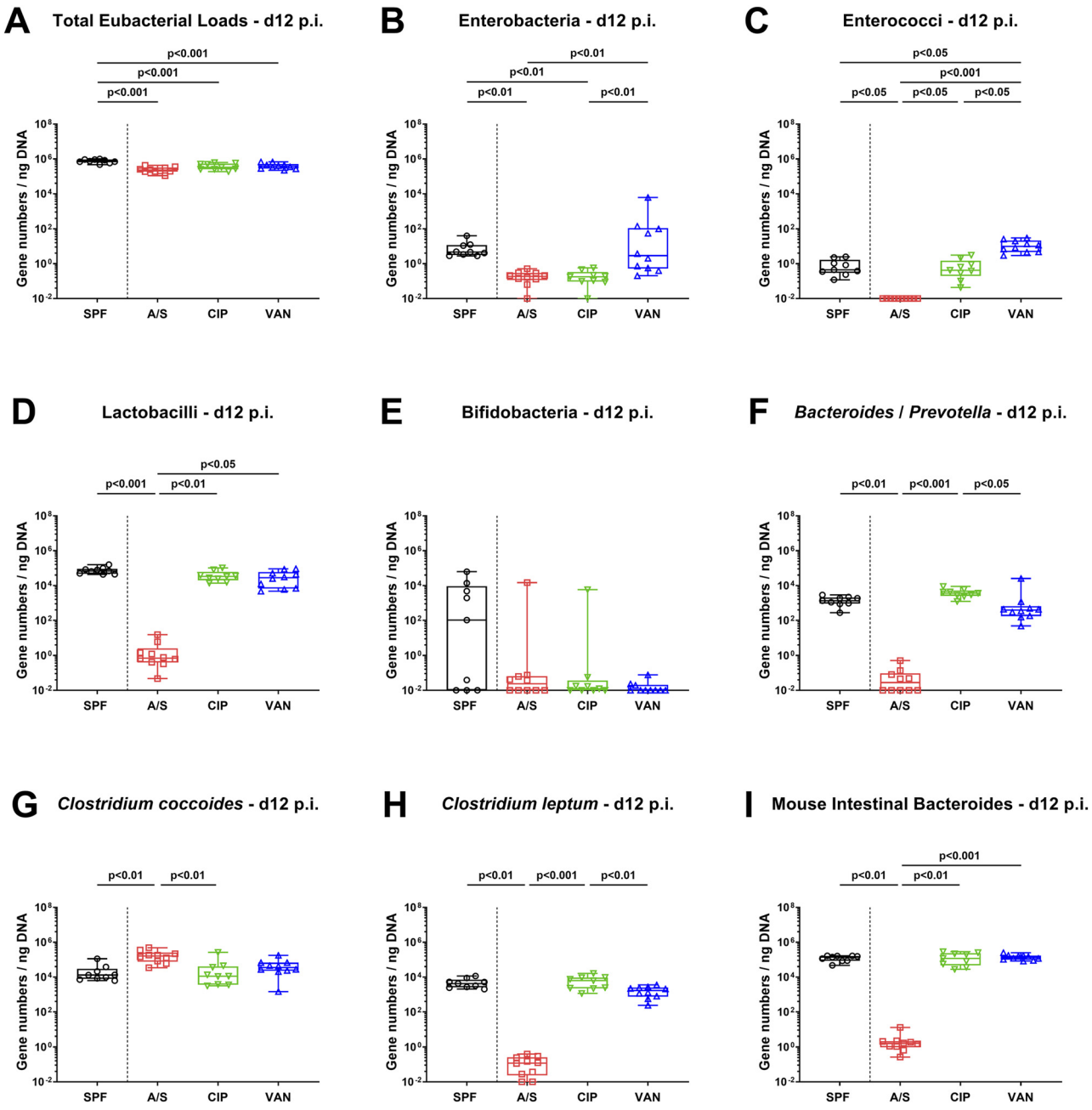

Fig. 8. **Fecal microbiota composition of mice that had been subjected to differential antibiotic pretreatment 12 days post *C. jejuni* infection** Conventional C57BL/6j mice (naive) harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S; red squares), ciprofloxacin (CIP; green inverted triangels) or vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 until d-2) or remained untreated (SPF; black circles). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain. Upon sacrifice on day 12 post-infection (d12 p.i.), the gut microbiota composition was surveyed in fecal samples applying culture-independent (16S rRNA-based molecular) methodology. Individual gene copy numbers per ng DNA are given for **(A)** total eubacterial loads, **(B)** enterobacteria, **(C)** enterococci, **(D)** lactobacilli, **(E)** bifidobacteria, **(F)** *Bacteroides/Prevotella* species, **(G)** *Clostridium coccoides* group, **(H)** *Clostridium leptum* group, and **(I)** Mouse Intestinal Bacteroides. Box plots indicating the 25th and 75th percentiles of the median (bar within the box), the total range, and the significance levels (*P* values) determined by the one-sided ANOVA with Tukey post-hoc test (A,H) or the Kruskal-Wallis test with Dunn's post-hoc test (B–G, I) are indicated. Shown data were pooled from 3 independent experiments

C. jejuni infected mice from the VAN cohort, however, displayed more enterococci gene copies ($P < 0.05$; Fig. 8C), whereas CIP pretreated mice exhibited lower enterobacterial copy numbers in their feces on day 12 p.i. if compared to untreated and infected SPF control animals ($P < 0.01$; Fig. 8B). Hence, the antibiotics-induced differences in numbers of distinct commensal gut bacterial genes on day 12 post *C. jejuni* infection were most prominent following A/S pretreatment, but only minor upon VAN or CIP challenge as summarized in Figs 9 and 10).

