European Journal of Microbiology and Immunology 1 (2011) 3, pp. 237–248
DOI: 10.1556/EuJMI.1.2011.3.8

WHAT YOU EAT IS WHAT YOU GET: NOVEL *CAMPYLOBACTER* MODELS IN THE QUADRANGLE RELATIONSHIP BETWEEN NUTRITION, OBESITY, MICROBIOTA AND SUSCEPTIBILITY TO INFECTION

S. Bereswill[1], R. Plickert[1], A. Fischer[1], A. A. Kühl[2], C. Loddenkemper[2], A. Batra[3], B. Siegmund[3], U. B. Göbel[1] and M. M. Heimesaat[1]*

[1]Department of Microbiology and Hygiene, Charité – University Medicine Berlin, Berlin, Germany
[2]Department of Pathology/ Research Center ImmunoSciences (RCIS), Charité – University Medicine Berlin, Berlin, Germany
[3]Department of Internal Medicine, Charité – University Medicine Berlin, Berlin, Germany

Received: June 29, 2011; Accepted: July 8, 2011

Enterocolitis caused by *Campylobacter jejuni*-infections represents an important socioeconomic burden worldwide. Recent results from novel murine infection models reveal that the intestinal microbiota is essential for maintaining colonization resistance against *C. jejuni*. We extended these studies to investigate the role of nutrition and obesity in susceptibility to *C. jejuni*-infection. Gnotobiotic (GB) mice generated by antibiotic treatment, which were fed with a human cafeteria diet (CAF), as well as obese (*ob/ob*) mice with a conventional microbiota harbored higher *Escherichia coli* loads in their colon as compared to respective controls. Following oral infection, *C. jejuni* 43431 ATCC readily colonized the intestines of CAF and *ob/ob* mice, whereas GB mice fed with a standard chow (MUD) eradicated the pathogen within days. Furthermore, live *C. jejuni* translocated into mesenteric lymph nodes of CAF, but not MUD mice. Strikingly, stably infected animals developed enterocolitis as indicated by increased numbers of immune and apoptotic cells in the colon *in situ*.

We conclude that a specific human diet and obesity render mice susceptible to *C. jejuni* infection. The corresponding murine models are excellently suited for the study of *C. jejuni* pathogenesis and will help to get further insights into interplays between *C. jejuni*, microbiota, diet, obesity and immunity.

Keywords: *Campylobacter jejuni*, gnotobiotic mice, colonization resistance, nutrition, cafeteria diet, obesity, *ob/ob* mice, human microbiota, host–pathogen interaction, innate immunity

Introduction

The Gram-negative bacterial pathogen *Campylobacter jejuni* is transmitted via the food chain from farm animals to humans [1, 2]. Infected patients present with abdominal pain, fever, myalgia, and watery or bloody diarrhea. The acute stage of *C. jejuni* enteritis is characterized histologically by crypt abscesses, ulcerations, and proliferation of immune cells in the colon *in situ* [1, 3–5]. The vast majority of infections is self-limited and not followed by further complications. However, in rare cases *C. jejuni* infection triggers chronic sequelae such as Reiter's syndrome, reactive polyarthropathy, and Guillain–Barré syndrome [1, 6].

Virulence factors, pathogenicity, and epidemiology of *C. jejuni* have been intensively investigated since the discovery of the disease in the 1970s [7]. Mechanisms mediating intestinal colonization resistance and the role of nutrition in susceptibility to *C. jejuni* infection, however, are not well understood. In addition, immunopathological responses within the vertebrate colon following *C. jejuni* infection are still poorly investigated. These limitations are mainly due to the scarcity of suitable experimental *in vivo* models [8]. Whereas mice are highly convenient for the study of colonization capacity of bacterial pathogens and can help to overcome the limitations in studying immunopathology, murine infection models have the disadvantage of sporadic colonization and/or absence of disease manifestations [1, 8, 9]. This is due to the fact that conventional mice with a normal intestinal microbiota are highly resistant to *C. jejuni* colonization. Very recent work from our group revealed that gnotobiotic (GB) mice and GB mice harboring a modified microflora are well suited for the study of *C. jejuni* infection [9]. GB mice gener-

*Corresponding author: Markus M. Heimesaat; Charité – University Medicine Berlin, CC5, Department of Microbiology and Hygiene, Campus Benjamin Franklin, Hindenburgdamm 27, D-12203 Berlin, Germany; Phone: +49-30-8445-3739; Fax: +49-30-450-524-902; E-mail: markus.heimesaat@charite.de

ISSN 2062-509X / $ 20.00

ated by quintuple antibiotic treatment, GB mice reconstituted with a human flora, and IL-10-deficient mice exerting chronic colitis could be readily infected by *C. jejuni,* and they developed immune as well as inflammatory responses against the pathogen [9–11]. Furthermore, *C. jejuni* was shown to colonize the entire gastrointestinal (GI) tract of isolator-raised germfree (GF) mice and to induce clinical signs of disease including granulocyte infiltrates, bloody diarrhea, and humoral immune responses, reproducibly occurring after infection [12–16]. Given that GF mice, however, are immuno-compromised due to the lack of an intact immune system [17, 18], they do not represent a suitable experimental model of *C. jejuni* infections in humans [9].

In order to further optimize murine models for *C. jejuni* enteritis by mimicking the patho-physiological conditions in humans, we used in this study our GB mouse model in which the intestinal flora was completely abrogated by quintuple antibiotic treatment for 6–8 weeks [9, 19–21]. Given that the physiological intraluminal milieu within the GI tract including the gut bacteria composition is mainly determined by host-specific nutrition, GB mice were fed with a human cafeteria diet (CAF) [22–24]. The results obtained after *C. jejuni* infection prove that the host-specific diet plays a crucial role in colonization resistance against or susceptibility to *C. jejuni* infection. Furthermore, obese (*ob/ob*) mice with a conventional murine microbiota were highly susceptible to *C. jejuni* infection. The fact that *C. jejuni*-infected animals developed characteristic immune responses similar to those seen in humans indicates that GB mice fed with a human diet as well as obese mice with a conventional microbiota represent valuable models for studying *C. jejuni* infection. Thus, the study presented here indicates for the first time that the quadrangle relationship between diet, obesity, intestinal microbiota, and infection is crucial for the establishment of campylobacteriosis in a vertebrate host.

Materials and methods

Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, Germany). Animal welfare was monitored twice daily by assessment of clinical conditions. Fresh human fecal samples for recolonizing GB mice (hfa) were collected from healthy volunteers. Before sample collection, written informed consent was obtained from all volunteers. Since fecal samples were obtained from coworkers of our laboratory and thus outside a clinical environment and used for recolonization of mice only, experiments were exempted from approval by the Charité – Universitätsmedizin ethical committee according to German legacy (§15, Legal Basis for Clinical Trials).

Mice

All animals were bred and maintained in the facilities of the "Forschungsinstitut für Experimentelle Medizin" (FEM, Charité – Universitätsmedizin, Berlin, Germany), under specific-pathogen-free (SPF) conditions. Mice homozygous for the obese spontaneous mutation (Lep^{ob} commonly referred to as *ob* or *ob/ob*; in the KS background) and C57BL/6 (C6) mice were used in the experiments. Age- and sex-matched mice between 10 and 12 weeks of age were used.

Generation of gnotobiotic mice

To eradicate the commensal gut flora, mice were transferred to sterile cages and treated by adding ampicillin (1 g/L; Ratiopharm), vancomycin (500 mg/L; Cell Pharm), ciprofloxacin (200 mg/L; Bayer Vital), imipenem (250 mg/L; MSD), and metronidazole (1 g/L; Fresenius) to the drinking water *ad libitum* for 6–8 weeks as described earlier in detail [9, 19–21].

Generation of gnotobiotic mice with a defined microbiota

For recolonization experiments, *E. coli*, *Lactobacillus johnsonii*, and a mix of *Lactobacillus* spp. (*L. johnsonii*, *L. acidophilus*, *L. delbrückii*, *L. murinus*, *L. reuteri*, and other taxonomically uncharacterized *Lactobacillus* spp.) were isolated from naïve mice, grown, and characterized as described in detail earlier [19]. Four days prior to the recolonization experiments, the antibiotic cocktail was replaced by sterile drinking water. For recolonization, GB mice received the respective strain(s) in a volume of 0.3 mL on three consecutive days by gavage with a load of approximately 10^9 CFU/mL. After further 7 days (to assure proper colonization of the entire GI tract), and right before *C. jejuni* infection, fecal samples from individual animals were taken to quantitate the bacterial loads in serial dilutions on appropriate solid culture media and to rule out secondary contamination.

