Microbiota composition and inflammatory immune responses upon peroral application of the commercial competitive exclusion product Aviguard® to microbiota-depleted wildtype mice

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ABSTRACT

Non-antibiotic feed additives including competitive exclusion products have been shown effective in reducing pathogen loads including multi-drug resistant strains from the vertebrate gut. In the present study we surveyed the intestinal bacterial colonization properties, potential macroscopic and microscopic inflammatory sequelae and immune responses upon peroral application of the commercial competitive exclusion product Aviguard® to wildtype mice in which the gut microbiota had been depleted by antibiotic pre-treatment. Until four weeks following Aviguard® challenge, bacterial strains abundant in the probiotic suspension stably established within the murine intestines. Aviguard® application did neither induce any clinical signs nor gross macroscopic intestinal inflammatory sequelae, which also held true when assessing apoptotic and proliferative cell responses in colonic epithelia until day 28 post-challenge. Whereas numbers of colonic innate immune cell subsets such as macrophages and monocytes remained unaffected, peroral Aviguard® application to microbiota depleted mice was accompanied by decreases in colonic mucosal counts of adaptive immune cells such as T and B lymphocytes. In conclusion, peroral Aviguard® application results i.) in effective intestinal colonization within microbiota depleted mice, ii.) neither in macroscopic nor in microscopic inflammatory sequelae and iii.) in lower colonic mucosal T and B cell responses.

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INTRODUCTION

Human infections with food-borne pathogens particularly with multi-drug resistant bacterial strains are progressively rising and constitute significant health and socioeconomic burdens worldwide [1–3]. Combinations of distinct probiotic bacteria have been shown to suppress pathogenic bacteria from the vertebrate gut due to competitive exclusion [4, 5]. These competitive exclusion products have been successfully applied in animal feeding, particularly in poultry as promising antibiotics-independent approaches "from farm to fork" in order to reduce prevalences of human infections with enteropathogens such as *Salmonella* [6, 7]. Among the commercially available products, Aviguard® has been developed as a compound with longer shelf life compared to other competitive exclusion products for the application in poultry such as chicken and turkeys as drinking water additive or for spray treatment [8].

Aviguard® constitutes a freeze-dried fermentation product containing a mixture of viable commensal bacterial strains which represent the main commensal bacterial populations in the ceca of adult chicken [8].

Like Salmonella, Campylobacter jejuni are part of the commensal gut microbiota in livestock including poultry. Humans become infected by ingestion of contaminated (mostly undercooked or raw) meat products and surface water [9, 10] and display symptoms of varying degree after an incubation period of 2–5 days. Whereas some patients complain about rather mild discomfort, others suffer from severe campylobacteriosis characterized by abdominal cramps, watery or even inflammatory, bloody diarrhea and fever [11]. Even though C. jejuni constitute the most common bacterial causative agents of food-borne gastroenteritis in humans worldwide [1–3], only little is known regarding the molecular mechanisms underlying pathogen-host interactions. One of the reasons for this dilemma is the fact that reliable experimental in vivo models have been missing for a long time. Conventional laboratory mice, for instance, are protected from C. jejuni infection even after peroral challenge with high bacterial loads due to the distinct complex murine gut microbiota composition providing an effective colonization resistance to the host [12, 13]. Upon depletion of the gut microbiota by broad-spectrum antibiotic treatment and after reconstitution of microbiota depleted mice with a complex human as opposed to murine gut microbiota by fecal microbiota transplantation, however, the pathogen can stably establish within the gastrointestinal tract and induce inflammatory key features observed in human campylobacteriosis [13, 14].

Given that we are currently on the way to treatment studies applying Aviguard® in C. jejuni infected mice, we addressed in the present study whether application of the competitive exclusion product would result in stable colonization of the probiotic bacterial mixture in the murine gastrointestinal tract and whether Aviguard® per se may induce immune responses resulting in potential macroscopic and/or microscopic inflammatory sequelae in the murine host.