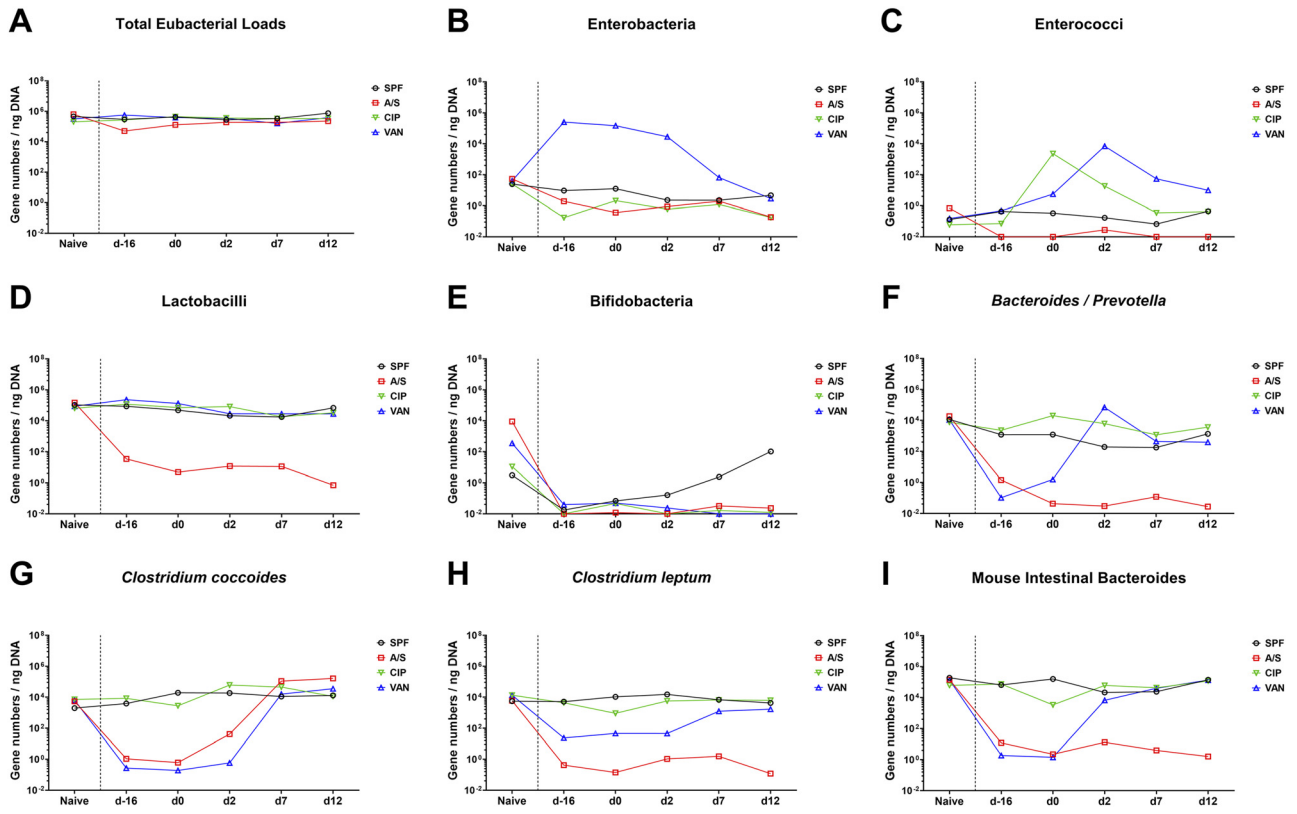

Fig. 9. **Overview of fecal microbial groups over time post *C. jejuni* infection**
Conventional C57BL/6j mice (naive) harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S; red squares), ciprofloxacin (CIP; green inverted triangels) or vancomycin (VAN; blue triangles) for 4 weeks (from day (d)-30 (i.e., naive) until d-2) or remained untreated (SPF; black circles). Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain. Commensal gut microbial groups were surveyed in fecal samples over time before and after infection applying culture-independent (16S rRNA-based molecular) methodology. The medians of gene copy numbers per ng DNA are given for **(A)** total eubacterial loads, **(B)** enterobacteria, **(C)** enterococci, **(D)** lactobacilli, **(E)** bifidobacteria, **(F)** *Bacteroides/Prevotella* species, **(G)** *Clostridium coccoides* group, **(H)** *Clostridium leptum* group, and **(I)** Mouse Intestinal Bacteroides. Shown data were pooled from 3 independent experiments

DISCUSSION

The complex commensal gut microbiota provides a protective shield against invading pathogens potentially causing harm to the host [14]. In our present study we addressed to what extent oral pretreatment of mice with antibiotic agents from different classes and with different antimicrobial spectra that are commonly used in medical practice rendered mice susceptible to oral *C. jejuni* infection. Our results revealed that i.) antibiotic pretreatment abrogated CR that was most prominent following A/S challenge