Fresh human and murine fecal samples free of enteropathogenic bacteria, parasites, and viruses were collected from five individual healthy volunteers and animals, respectively, pooled and dissolved in an equal volume of sterile PBS, aliquoted, and stored at –80 °C until use. For recolonization experiments, aliquots were thawed and bacterial communities quantified by cultural methods [9] before gavage of mice with 0.3 mL of the respective suspension. Between independent experiments, bacterial counts of groups varied by < 0.5 log orders of magnitude.

Cafeteria diet

Mice were given free access to human food (cafeteria diet, CAF; [22–24]) *ad libitum* for 10 weeks. The CAF consisted of typical local specialties from Berlin such as meat balls

("Buletten"), curry sausages ("Currywurst"), Döner Kebab, fried chicken nuggets, pizza, ham, salami, cheese, spaghetti, French fries, walnuts, apple, carrots, paprica, salad, chocolate cookies, crackers, bread, toast bread, and many others. The food was handled under regular, common human hygiene conditions (thus not in a sterile environment, no use of gloves, regular hand hygiene while handling food). In addition, mice could choose between tap water and alcohol-free beer (mostly preferring the latter). Control mice were fed with regular chow (murine diet, MUD) and tap water *ad libitum*.

C. jejuni *infection of mice*

Mice were infected with approximately 10^9 viable CFU of *C. jejuni* strains ATCC 43431 by gavage in a total volume of 0.3 mL PBS (phosphate buffered saline) on three consecutive days as described earlier in detail [9].

Sampling procedures

Mice were sacrificed by isofluran treatment (Abbott, Germany). Cardiac blood and tissue samples from spleen, liver, abdominal fat, mesenteric lymph nodes (MLNs), and GI tract (stomach, duodenum, ileum, and colon) were removed under sterile conditions. GI samples from each mouse were collected in parallel for microbiological and immunohistochemical analyses. For immunohistochemical stainings, colon samples were immediately fixed in 5% formalin and embedded in paraffin, and sections (5 µm) were stained with the respective antibodies as described below.

Immunohistochemistry

In situ immunohistochemical analysis of colon paraffin sections was performed as described previously [9, 25]. Primary antibodies against CD3 (#N1580, Dako, Denmark, dilution 1:10), myeloperoxidase-7 (MPO-7, # A0398, Dako, 1:10000), FOXP-3 (FJK-16s, eBioscience, 1:100), B220 (eBioscience, San Diego, CA, USA, 1:200), and cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:200) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, 400× magnification) were determined light-microscopically by three independent investigators (MMH, RP, AAK).

Quantitative analysis of C. jejuni *translocation*

Live *C. jejuni* were detected in MLNs, spleen, liver, abdominal fat tissue, and cardiac blood by culture as described earlier [9]. In brief, tissues were homogenized in sterile PBS and analyzed in dilution series on karmali agar (Oxoid, Wesel, Germany) in a microaerophilic atmosphere at 37 °C for at least 48 h. Cardiac blood (0.2 mL) was immediately streaked out on karmali agar plates.

Analysis of the intestinal microflora

Cultural analyses and biochemical identification of luminal bacterial communities from stomach, duodenum, ileum, and colon were performed as previously described [19, 20, 26].

Statistical analysis

Mean values, medians, standard deviations, and levels of significance were determined using appropriate tests as indicated (two-tailed Student's *t*-test, Mann–Whitney-*U* test). Two-sided probability (*P*) values ≤ 0.05 were considered significant. All experiments were repeated at least twice.

Results

Association of gnotobiotic mice with distinct bacterial species alone does not affect susceptibility to C. jejuni *infection*

We have recently shown that the host-specific intestinal microbiota plays a key role in maintaining colonization resistance against *C. jejuni*. Conventional mice and GB mice reconstituted with a specific murine intestinal flora were resistant to *C. jejuni* colonization and eradicated the pathogen within two days after oral infection [9]. In contrast, GB animals colonized with a complex human gut microbiota were highly susceptible to *C. jejuni* infection and developed significant immunopathology in the colon. When compared to the murine gut microbiota, the intestinal human flora harbored higher enterobacteria but lower lactobacilli numbers. Thus, we aimed at investigating whether these respective bacterial species might influence colonization resistance against *C. jejuni*. We hypothesized that lactobacilli might be causatively involved in colonization resistance against *C. jejuni*, whereas *E. coli* might be beneficial for establishment of *C. jejuni* in the GI tract and thus counteract colonization resistance. GB mice (generated by quintuple antibiotic treatment for 8 weeks, see methods) were perorally recolonized with approximately 10^9 CFU of *E. coli*, *L. johnsonii*, a lactobacilli mix (consisting of *L. johnsonii*, *L. acidophilus*, *L. delbrückii*, *L. murinus*, *L. reuteri*, and other taxonomically uncharacterized *Lactobacillus* spp. [19]), or a combination of *E. coli* and *L. johnsonii* on three consecutive days (data not shown). Mice recolonized with a conventional murine SPF flora served as negative controls. One week following reconstitution, mice harboring comparable loads of the respective species within the ileum and colon were then orally infected with

C. jejuni ATCC 43431 on three consecutive days. Twelve days following infection (post infection, p.i.), *C. jejuni* ATCC 43431 was found at comparable levels within the entire GI tract (stomach, duodenum, ileum, and colon), with the highest numbers in the colon, irrespective of the bacterial species established in the GB mice *(Fig. 1A–D)*. Control animals with a conventional flora serving as negative controls had eradicated *C. jejuni* ATCC 43431 from their intestines until day 12 *(Fig. 1A–D)*. In addition, *C. jejuni* ATCC 43431 translocation into local lymphoid organs was not prevented by lactobacilli colonization of the GI tract alone, as live *C. jejuni* ATCC 43431 could be detected at comparable levels in MLNs of all recolonized GB mice. Conventional mice with a normal microbiota, however, did not display any translocated *C. jejuni* ATCC 43431 in their MLNs *(Fig. 1E)*.

Taken together, colonization resistance against *C. jejuni* could not be attributed to murine lactobacilli alone and is rather mediated by the complex interplay of bacterial communities in the gut microbiota and/or by distinct physiological conditions of the intraluminal milieu, which need to be further investigated in detail.

Nutrition affects susceptibility to C. jejuni *infection, composition of the gut microbiota, and the intraluminal milieu*

The intraluminal milieu of the GI tract and the composition of the gut microbiota are predominantly influenced by nutrition. Therefore, we investigated whether feeding GB mice with a human "Western-style" diet would induce a