MATERIAL AND METHODS

Mice, gut microbiota depletion

Conventional 6-week-old C57BL/6J wildtype mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and maintained in the Forschungsinstitut für Experimentelle Medizin, Charité – University Medicine Berlin (Berlin, Germany). Mice were maintained in cages including filter tops within an experimental semi-barrier (accessible only with lab coat, overshoes, caps, and sterile gloves) under standard conditions (22–24 °C room temperature, 55 ± 15% humidity, 12 h light/12 h dark cycle) and had free access to autoclaved standard chow (food pellets: ssniff R/M-H, V1534-300, Sniff, Soest, Germany). By the age of 7 weeks, female and male mice were subjected to broad-spectrum antibiotic treatment in order to deplete the

commensal gut microbiota as described earlier [13, 15]. In brief, mice were transferred to sterile cages (maximum of 4 animals per cage) and treated with an antibiotic cocktail for eight weeks by adding ampicillin plus sulbactam (1 g/L; Dr. Friedrich Eberth Arzneimittel, Ursensollen, Germany), vancomycin (500 mg/L; Hikma Pharmaceuticals, London, UK), ciprofloxacin (200 mg/L; Fresenius Kabi, Bad Homburg, Germany), imipenem (250 mg/L; Fresenius Kabi) and metronidazole (1 g/L; B. Braun, Melsungen, Germany) to the drinking water (ad libitum). Microbiota-depleted mice were continuously kept and handled under strict aseptic conditions and received autoclaved food and drinking water in order to minimize the risk of contaminations.

Application of the commercial exclusion product Aviguard®

The commercial exclusion product Aviguard® was purchased from Lallemand Animal Nutrition (Worcestershire, UK). Approximately 30 min before oral challenge, 1 g of the compound was dissolved in 10 mL phosphate buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA). On three consecutive days (namely, days 0, 1 and 2), 0.3 mL of the bacterial suspension were perorally applied to mice by gavage. According to the manufacturer's instructions, the compound contains the following bacterial species (approximately 10^9 colony forming units (CFU) per g): *Escherichia coli*, *Citrobacter* species, *Enterococcus* species (*E. faecalis*, *E. faecium*), *Lactobacillus* species (*L. casei*, *L. plantarum*), *Bacteroides* species, *Clostridium* species (*C. sporogenes*), *Eubacterium* species, *Propionibacterium* species, *Fusobacterium* species, *Ruminococcus* species [16].

Cultural analysis of the gut microbiota composition

For quantitative cultural assessment of the microbiota composition in the Aviguard® suspensions and in feces, respective samples were homogenized in sterile PBS (Thermo Fisher Scientific) and analyzed in serial dilutions on respective solid media as described earlier [15]. Bacteria were grown at 37 °C for at least two days under aerobic, microaerobic and anaerobic conditions as stated elsewhere [15, 17, 18].

Culture-independent (molecular) analysis of the gut microbiota composition

In order to additionally assess fastidious and even uncultivable bacteria quantitatively we performed culture-independent, molecular (i.e., 16S rRNA based) analyses of the bacterial suspensions and fecal samples. Therefore, the total genomic DNA was extracted from respective samples, quantitated by Real-time polymerase chain reaction (RT-PCR) using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng per μL as described previously [15]. Then, total eubacterial loads as well as the main bacterial groups abundant in the murine intestinal microbiota including enterobacteria, enterococci, lactobacilli, bifidobacteria, *Bacteroides/Prevotella* species, *Clostridium leptum*

group, and *Clostridium coccoides* group were assessed by quantitative RT-PCR (qRT-PCR) with species-, genera- or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as described previously [19–21], and numbers of 16S rRNA gene copies per ng DNA of each sample were determined.

Clinical conditions

Before and at defined time points after Aviguard® application, we quantitatively surveyed the clinical conditions of mice on a daily basis by using a standardized cumulative clinical score (maximum 12 points), addressing the clinical aspect/wasting (0: normal; 1: ruffled fur; 2: less locomotion; 3: isolation; 4: severely compromised locomotion, pre-final aspect), the abundance of blood in feces (0: no blood; 2: microscopic detection of blood by the Guajac method using Haemoccult, Beckman Coulter/PCD, Germany; 4: macroscopic blood visible), and stool consistency (0: formed feces; 2: pasty feces; 4: liquid feces) as described earlier [22].