given highest fecal *C. jejuni* loads throughout the observation period p.i. that were accompanied by ii.) most pronounced apoptotic and distinct adaptive immune cell responses, and iii.) most prominently enhanced expression of cellular matrix-degrading gelatinases in the colon. iv.) Importantly, mice that had been subjected to A/S pretreatment displayed the most prominent gut microbiota changes immediately before *C. jejuni* infection, whereas VAN and particularly CIP only resulted in moderate to rather subtle fecal microbiota changes if compared to untreated SPF controls (as summarized in Figs 9 and 10).

Antibiotic-induced dysbiosis represents a significant disruption to the intestinal microbial ecosystem, profoundly impacting CR. This was evident by the strong contrast in fecal *C. jejuni* shedding observed over time between the different groups. As expected, SPF mice, which maintained an intact gut microbiota, exhibited robust CR, effectively clearing *C. jejuni* within 48 h after infection and maintaining undetectable levels of the pathogen throughout the experimental period. This finding aligns with previous observations of CR not only against *C. jejuni* but also against other bacterial enteropathogens [11, 14]. In our study, we observed varying degrees of CR against *C. jejuni* across the distinct antibiotic regimens used to pretreat the experimental groups. A/S pretreated mice exhibited the most profound loss of CR, as evidenced by persistently high and consistent fecal pathogen shedding throughout the experiment.