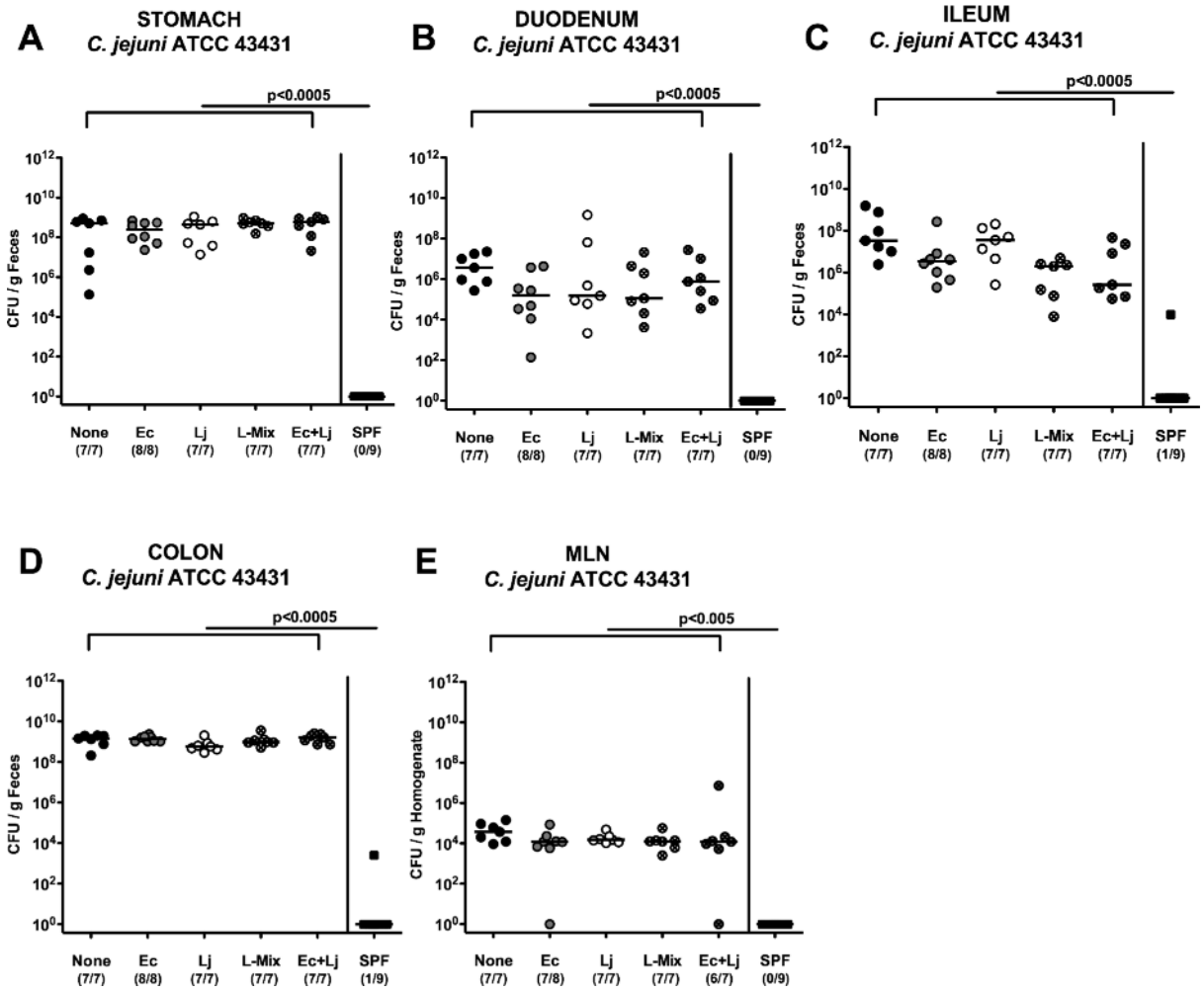

Fig. 1. *C. jejuni* colonization along the gastrointestinal tract and translocation into MLNs of defined recolonized gnotobiotic mice. Gnotobiotic mice were generated by antibiotic gut decontamination, recolonized with *E. coli* (Ec, gray circles), *Lactobacillus johnsonii* (Lj, white circles), a Lactobacilli-mix (L-mix, crossed circles), a mix of *E. coli* and *L. johnsonii* (Ec + Lj, crossed gray circles), sterile PBS (none, solid circles), or a complex conventional murine SPF microbiota (solid squares), and orally infected with *C. jejuni* strain ATCC 43431 as described in the section titled Materials and methods. The pathogen densities in distinct compartments of the gastrointestinal tract (A–D) and mesenteric lymph nodes (MLNs, E) were determined by quantification of live *C. jejuni* in luminal samples taken from (A) stomach, (B) duodenum, (C) ileum, and (D) colon at day 12 p.i. by cultural analysis (CFU, colony forming units). Numbers of animals harboring *C. jejuni* out of the total number of analyzed animals are given in parentheses. Medians (black bars) and levels of significance (*P*-values) determined by Mann–Whitney-*U* test are indicated. Data shown are representative for three independent experiments

human microbiota composition, which renders GB mice susceptible to *C. jejuni* infection. In order to mimick the intraluminal milieu of the GI tract following human nutrition, GB mice were fed with a cafeteria diet (CAF) whereas controls received a standard murine diet (MUD). CAF mice with free access to "human food" *ad libitum* for 10 weeks (for details and handling conditions refer to methods) gained between 40% and 50% of their individual body weights whereas MUD mice did not (< 5% weight gain, data not shown).

After 10 weeks of feeding, CAF and MUD mice were orally infected with *C. jejuni* ATCC 43431. At day 12 p.i., CAF mice harbored *C. jejuni* ATCC 43431 throughout the entire GI tract at high numbers, with the highest counts of 10^9 CFU/g luminal content in the colon, whereas the MUD animals had expelled the pathogen until then *(Fig. 2A)*. In addition, in more than half of the infected mice fed with CAF, but none of MUD animals, live *C. jejuni* ATCC 43431 could be detected in MLNs at day 12 p.i. *(Fig. 2B)*. *C. jejuni* ATCC 43431, however, did not translocate into other organs such as blood, spleen, liver, or abdominal fat of mice, irrespective of the dietary regimen *(Fig. 2B)*.

Impact of nutrition on intestinal microbiota composition

Given that nutrition influences the gut microbiota composition, and the gut microbiota determines the susceptibility to and resistance against *C. jejuni* infection, we next performed a quantitative survey of the intraluminal bacterial species composition of the colon of mice on CAF or MUD by culture and compared the respective loads with those obtained in initially GB mice which had been reconstituted with a complete human microbiota (i.e. human-flora-associated mice, HFA).

Interestingly, CAF mice harbored significantly higher *E. coli* loads (of more than 6 orders of magnitude) in their large intestines as compared to MUD animals, whereas the difference to HFA animals was rather subtle (< 1 order of magnitude; *Fig. 3A*). The enterococci counts in the CAF group, however, were approximately 2 and 3 log orders lower than those in MUD and HFA mice, respectively *(Fig. 3B)*. As expected, MUD mice harbored approximately 2 and 5 orders of magnitude higher *Lactobacillus* spp. loads as compared to CAF and HFA mice *(Fig. 3C)*. Surprisingly, no *Bacteroides/Prevotella* spp. at all could be detected in the colons of mice following CAF, whereas HFA mice displayed higher loads of these obligate anaerobic Gram-negative rods (1 order of magnitude) than mice on MUD *(Fig. 3D)*. Finally, CAF and HFA mice displayed approximately 1.5 orders and < 1 order of magnitude higher counts of *Clostridium/Eubacterium* spp. and total bacterial loads as compared to mice from the MUD group *(Figs. 3E, F)*, respectively. Taken together, 10 weeks following CAF, initially GB mice harbored higher total bacteria loads as well as higher numbers of anaerobic Gram-positive rods and, most pronounced, *E. coli*, but lower *Lactobacillus* spp. counts when compared to SPF