Sampling Procedures

On day 28 post-application, mice were sacrificed by CO_2 asphyxiation. *Ex vivo* biopsies and luminal samples were taken from the colon under sterile conditions. Large intestinal samples were collected from each mouse in parallel for microbiological and immunohistopathological analyses. The colonic lengths were measured with a ruler.

Quantitative *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 recently reported [19, 23]. In brief, in order to detect apoptotic epithelial cells, proliferating epithelial cells, macrophages/monocytes, T lymphocytes, regulatory T cells, and B lymphocytes, 5 μm thin colonic paraffin sections were stained with primary antibodies directed against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA, 1:200), Ki67 (TEC3, Dako, Glostrup, Denmark, 1:100), F4/80 (no. 14-4801, clone BM8, eBioscience, San Diego, CA, USA, 1:50), CD3 (no. N1580, Dako, 1:10), FOXP3 (clone FJK-165, no. 14-5773, eBioscience, 1:100), and B220 (no. 14-0452-81, eBioscience; 1:200), 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 mm², 400 × magnification).

Statistical Analyses

Medians and levels of significance were determined with GraphPad Prism v8, USA. For statistical analyses the Kruskal–Wallis test with Dunn's post-correction for not normally distributed data was applied. Two-sided probability (*P*) values ≤0.05 were considered significant. Data were pooled from four independent experiments.

Ethical statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) following approval by the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration number G0039/15). Animal welfare was monitored daily by assessment of clinical conditions.

RESULTS AND DISCUSSION

## Bacterial composition of suspensions containing the commercial competitive exclusion product Aviguard® for peroral application to microbiota depleted wildtype mice

On three consecutive days (namely days 0, 1 and 2), microbiota depleted C57BL/6j wildtype mice were perorally challenged with the commercial competitive exclusion product Aviguard® by gavage. Our cultural analyses revealed that the applied suspensions contained in total almost 10^9 viable bacteria per mL, including approximately 10^6 CFU enterobacteria per mL and between 10^7 and 2.5×10^8 CFU lactobacilli, enterococci, *Bacteroides/Prevotella* and *Clostridium/Eubacterium* species per mL suspension (Fig. 1A). In order to additionally assess fastidious and non-cultivable bacteria we applied culture-independent, 16S rRNA based analyses. In support of the cultural data, our molecular analyses revealed abundances of enterobacteria, enterococci, lactobacilli, bifidobacteria, *Bacteroides/Prevotella* species, *C. coccoides* and *C. leptum* groups (Fig. 1B). Hence, the vast majority of cultivable, fastidious and uncultivable probiotic bacterial species abundant in the commercial competitive exclusion product Aviguard® (except for fusobacteria and propionibacteria) could be assessed by culturomics and molecular approaches.

Gut microbiota composition following peroral Aviguard® application to microbiota depleted mice

Next, we addressed whether the applied bacteria were able to establish within the intestinal tract and surveyed respective bacterial groups, genera and species in fecal samples obtained until four weeks post-challenge by culture and culture-independent methods. As compared to day 7 post-challenge, numbers of enterobacteria and enterococci were slightly lower at the end of the observation period (approximately 1.0–1.5 orders of magnitude; $P < 0.001$), whereas lactobacilli, *Bacteroides/Prevotella* species, *Clostridium/Eubacterium* species as well as total bacterial loads did not differ between day 7 and 28 post-challenge (n.s.; Fig. 2A).