In our study, A/S pretreatment induced the highest shifts in the gut microbiota communities, which was mirrored by the highest susceptibility to *C. jejuni* colonization. This was reflected by both, the high pathogen densities and pronounced immune response observed in this group. The severe depletion of bacterial groups likely contributed

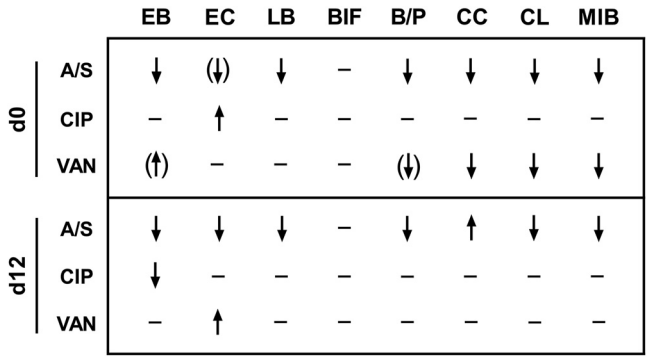

Fig. 10. **Summary of fecal microbiota compositions in mice pretreated with different antibiotics immediately before *C. jejuni* infection and at the end of the experiment**
Conventional C57BL/6j mice (naive) harboring a specific pathogen free (SPF) gut microbiota were pretreated with different antibiotics such as ampicillin plus sulbactam (A/S), ciprofloxacin (CIP) or vancomycin (VAN) for 4 weeks (from day (d)-30 until d-2) or remained untreated. Two days prior infection, the antibiotics were replaced by sterile tap water (d-2). On d0 and d1, mice were perorally infected with *C. jejuni* 81-176 strain and sacrifized on day 12 post-infection. The fecal microbiota compositions were surveyed immediately before infection (d0) and at the end of the experimental period (i.e., d12) applying culture-independent (16S rRNA-based molecular) methodology assessing enterobacteria (EB), enterococci (EC), lactobacilli (LB), bifidobacteria (BIF), *Bacteroides/Prevotella* species (B/P), *Clostridium coccoides* group (CC), *Clostridium leptum* group (CL), and Mouse Intestinal Bacteroides (MIB). Arrows indicated changes if compared to SPF counterparts: higher; (trend towards higher); lower, (trend towards lower); -: unchanged

to the availability of ecological niches and nutrients required for *C. jejuni*'s catabolic functions, such as amino acids that are the primary carbon sources for this pathogen [14, 47]. On the other hand, CIP and VAN pretreatment had a less pronounced impact on gut microbial composition, which may explain the intermediate CR phenotype observed in these groups. Notably, beneficial bacterial groups such as the lactobacilli were preserved in CIP and VAN treated mice. Lactobacilli have been strongly implicated in CR against *C. jejuni* as they are known to exert protective effects [14]. These protective properties, exerted via competing for adhesion sites, producing antimicrobial metabolites, and modulating immune responses, are consistent with previous studies reporting lactobacilli to have potent protective effects against *C. jejuni* infections [48–50], as well as infections caused by other enteropathogens, including *Salmonella*, *E. coli* and *L. monocytogenes* [51–53].

Apart from lactobacilli, the MIB group appears to play a role in CR. In fact, in a previous study, the variable degree of CR observed among *Citrobacter rodentium* infected SPF mice was attributed to differences in the relative abundance of MIB [54]. Interestingly, in our study, MIB were preserved in CIP as opposed to VAN pretreated mice, but reemerged in the latter, whereas the bacteria were almost completely abrogated in the A/S group. The absence of MIB in A/S pretreated mice may at least partially explain the heightened susceptibility to *C. jejuni* colonization compared to the intermediate CR observed in the CIP and VAN groups.

The preservation of key microbial groups in CIP pretreated mice aligns with prior studies reporting that CIP causes less pronounced disruptions to gut microbial diversity and composition [55]. In our study, we observed that lactobacilli and *C. leptum* of the gut microbiota remained largely unaffected which is consistent with earlier studies highlighting their resilience to CIP treatment [56]. These bacterial groups have been reported to play important roles in preventing pathogen infections, including *Clostridioides difficile* infections [57, 58].