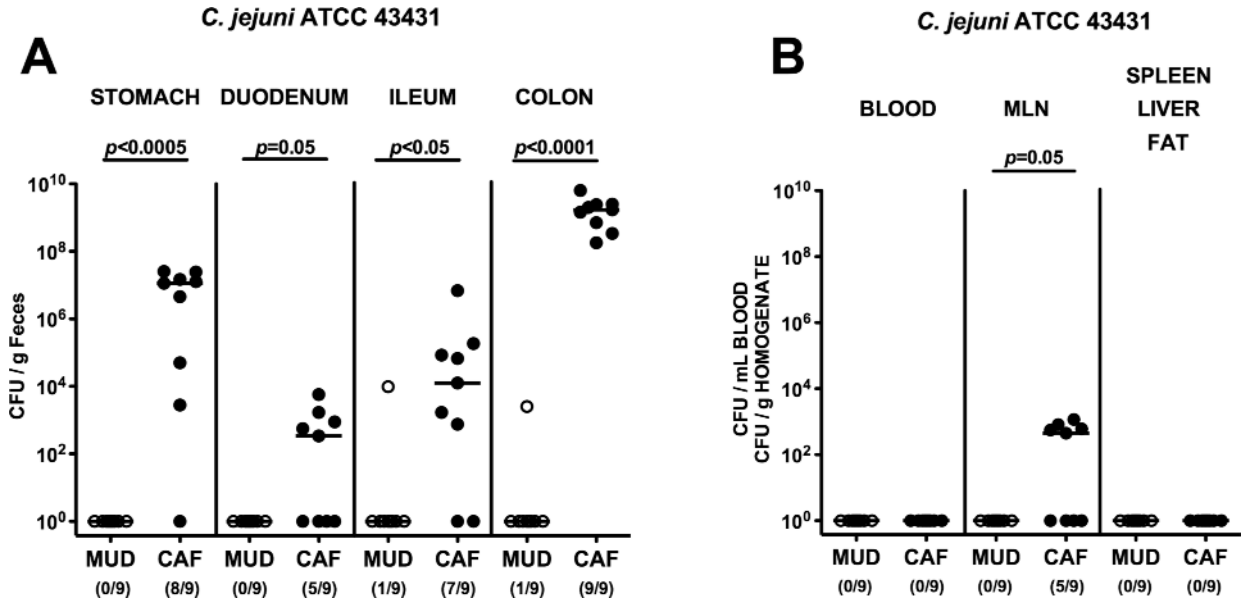

Fig. 2. *C. jejuni* colonization along the gastrointestinal (GI) tract and translocation in mice with cafeteria diet. Gnotobiotic mice were generated by antibiotic gut decontamination, fed either a cafeteria diet (CAF, solid circles) or conventional murine diet (MUD, open circles) for 10 weeks, and orally infected with *C. jejuni* strain ATCC 43431 as described in methods. The pathogen densities in distinct compartments of the GI tract (stomach, duodenum, ileum, and colon; A) or cardiac blood and organs such as mesenteric lymph nodes (MLNs), spleen, liver, and abdominal fat (B) were determined by quantification of live *C. jejuni* in luminal GI samples, blood and organ homogenates, respectively, taken at day 12 p.i. by cultural analysis (CFU, colony forming units). Numbers of animals harboring *C. jejuni* out of the total number of analyzed animals are given in parentheses. Medians (black bars) and levels of significance (*P*-values) determined by Mann–Whitney-*U* test are indicated. Data shown are representative for three independent experiments

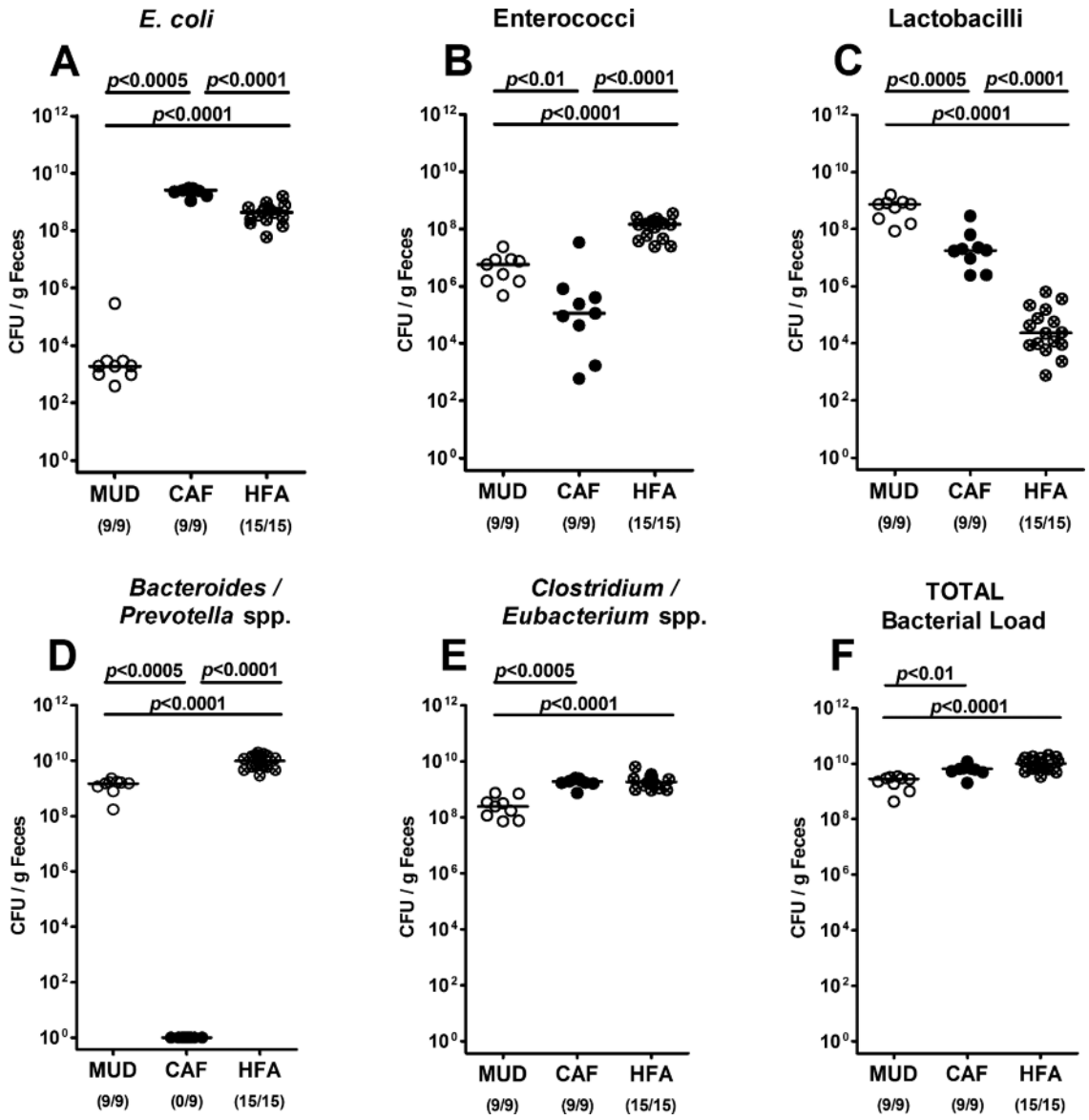

Fig. 3. Bacterial colonization of intestines in mice following different dietary regimens. Gnotobiotic mice were generated by antibiotic gut decontamination, fed either a cafeteria diet (CAF) or conventional murine diet (MUD) for 10 weeks, and compared to gnotobiotic mice harboring a human gut microbiota (HFA) following oral recolonization as described in methods. Numbers of (A) *E. coli*, (B) *Enterococcus* spp., (C) *Lactobacillus* spp., (D) *Bacteroides/Prevotella* spp., (E) *Clostridium/Eubacterium* spp., and (F) total bacterial loads were determined in feces samples of mice right before *C. jejuni* infection (day 0) by detection of colony forming units (CFU) per gram feces on appropriate culture media (see methods). Bacterial species were identified by biochemical analysis and reconfirmed by comparative sequence analyses of 16S rRNA genes. Numbers of animals harboring the respective bacterial species are given in parentheses. Medians and significance levels (*P*-values) determined by Mann–Whitney-*U* test are indicated. Data shown were pooled from three independent experiments

mice on MUD. Most importantly, the gut microbiota composition following CAF was more comparable to the flora of "humanized" animals than to the one displayed by SPF mice on MUD.

C. jejuni *induced intestinal immunopathology in mice following Cafeteria diet*

Given that in humans *C. jejuni* induces the recruitment of pro-inflammatory immune cell populations at sites of inflammation in the large intestine, we next quantitated inflammatory cells as well as immune cell recruitment by immunohistochemical staining of colon paraffin sections of *C. jejuni* ATCC 43431-infected animals with antibodies against caspase-3 (apoptotic cells), MPO7 (neutrophils), CD3 (T-lymphocytes), FOXP3 (regulatory T-cells, Treg), and B220 (B-lymphocytes).