Additional molecular analyses revealed that fecal samples taken at day 28 as compared to day 7 post-challenge contained
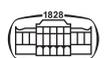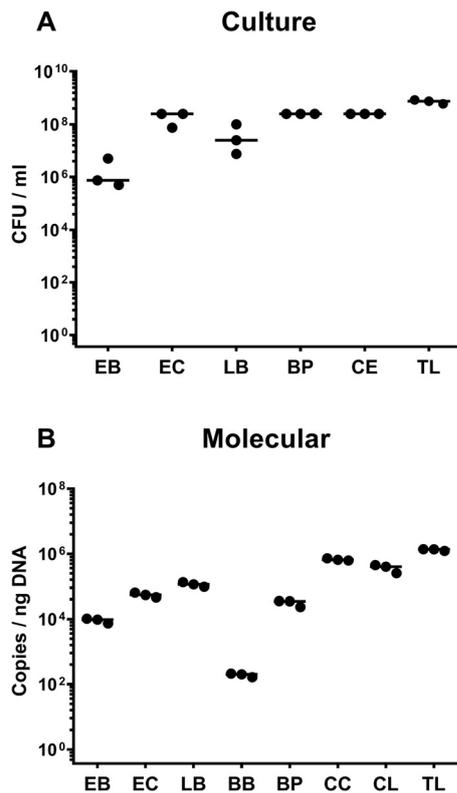
Fig. 1. Microbiota composition of the perorally applied solutions containing the commercial competitive exclusion product Aviguard®. Microbiota depleted mice were perorally challenged with the commercial competitive exclusion product Aviguard® on days 0, 1 and 2. The microbiota composition of the applied solutions was assessed by (A) culture (expressed as colony forming units (CFU) per mL) and by (B) culture-independent 16S rRNA based methods (expressed as gene copies per ng DNA). Medians (black bars) are indicated. Shown data were derived from one representative experiment. EB, enterobacteria; EC, enterococci; LB, lactobacilli; BB, bifidobacteria; BP, *Bacteroides/Prevotella* species; CE, *Clostridium/Eubacterium* species; CC, *Clostridium coccoides* group; CL, *Clostridium leptum* group; TL, total eubacterial load

Fig. 2. Cultural survey of the gut microbiota composition following peroral application of the commercial competitive exclusion product Aviguard® to microbiota depleted mice. Microbiota depleted mice were perorally challenged with the commercial competitive exclusion product Aviguard® on days 0, 1 and 2. The gut microbiota composition was assessed at distinct time points post-challenge by culture and indicated as colony forming units (CFU) per g feces. Medians (black bars), levels of significance (P-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed mice (in parentheses) are indicated. Data were pooled from three independent experiments. EB, enterobacteria; EC, enterococci; LB, lactobacilli; BP, *Bacteroides/Prevotella* species; CE, *Clostridium/Eubacterium* species; TL, total bacterial load

up to two log orders of magnitude lower gene numbers of enterobacteria, enterococci, lactobacilli and bifidobacteria ($P < 0.01$–0.001; Fig. 3A–D), whereas the differences for *Bacteroides/Prevotella* species, *C. coccoides* and *C. leptum* groups as well as for the total eubacterial loads were rather subtle (i.e., less than one order of magnitude lower gene numbers at day 28 versus day 7 post-challenge; $P < 0.001$; Fig. 3E–H). Of note, fecal enterobacteria and lactobacilli were absent in more than 50% of mice when using molecular (Fig. 3A, C) as opposed to cultural analyses (Fig. 2).

Furthermore, even though the relatively slight differences in intestinal loads of distinct bacterial groups, genera and species observed between days 7, 14 and 28 post-challenge were highly significant, their biological relevance might be rather questionable. Given the presumably technical-related differences in quantitative detection of distinct genera such as enterobacteria and lactobacilli due to cultural as
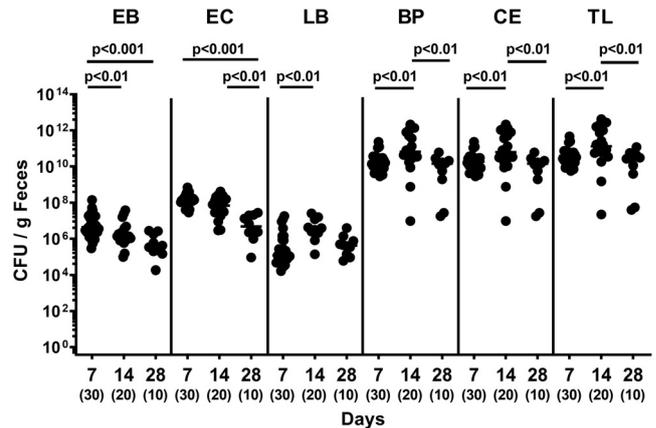
compared to molecular analyses further underlines the benefits of a combined analytic approach for a comprehensive survey of the complex gut microbiota composition. Hence, overall, the bacteria did, in fact, stably establish in the intestinal tract of mice until 4 weeks after peroral Aviguard® application.