In the case of VAN, despite not having a direct antimicrobial activity against obligate anaerobic bacteria, we here observed a decline in both, anaerobic Gram-negative (i.e., *Bacteroides/Prevotella* spp. and MIB) and Gram-positive groups (particularly *C. coccoides*) two days after cessation of antibiotic pretreatment (i.e., day 0 and immediately before *C. jejuni* infection) if compared to untreated SPF controls. The observed decline may represent a secondary effect driven by changes in the intraluminal milieu, which likely reversed within 12 days p.i. Notably, we detected a temporary blooming of enterobacteria in the feces at the end of the VAN pretreatment which is in line with a previous study showing enterobacterial growth following VAN-induced dysbiosis [59].

The lack of CR in A/S treated mice also correlated with the pronounced T cell and Treg responses and the most pronounced upregulation of matrix-degrading gelatinases and their counter-regulatory Timps in the infected colon. The increase in Tregs, alongside the elevated Timp expression, suggest an active attempt to mitigate inflammatory tissue damage in the colon. The enhanced pro-inflammatory response in the colon of A/S pretreated *C. jejuni* infected mice was further characterized by the higher upregulation of MMP9, known to be associated with increased activation of the TLR4-driven NF-kB pathway, a hallmark of *C. jejuni*-induced enterocolitis [60, 61], whereas an upregulated expression of MMPs and related Timps is a recognized feature of the immunopathogenesis of enterocolitis [61, 62]. These findings indicate heightened epithelial damage and tissue remodeling in the colon of A/S pretreated mice, a phenotype not observed in CIP and VAN treated mice, as reflected by their lower pathogen burdens and less pronounced inflammatory responses compared to the resistant SPF mice.

CONCLUSIONS AND OUTLOOK

Collectively, our findings support A/S pretreated mice as very well suited *C. jejuni* infection model and highlight the importance of the species-specific microbiota composition in maintaining CR against *C. jejuni*. The differential effects of commonly applied antibiotics from different classes (like A/S, VAN, and CIP in our study) on microbiota community structure further emphasizes the critical role of distinct gut microbial groups such as lactobacilli and

C. leptum in preventing pathogen colonization and immune dysregulation, and adds another layer of complexity into the mechanisms associated with CR and the gut microbiota's functions in general. The precise mechanisms underlying these interactions between antibiotics, the gut microbiota, and host immunity still need further exploration, however. Future studies should employ metagenomic and metabolomic analyses to comprehensively characterize not only the shifts in the community structure, but also the functional shifts in response to antibiotics. Additionally, the effects of antibiotics on immune cell profiles and differentiation also need further investigation. These approaches would pave the way for identifying specific metabolic pathways and metabolites that are disrupted as a result of antibiotic-induced dysbiosis and their impact on pathogen colonization. Moreover, longitudinal analyses of these changes over time could provide insights into the interplay between microbial recovery and immune reprogramming, and thus offer potential targets for microbiota-based therapeutic interventions.

Ethics statement: All animal experiments were carried out according to the European animal welfare guidelines (2010/63/EU) following approval by the commission for animal experiments ("Landesamt für Gesundheit und Soziales", LaGeSo, Berlin). The clinical conditions of mice were monitored daily.

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Authors' contributions: NWS: Analyzed data, wrote the paper.

SM: Analyzed data, edited the paper.

KS: Provided advice in experimental design, critically discussed results, edited the paper.

SB: Provided advice in experimental design, critically discussed results, edited the paper.

MMH: Designed and performed experiments, analyzed data, co-wrote the paper.

Conflict of interests: SB and MMH are members of the Editorial Board of the journal, therefore they did not take part in the review process in any capacity and the submission was handled by a different member of the editorial board. The submission was subject to the same process as any other manuscript and editorial board membership had no influence on editorial consideration and the final decision.

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