At day 12 following oral infection with *C. jejuni* ATCC 43431, GB mice as well as mice fed with CAF displayed significantly higher numbers of apoptotic cells as well as neutrophils, T- and B-lymphocytes, and Tregs in their large intestines as compared to infected SPF mice *(Fig. 4)*. In addition, CAF mice displayed twice as

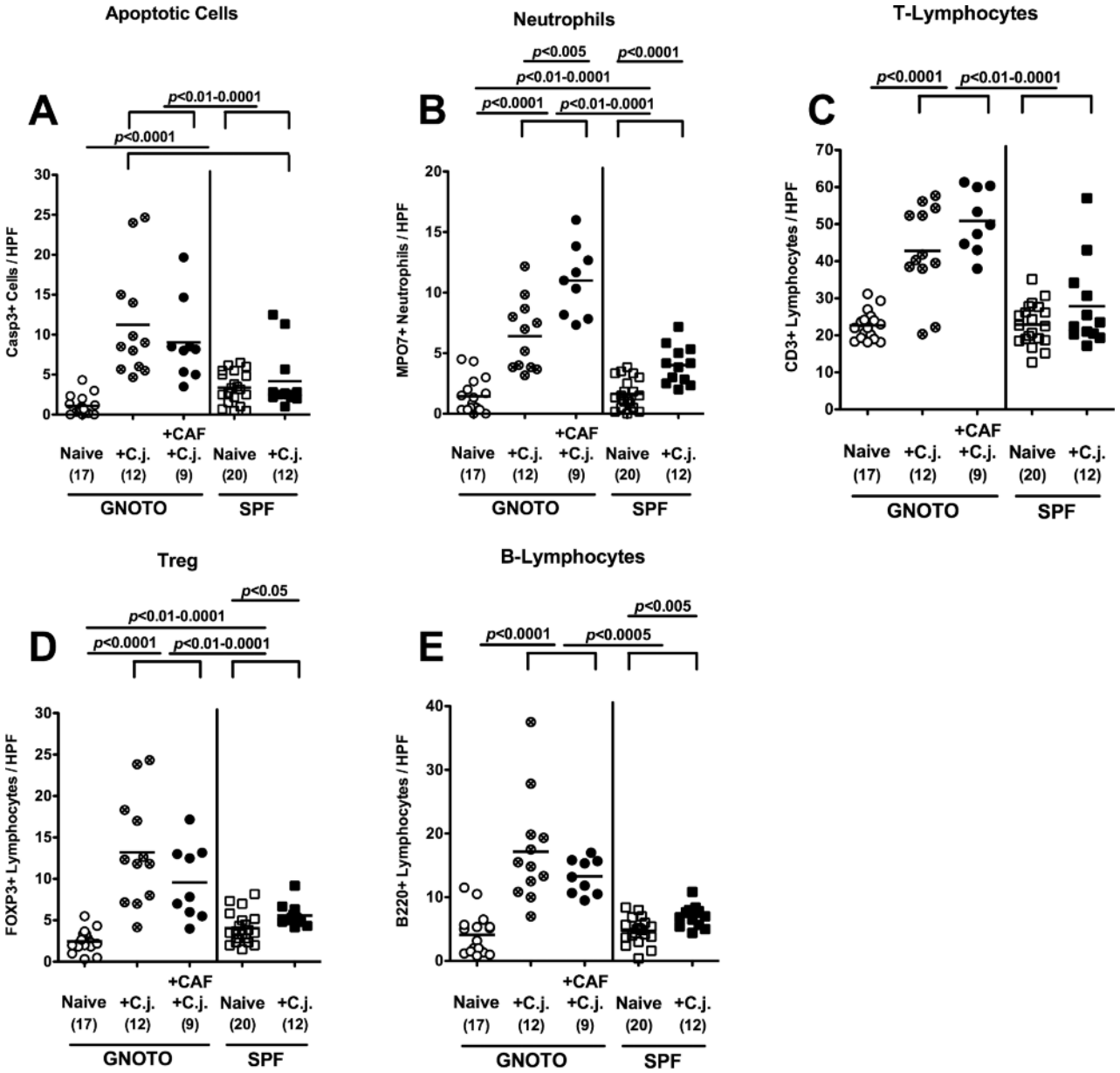

Fig. 4. Immunopathology in the colons of *C. jejuni*-infected mice following different dietary regimens. Gnotobiotic mice (GNOTO, circles) were generated by antibiotic gut decontamination and fed either cafeteria diet (CAF, solid circles) or conventional murine diet (sterile pellets, open and crossed circles), orally infected with *C. jejuni* strain ATCC 43431 (C.j.), and compared to noninfected gnotobiotic controls (open circles) and to naïve (open squares) or *C. jejuni* strain ATCC 43431 (C.j., solid squares)-infected animals harboring a conventional murine microbiota (SPF). The average numbers of apoptotic cells (positive for caspase-3, panel A), neutrophilic granulocytes (neutrophils, positive for MPO-7, panel B), T-lymphocytes (positive for CD3, panel C), regulatory T-cells (Treg, positive for FOXP3, panel D), and B-lymphocytes (positive for B220, panel E) from at least six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained colon sections. Numbers of animals of the respective genotype analyzed are given in parentheses. Means (black bars) and levels of significance (*P*-values) as compared to the respective groups (determined by the Student's *t*-test) are indicated. Data shown were pooled from at least three independent experiments

many neutrophils in the colon as GB mice at day 12 p.i. *(Fig. 4B)*.

Taken together, mice fed with CAF and as a consequence "suffering" from obesity not only harbored a gut flora which rendered them susceptible to *C. jejuni* infection but also displayed pro-infammatory immune responses in their colon following *C. jejuni* ATCC 43431 infection.

Impact of obesity on susceptibility to C. jejuni *infection*

To confirm our results obtained in CAF mice (with obesity) and further investigate the quadrangle relationship between nutrition, obesity, gut flora changes, and susceptibility to *Campylobacter* infection, another model was employed to assess the role of obesity in our findings. Mice

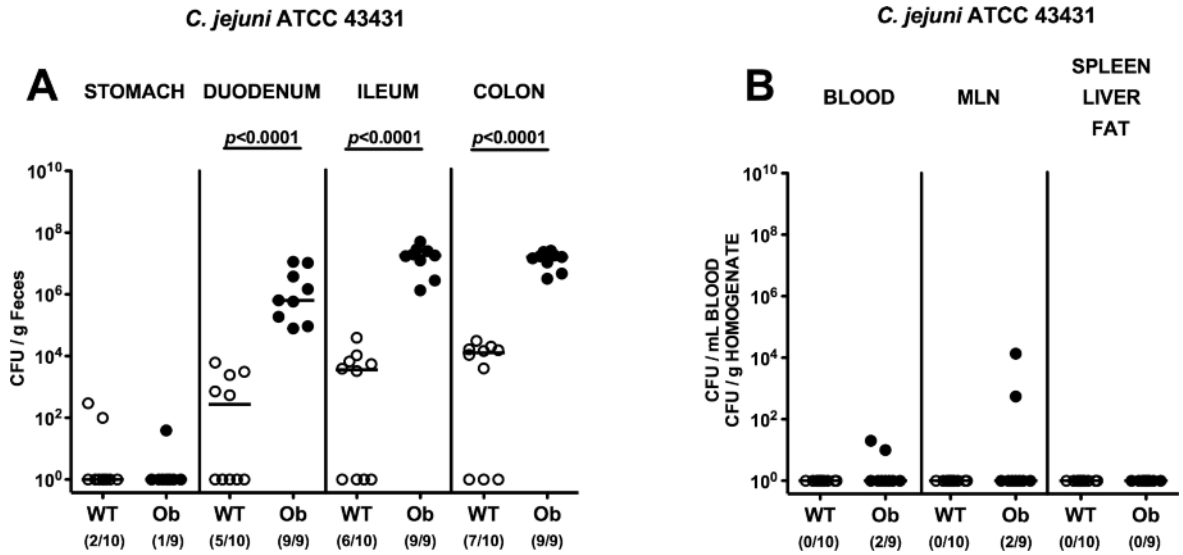

Fig. 5. *C. jejuni* colonization along the gastrointestinal (GI) tract and translocation in obese mice. Mice homozygous for the obese spontaneous mutation (*ob*/*ob*, solid circles) and wild-type controls (WT, KS background; open circles) were orally infected with *C. jejuni* strain ATCC 43431 as described in methods. The pathogen densities in distinct compartments of the GI tract (stomach, duodenum, ileum, and colon; A), or cardiac blood and organs such as mesenteric lymph nodes (MLNs), spleen, liver, and abdominal fat (B) were determined by quantification of live *C. jejuni* in luminal GI samples, blood, and organ homogenates, respectively, taken at day 12 p.i. by cultural analysis (CFU, colony forming units). Numbers of animals harboring *C. jejuni* out of the total number of analyzed animals are given in parentheses. Medians (black bars) and levels of significance (*P*-values) determined by Mann–Whitney-*U* test are indicated. Data shown were pooled from three independent experiments

homozygous for the obese spontaneous mutation Lep^{ob}, referred to as *ob*/*ob*, and wild-type (WT) control animals (both in KS background), all of them harboring a conventional gut flora, were orally infected with *C. jejuni* ATCC 43431 on three consecutive days. *Ob/ob* mice harbored up to 4 orders of magnitude higher *C. jejuni* ATCC 43431 loads in their GI tract (duodenum, ileum, and colon), with the highest counts of $>10^7$ CFU/g luminal content in ileum and colon as compared to WT controls *(Fig. 5A)*. Furthermore, live *C. jejuni* ATCC 43431 could be cultured from cardiac blood and MLNs from 22.2% of *ob/ob*, but none of WT, mice, whereas no translocation of *C. jejuni* ATCC 43431 into spleen, liver, or abdominal fat could be detected *(Fig. 5B)*.