Macroscopic sequelae upon peroral Aviguard® application to microbiota-depleted mice

We further addressed whether Aviguard® application to microbiota depleted mice resulted in any macroscopic sequelae. We therefore quantitatively surveyed clinical conditions in mice before and at distinct time points after Aviguard® challenge by using a standardized clinical scoring system assessing wasting, abundance of fecal blood and stool consistency [22]. However, neither at day 0 nor at days 7, 14 and 28 following Aviguard® or mock application any clinical signs could be observed (Fig. 4).

Given that intestinal inflammation is accompanied by a shortening of the affected intestinal compartment [15], we measured colonic lengths upon necropsies. On days 7, 14 and 28 following Aviguard® challenge, colonic lengths were comparable to those obtained from mock controls (n.s.; Fig. 5). Hence, Aviguard® application did neither induce any clinical signs nor gross macroscopic inflammatory sequelae in the colon.
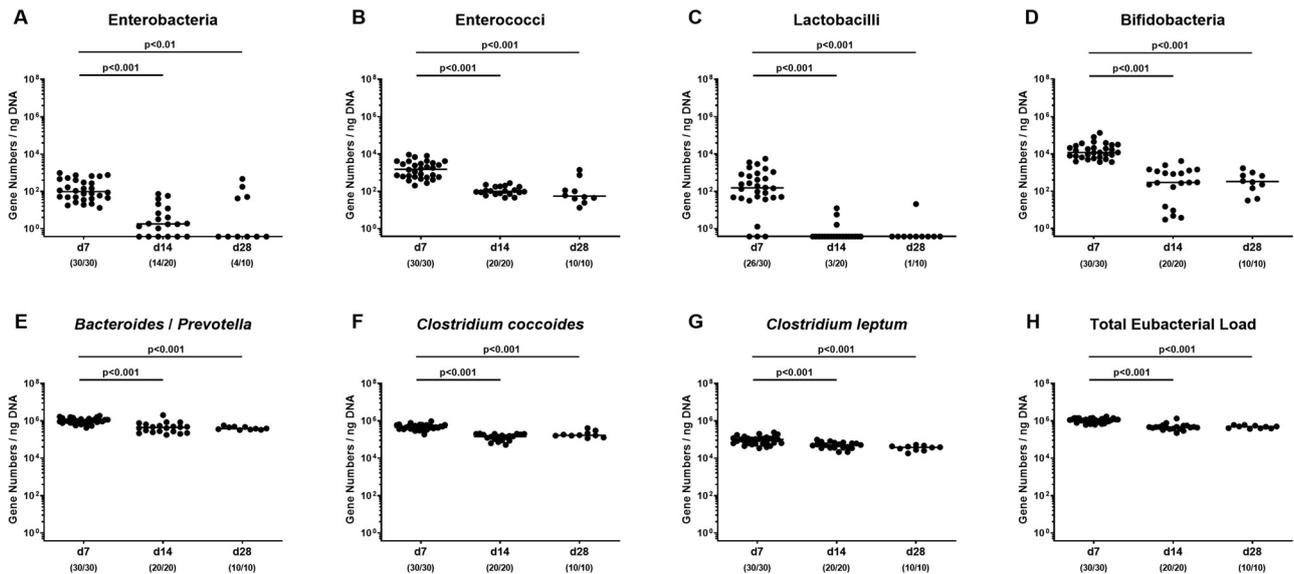
Fig. 3. Culture-independent, molecular survey of the gut microbiota composition following peroral application of the commercial competitive exclusion product Aviguard® to microbiota depleted mice. Microbiota depleted mice were perorally challenged with the commercial competitive exclusion product Aviguard® on day (d) 0, d1 and d2. The gut microbiota composition was assessed at distinct time points post challenge by culture-independent, 16S rRNA based methods quantitating the main commensal bacterial groups and the total eubacterial load (expressed as gene copies per ng DNA). Medians (black bars), levels of significance (*P*-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of mice harboring respective bacteria out of the total number of analyzed mice (in parentheses) are indicated. Data were pooled from three independent experiments