These findings independently demonstrate in another experimental model that obese mice kept under standard conditions and harboring a conventional microbiota were more susceptible to *C. jejuni* infection as compared to lean WT controls with the same diet.

Obese mice harbor higher E. coli *and enterococci loads in the colon lumen*

Next, we were interested in differences in the gut microflora composition of obese as compared to lean animals, which might render *ob/ob* mice susceptible to *C. jejuni* infection. Most interestingly, naive *ob/ob* mice harbored significantly higher *E. coli* and enterococci loads (approximately 2 orders of magnitude) in the colons as compared to naive WT controls, whereas *Clostridium/Eubacterium* spp. numbers were slightly lower *(Fig. 6)*. At day 12 following *C. jejuni* ATCC 43431 infection, *ob/ob* mice harbored higher total bacterial numbers as well as higher loads of *E. coli*, *Bacteroides/Prevotella* spp., *Clostridium/Eubacterium* spp. in the colon *(Fig. 6)*. Taken together, higher *E. coli* counts could uniquely be detected in mice prone to *C. jejuni* infection – irrespective of whether "humanized" mice, CAF-fed animals, or *ob/ob* mice with conventional flora had been examined.

C. jejuni*-induced intestinal inflammation in obese mice*

In the naïve, uninfected condition, Casp3+, MPO7+, CD3+, B220+, and FOXP3+ cell numbers in the colons *in situ* did not differ when comparing *ob/ob* and WT control mice *(Fig. 7)*. Twelve days following oral *C. jejuni* ATCC 43431 infection, however, a substantial increase (more than or equal to twofold) of apoptotic cells, neutrophilic granulocytes, T- and B-lymphocytes as well as Tregs in colon sections of *ob/ob*, but not WT, animals could be detected *(Fig. 7)*. Thus, obese mice displayed a gut flora composition rendering them susceptible to *C. jejuni* infection; also, inflammatory changes in the colon as a consequence of high *C. jejuni* loads could be determined. Taken together, we could extend the murine models for campylobacteriosis by the use of obese mice and mice fed with a human diet, indicating that both diet and obesity are sufficient to render mice susceptible to *C. jejuni* infection.

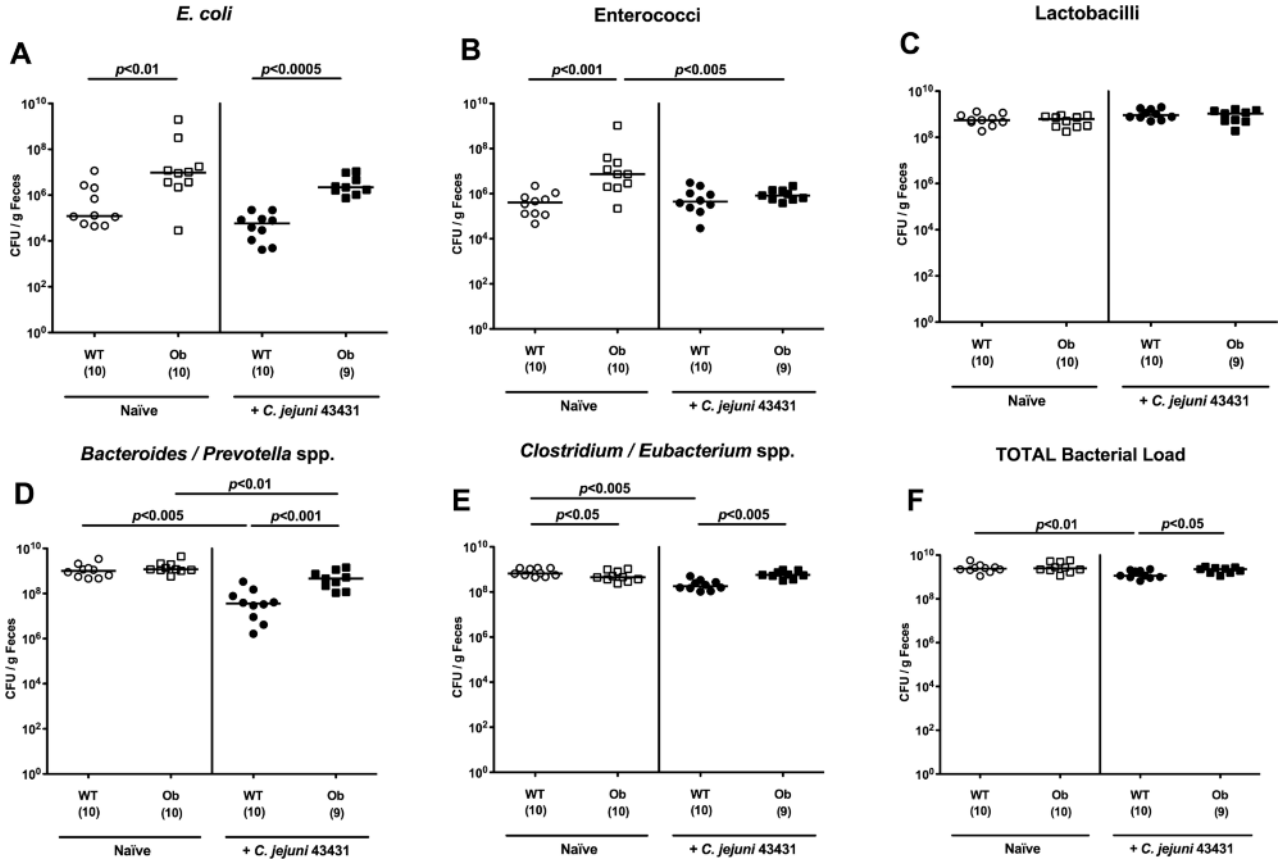

Fig. 6. Bacterial colonization status of intestines in obese mice. Mice homozygous for the obese spontaneous mutation (*ob/ob,* squares) and wild-type controls (WT, KS background; circles), both harboring a conventional gut microbiota, were orally infected with *C. jejuni* strain ATCC 43431 (open symbols) as described in methods. Numbers of (A) *E. coli,* (B) *Enterococcus* spp., (C) *Lactobacillus* spp., (D) *Bacteroides/Prevotella* spp., (E) *Clostridium/Eubacterium* spp., and (F) total bacterial loads were determined in feces samples of mice at day 12 p.i. (filled symbols) and compared to the respective uninfected control animals (open symbols) by detection of colony forming units (CFU) per gram feces on appropriate culture media (see methods). Bacterial species were identified by biochemical analysis and reconfirmed by comparative sequence analyses of 16S rRNA genes. Numbers of animals harboring the respective bacterial species are given in parentheses. Medians and significance levels (P-values) determined by Mann–Whitney-U test are indicated. Data shown were pooled from three independent experiments

Discussion

Valid experimental data concerning intestinal colonization capacities of *C. jejuni*, mechanisms of its host interactions, and immunopathological responses in the course of *C. jejuni* infections of vertebrates are scarce. This is mainly due to the fact that mice harboring a conventional gut microbiota display a strong colonization resistance against *C. jejuni* and are thus not suitable as standardized vertebrate model for the study of *C. jejuni* infection *in vivo*. We have successfully overcome these limitations and developed novel murine *C. jejuni* infection models by modification of the commensal gut microbiota [9]. GB mice in which the gut flora had been completely eradicated by antibiotic treatment as well as mice colonized with a complex human intestinal flora were highly susceptible to *C. jejuni* infection, harbored high *C. jejuni* loads within their intestines, displayed significant host immune and inflammatory responses, and can thus excellently serve as novel experimental models for *C. jejuni*-infection and -mediated immunopathology (summarized in Ref. [9]).