Apoptotic and proliferative cell responses in colonic epithelia following peroral Aviguard® application to microbiota depleted mice

We next surveyed potential microscopic cellular sequelae of Aviguard® application to microbiota depleted mice. Given

that apoptosis is considered a reliable marker for the grading of intestinal inflammation [13], we stained colonic paraffin sections with an antibody against cleaved caspase-3. Our *in situ* immunohistochemical analyses revealed that numbers of apoptotic colonic epithelial cells enumerated at
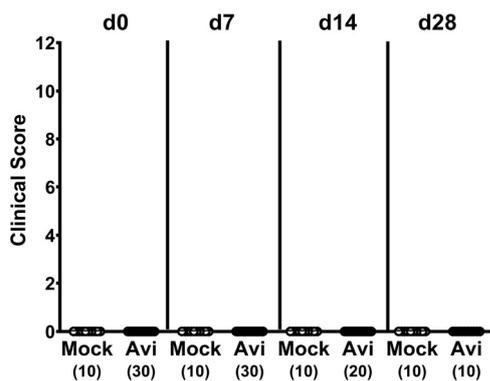

Fig. 4. Clinical conditions following peroral application of the commercial competitive exclusion product Aviguard® to microbiota depleted mice. Microbiota depleted mice were perorally challenged with the commercial competitive exclusion product Aviguard® (Avi) on day (d) 0, d1 and d2 or received vehicle (mock). Immediately before and at defined time points after either peroral challenge, the clinical conditions of mice were quantitative assessed applying a standardized clinical scoring system (see methods). Numbers of analyzed animals are indicated in parentheses. Data were pooled from three independent experiments
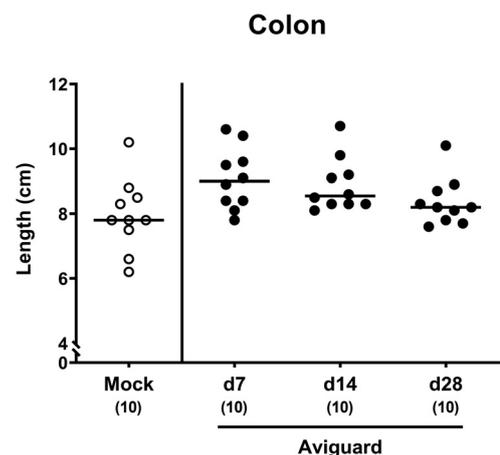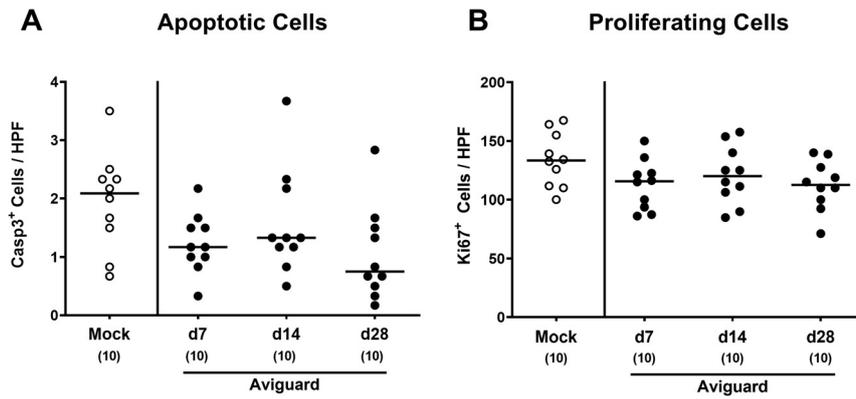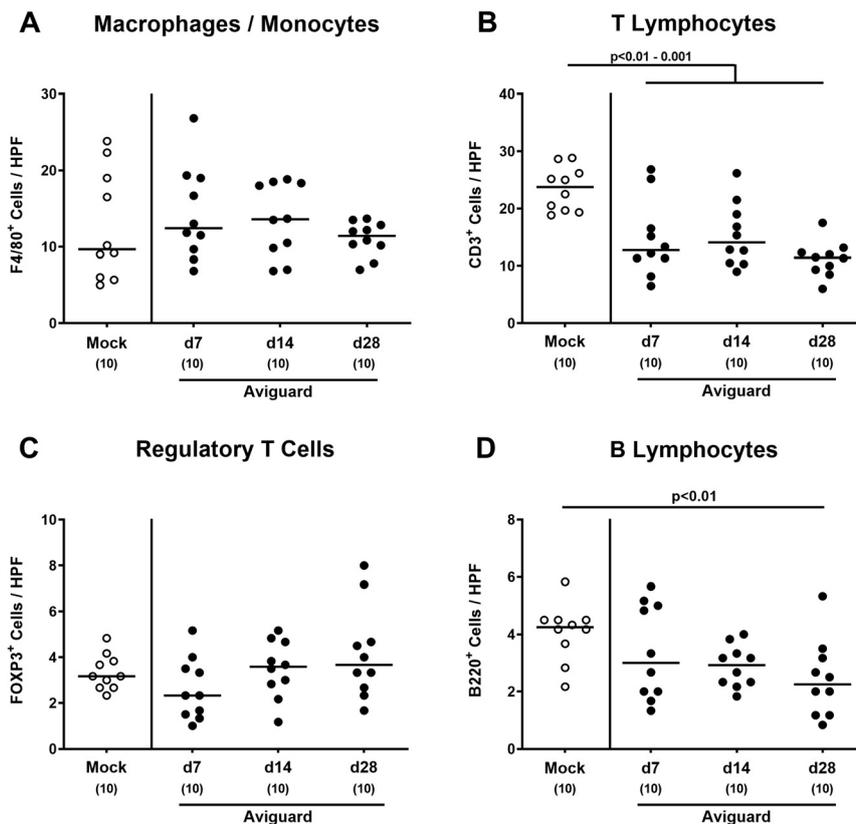

Fig. 5. Colonic lengths following peroral application of the commercial competitive exclusion product Aviguard® to microbiota depleted mice. Microbiota depleted mice were perorally challenged with the commercial competitive exclusion product Aviguard® on day (d) 0, d1 and d2 or received vehicle (mock). The colonic lengths were measured at distinct time points post-challenge with a ruler (expressed in cm). Numbers of analyzed mice (in parentheses) are indicated. Data were pooled from three independent experiments

Fig. 6. Colonic epithelial cell apoptosis and cell proliferation following peroral application of the commercial competitive exclusion product Aviguard® to microbiota depleted mice. Microbiota depleted mice were perorally challenged with the commercial competitive exclusion product Aviguard® on day (d) 0, d1 and d2 or received vehicle (mock). At defined time points post-challenge, the average numbers of colonic epithelial (A) apoptotic (Casp3+) and (B) proliferating (Ki67+) cells were assessed microscopically from six high power fields (HPF, 400 × magnification) per animal in immunohistochemically stained colonic paraffin sections. Medians (black bars) and numbers of analyzed mice (in parentheses) are indicated. Data were pooled from three independent experiments.

days 7, 14 and 28 following Aviguard® application were comparable to mock control mice (n.s.; Fig. 6A), which also held true for Ki67+ colonic epithelial cells indicative for cell proliferation (n.s.; Fig. 6B). Hence, Aviguard® did neither induce apoptotic nor proliferative cell responses in colonic epithelia.