In the study presented here, comparable and stable *C. jejuni* infection of intestines in GB mice which had been mono- and co-colonized with defined bacterial species from the commensal gut microbiota further confirms the GB mouse model as a valuable tool for the study of pathogen–bacteria interactions *in vivo*.

Given that the host-specific gut microbiota composition determines colonization resistance against *C. jejuni* infection and that the murine differed from the human intestinal microbiota by higher lactobacilli but lower enterobacteria numbers [9], we had raised the simple hypothesis that colonization resistance might be due to relatively high lactobacilli (and lower *E. coli* loads), whereas susceptibility to *C. jejuni* infection might be facilitated by higher *E. coli* (and lower lactobacilli) counts. Recolonization experiments of GB mice, however, indicate that colonization resistance against and susceptibility to *C. jejuni* infection are rather due to the complex microflora composition, physiological intraluminal milieu of the intestine, or so-far unknown factors and cannot be simply attributed to absolute loads or relative distribution of single bacterial species alone. Thus,

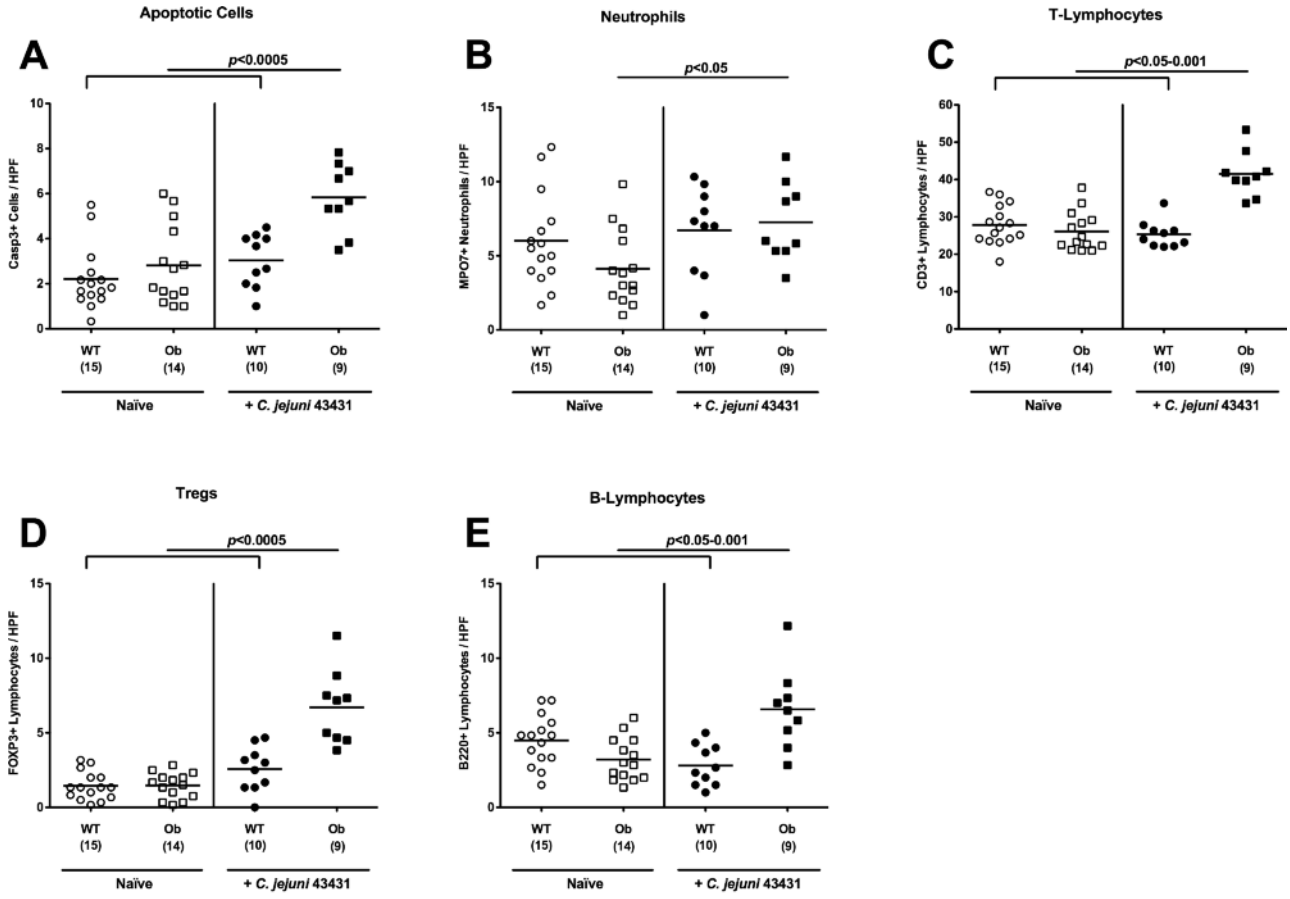

Fig. 7. Induction of immunopathology in the colon of obese mice following *C. jejuni* infection. Mice homozygous for the obese spontaneous mutation (*ob/ob,* squares) and wild-type controls (WT, KS background; circles) were orally infected with *C. jejuni* strain ATCC 43431 (filled symbols) as described in methods and compared to noninfected control animals (open symbols). The average numbers of apoptotic cells (positive for caspase-3, panel A), neutrophilic granulocytes (Neutrophils, positive for MPO-7, panel B), T-lymphocytes (positive for CD3, panel C), regulatory T-cells (Treg, positive for FOXP3, panel D), and B-lymphocytes (positive for B220, panel E) from at least six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained colon sections. Numbers of animals of the respective genotype analyzed are given in parentheses. Means (black bars) and levels of significance (*P*-values) as compared to the respective groups (determined by the Student's *t*-test) are indicated. Data shown were pooled from three independent experiments

we can only conclude that mechanisms underlying murine colonization resistance against *C. jejuni* are multifactorial.

Given that the gut microbiota composition and, in turn, the intestinal intraluminal milieu are mainly determined by dietary habits, we were interested in knowing whether nutrition can predispose and render a host susceptible to or resistant against *C. jejuni* infection. Interestingly, GB mice fed with a human CAF exhibited a gut microbiota composition which was comparable to the one seen in GB mice following reconstitution with a complex human flora (HFA): As compared to mice with a conventional gut microbiota, which was fed with a regular chow (MUD), CAF and HFA mice exhibited higher total bacterial, enterobacterial (*E. coli*), and *Clostridium/Eubacterium* spp. loads but lower *Lactobacillus* spp. numbers in their colon, indicating that the human diet favors the establishment of a human-like microflora in mice. Furthermore, CAF and HFA, but not MUD, mice were highly susceptible to *C. jejuni* infection, as indicated by high pathogen densities throughout the entire GI tract at day 12 p.i., subsequent immune and inflammatory host responses within the colon, and translocation of live *C. jejuni* into MLNs. Thus, we show for the first time that nutritional modification plays a crucial role in murine colonization resistance against and susceptibility to *C. jejuni* infection. In this context, it is interesting to note that results from a recent study in dogs revealed that dogs fed with a human diet were more susceptible to *C. jejuni* infection as compared to animals fed with a standard diet [27].

To confirm our results obtained in CAF mice subjected to 10 weeks of human "Western" diet that resulted in a significant gain of >40% body weight and thus obesity, we used another well-established model of obesity. Interestingly, *ob/ob* mice fed with a regular chow harbored a "normal" flora with higher *E. coli* and enterococci counts as compared to the conventional microbiota of lean WT controls on MUD. Following oral infection, *ob/ob* mice could be readily colonized by *C. jejuni*, and they exhibited significant immune and inflammatory responses in the intestine, as indicated by increased numbers of T- and B-lymphocytes,

Tregs, neutrophils, and apoptotic cells in the colon *in situ*. Thus, similar to human campylobacteriosis, *C. jejuni* triggers both innate and adaptive immune responses in HFA, CAF, and *ob/ob* mice.