Fig. 7. Colonic immune cell responses following peroral application of the commercial competitive exclusion product Aviguard® to microbiota depleted mice. Microbiota depleted mice were perorally challenged with the commercial competitive exclusion product Aviguard® on day (d) 0, d1 and d2 or received vehicle (mock). At defined time points post-challenge, the average numbers of (A) macrophages and monocytes (F4/80+), (B) T lymphocytes (CD3+), (C) regulatory T cells (FOXP3+) and (D) B lymphocytes (B220+) were assessed microscopically from six high power fields (HPF, 400 × magnification) per animal in immunohistochemically stained colonic paraffin sections. Medians (black bars), levels of significance (P-values) assessed by the Kruskal-Wallis test and Dunn's post-correction and numbers of analyzed mice (in parentheses) are indicated. Data were pooled from three independent experiments.

Colonic immune cell responses following peroral Aviguard® application to microbiota depleted mice

We further addressed whether peroral Aviguard® application to microbiota depleted mice was associated with distinct innate and adaptive immune cell responses in the large intestinal tract and therefore, applied quantitative *in situ* immunohistochemistry. Numbers of F4/80+ innate immune cell subsets such as macrophages and monocytes enumerated in the colonic mucosa and lamina propria of mice 7, 14 and 28 days following Aviguard® application were comparable and did not differ from mock control animals (n.s.; Fig. 7A), which also held true for FOXP3+ regulatory T cells (n.s.; Fig. 7C). At either time point following Aviguard® challenge colonic numbers of CD3+ T lymphocytes were lower as compared to those assessed in mock counterparts ($P < 0.01$–0.001; Fig. 7B), whereas in case of B220+ B lymphocytes, cell counts were lower at day 28 following Aviguard® application as compared to the mock cohort ($P < 0.01$; Fig. 7D). Hence, peroral Aviguard® application to microbiota depleted mice was accompanied by decreased colonic mucosal numbers of adaptive immune cells such as T and B lymphocytes.

SUMMARY

In our present study we show for the first time that peroral application of the commercial competitive exclusion product Aviguard® to microbiota depleted mice results i.) in effective intestinal colonization within microbiota depleted mice, ii.) neither in macroscopic nor in microscopic inflammatory sequelae and iii.) in lower colonic mucosal T and B cell responses.

We are currently on the way to performing comprehensive *in vivo* studies applying murine *C. jejuni* infection and inflammation models in order to shed further light onto the triangle relationship ("Ménage à trois") between the pathogen, the commensal probiotic gut bacteria and host immunity. Therefore, the here obtained results constitute important prerequisites to interpret the data sets we will derive from our further surveys. Furthermore, colonization data reassured that the probiotic bacteria within the competitive exclusion product can establish within the intestinal tract of the murine host and that the combination of culturomics and molecular approaches are highly reliable in order to quantitatively assess the vast majority of bacterial species that are abundant in the probiotic suspension and in the intestinal tract of the vertebrate host after peroral application.

Given that *C. jejuni* infection of both, microbiota depleted wildtype and IL-10-/- mice was accompanied by increased numbers of T and B lymphocytes in the colonic mucosa and lamina propria [13, 24], the T and B cell lowering effects upon Aviguard® application observed here might point towards a potential immune-modulatory effect that might dampen pro-inflammatory immune responses induced by *C. jejuni* and needs to be addressed in more detail in future studies. In a previous report, Aviguard® has been shown to protect chicks from overwhelming intestinal colonization by *Salmonella* without interfering with their normal antibody production in response to pathogenic infection [8].

CONCLUSION

The here presented data provide evidence that microbiota depleted mice constitute valuable tools to further unravel the molecular mechanisms underlying the triangle relationship between enteropathogens such as *C. jejuni*, the commensal probiotic gut bacteria within commercial competitive exclusion products including Aviguard® and the vertebrate host immunity.

ACKNOWLEDGMENTS

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