In summary, the study presented here demonstrates that mono-colonized mice, CAF mice (with obesity), and *ob/ob* mice can serve as suitable vertebrate models for the study of *C. jejuni* infection. The obtained results further hint at a quadrangle relationship between nutrition, gut microbiota composition, obesity, susceptibility to *C. jejuni* infection, and – as a consequence – intestinal inflammation. It is tempting to speculate that dietary habits have a pivotal effect on pathogen susceptibility and changes therein might lower the risk of human campylobacteriosis.

Acknowledgements

This work was supported by grants from the German Research Foundation (DFG) to UBG (GO363/12–1, Campy Germ; SFB633, TP A7), SB and AF (SFB633, TP A7), AK and CL (SFB633, TP Z1), AB and BS (SFB633, TP A12), MMH (SFB633, TP B6), and from the German Federal Ministry of Education and Research (BMBF) to SB ("Lab in a hanky" projects TP 1.1 and TP 8.2). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Michaela Wattrodt, Ursula Rüschendorf, Gernot Reifenberger, Uwe Lohmann, and the staff of the animal research facility for excellent technical assistance, animal breeding, and genotyping of mice. We are grateful to Simone Spieckermann for immunohistochemistry staining of colon sections.

References

1. Kist M, Bereswill S: *Campylobacter jejuni*. Contrib Microbiol 8, 150–165 (2001)
2. Bereswill S, Kist M: Recent developments in Campylobacter pathogenesis. Curr Opin Infect Dis 16, 487–491 (2003)
3. van Spreeuwel JP, Duursma GC, Meijer CJ, Bax R, Rosekrans PC, Lindeman J: *Campylobacter colitis:* histological immunohistochemical and ultrastructural findings. Gut 26, 945–951 (1985)
4. Walker RI, Caldwell MB, Lee EC, Guerry P, Trust TJ, Ruiz-Palacios GM: Pathophysiology of *Campylobacter enteritis*. Microbiol Rev 50, 81–94 (1986)
5. Ketley JM: Pathogenesis of enteric infection by Campylobacter. Microbiology 143 (Pt 1), 5–21 (1997)
6. Tsang RS: The relationship of *Campylobacter jejuni* infection and the development of Guillain–Barre syndrome. Curr Opin Infect Dis 15, 221–228 (2002)
7. Dasti JI, Tareen AM, Lugert R, Zautner AE, Gross U: *Campylobacter jejuni:* a brief overview on pathogenicity-associated factors and disease-mediating mechanisms. Int J Med Microbiol 300, 205–211 (2010)
8. Dorrell N, Wren BW: The second century of Campylobacter research: recent advances, new opportunities and old problems. Curr Opin Infect Dis 20, 514–518 (2007)
9. Bereswill S, Fischer A, Plickert R, Haag LM, Otto B, Kühl AA, Dashti JI, Zautner AE, Munoz M, Loddenkemper C, Gross U, Göbel UB, Heimesaat MM: Novel murine infection models provide deep insights into the "Menage a Trois" of *Campylobacter jejuni,* microbiota and host innate immunity. PLoS One 6, e20953 (2011)
10. Mansfield LS, Bell JA, Wilson DL, Murphy AJ, Elsheikha HM, Rathinam VA, Fierro BR, Linz JE, Young VB: C57BL/6 and congenic interleukin-10-deficient mice can serve as models of *Campylobacter jejuni* colonization and enteritis. Infect Immun 75, 1099–1115 (2007)
11. Mansfield LS, Patterson JS, Fierro BR, Murphy AJ, Rathinam VA, Kopper JJ, Barbu NI, Onifade TJ, Bell JA: Genetic background of IL-10(–/–) mice alters host-pathogen interactions with *Campylobacter jejuni* and influences disease phenotype. Microb Pathog 45, 241–257 (2008)
12. Yrios JW, Balish E: Immune response of athymic and euthymic germfree mice to *Campylobacter* spp. Infect Immun 54, 339–346 (1986)
13. Yrios JW, Balish E: Colonization and infection of athymic and euthymic germfree mice by *Campylobacter jejuni* and *Campylobacter fetus* subsp. fetus. Infect Immun 53, 378–383 (1986)
14. Yrios JW, Balish E: Pathogenesis of *Campylobacter* spp. in athymic and euthymic germfree mice. Infect Immun 53, 384–392 (1986)
15. Jesudason MV, Hentges DJ, Pongpech P: Colonization of mice by *Campylobacter jejuni*. Infect Immun 57, 2279–2282 (1989)
16. Youssef M, Corthier G, Goossens H, Tancrede C, Henry-Amar M, Andremont A: Comparative translocation of enteropathogenic *Campylobacter* spp. and *Escherichia coli* from the intestinal tract of gnotobiotic mice. Infect Immun 55, 1019–1021 (1987)
17. Savidge TC, Smith MW, James PS, Aldred P: Salmonella-induced M-cell formation in germ-free mouse Peyer's patch tissue. Am J Pathol 139, 177–184 (1991)
18. Shroff KE, Cebra JJ: Development of mucosal humoral immune responses in germ-free (GF) mice. Adv Exp Med Biol 371A, 441–446 (1995)
19. Heimesaat MM, Bereswill S, Fischer A, Fuchs D, Struck D, Niebergall J, Jahn HK, Dunay IR, Moter A, Gescher DM, Schumann RR, Göbel UB, Liesenfeld O: Gram-negative bacteria aggravate murine small intestinal Th1-type immunopathology following oral infection with *Toxoplasma gondii.* J Immunol 177, 8785–8795 (2006)
20. Heimesaat MM, Fischer A, Jahn HK, Niebergall J, Freudenberg M, Blaut M, Liesenfeld O, Schumann RR, Göbel UB, Bereswill S: Exacerbation of murine ileitis by Toll-like receptor 4 mediated sensing of lipopolysaccharide from commensal *Escherichia coli*. Gut 56, 941–948 (2007)
21. Munoz M, Heimesaat MM, Danker K, Struck D, Lohmann U, Plickert R, Bereswill S, Fischer A, Dunay IR, Wolk K, Loddenkemper C, Krell HW, Libert C, Lund LR, Frey O, Hölscher C, Iwakura Y, Ghilardi N, Ouyang W, Kamradt T, Sabat R, Liesenfeld O: Interleukin (IL)-23 mediates *Toxoplasma gondii*-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. J Exp Med 206, 3047–3059 (2009)
22. Sclafani A, Springer D: Dietary obesity in adult rats: similarities to hypothalamic and human obesity syndromes. Physiol Behav 17, 461–471 (1976)

23. Rothwell NJ, Stock MJ: Combined effects of cafeteria and tube-feeding on energy balance in the rat. Proc Nutr Soc 38, 5A (1979)
24. Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, Newgard CB, Makowski L: Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. Obesity (Silver Spring) 19: 1109–1117 (2011)
25. Heimesaat MM, Nogai A, Bereswill S, Plickert R, Fischer A, Loddenkemper C, Steinhoff U, Tchaptchet S, Thiel E, Freudenberg MA, Göbel UB, Uharek L: MyD88/TLR9 mediated immunopathology and gut microbiota dynamics in a novel murine model of intestinal graft-versus-host disease. Gut 59, 1079–1087 (2010)
26. Heimesaat MM, Fischer A, Siegmund B, Kupz A, Niebergall J, Fuchs D, Jahn HK, Freudenberg M, Loddenkemper C, Batra A, Lehr HA, Liesenfeld O, Blaut M, Göbel UB, Schumann RR, Bereswill S: Shift towards pro-inflammatory intestinal bacteria aggravates acute murine colitis via Toll-like receptors 2 and 4. PLoS One 2, e662 (2007)
27. Stavisky J, Radford AD, Gaskell R, Dawson S, German A, Parsons B, Clegg S, Newman J, Pinchbeck G: A case-control study of pathogen and lifestyle risk factors for diarrhoea in dogs. Prev Vet Med 99, 185–192 (2011)