Therapeutic effects of oral benzoic acid application during acute murine campylobacteriosis

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ABSTRACT
Serious risks to human health are posed by acute campylobacteriosis, an enteritis syndrome caused by oral infection with the food-borne bacterial enteropathogen Campylobacter jejuni. Since the risk for developing post-infectious autoimmune complications is intertwined with the severity of enteritis, the search of disease-mitigating compounds is highly demanded. Given that benzoic acid is an organic acid with well-studied health-promoting including anti-inflammatory effects we tested in our present study whether the compound might be a therapeutic option to alleviate acute murine campylobacteriosis. Therefore, microbiota-depleted IL-10−/−/C0−/−/C0−/− mice were perorally infected with C. jejuni and received benzoic acid through the drinking water from day 2 until day 6 post-infection. The results revealed that benzoic acid treatment did not affect C. jejuni colonization in the gastrointestinal tract, but alleviated clinical signs of acute campylobacteriosis, particularly diarrheal and wasting symptoms. In addition, benzoic acid mitigated apoptotic cell responses in the colonic epithelia and led to reduced pro-inflammatory immune reactions in intestinal, extra-intestinal, and systemic compartments tested on day 6 post-infection. Hence, our preclinical placebo-controlled intervention trial revealed that benzoic acid constitutes a promising therapeutic option for treating acute campylobacteriosis in an antibiotic-independent fashion and in consequence, also for reducing the risk of post-infectious autoimmune diseases.

KEYWORDS
benzoic acid, organic acids, enteropathogenic infection, Campylobacter jejuni, anti-bacterial effects, anti-inflammatory effects, immune-modulatory effects, secondary abiotic IL-10−/−/C0−/−/C0−/− mice, acute campylobacteriosis model, host-pathogen interactions, antibiotics-independent treatment

INTRODUCTION
Campylobacter species particularly Campylobacter jejuni are recognized as leading infectious agents of food-related bacterial gastroenteritis in the world [1, 2]. Recent data from the European Food Safety Authority revealed over 246,000 reported cases of campylobacteriosis in the European Union in 2022 alone [3]. These numbers also highlight the significance of economic and health burdens imposed by C. jejuni infections, including medical expenses and productivity losses [4], thus underscoring the pressing need for effective prevention, detection, and management strategies for human campylobacteriosis [4, 5]. C. jejuni are rod-shaped Gram-negative bacteria that colonize the gastrointestinal tracts of livestock and preferably poultry as a commensal [6, 7]. This close association also serves as natural reservoirs for human infections, often transmitted by the consumption of contaminated poultry products, mainly undercooked chicken and Turkey meat [6, 8]. Once ingested, individuals are susceptible to develop an acute enteritis syndrome, characterized by abdominal pain,
bloody diarrhea, vomiting, and fever in more severe cases [9]. Among immune-competent individuals the C. jejuni induced symptoms usually last from a few days to maximum two weeks and the enteritis can be treated symptomatically [10, 11]. However, infected immune-compromised patients and children may face a more drastic and prolonged course of illness and require antibiotic treatment [12]. Even though campylobacteriosis usually resolves without residues, patients can protract post-infectious autoimmune complications weeks to months following C. jejuni infection; these include the Guillain-Barré syndrome, reactive arthritis, irritable bowel syndrome, and inflammatory bowel diseases, for instance [1, 13, 14].

The excessive human immune responses upon C. jejuni invasion of the intestinal tissues are mainly attributed to the major virulence factor lipo-oligosaccharide (LOS), a bacterial surface carbohydrate structure [15, 16]. LOS triggers an hyper-activation of the innate immune system via Toll-like receptor (TLR)-4, initiating a vicious inflammatory cascade: The rapid release of pro-inflammatory mediators leads to oxidative stress and apoptotic cell responses in the infected intestinal tract; in turn, intestinal epithelial barrier functions are compromised mounting in diarrhea and an malabsorptive disease [9, 15, 17, 18]. Furthermore, the pro-inflammatory immune reactions can even affect extra-intestinal including systemic organs [19]. Additionally, C. jejuni-LOS plays a pivotal role in the manifestation of post-infectious autoimmune sequelae due to the molecular resemblance between LOS and gangliosides found on peripheral nerves [20, 21].

While the majority of C. jejuni infections do not require antibiotic treatment [11], there has been a global rise of antibiotic resistance among Campylobacter species [22, 23]. As a result, research has increasingly shifted focus towards developing new control strategies and identifying alternative substances with anti-Campylobacter and/or anti-inflammatory properties following the golden goal to alleviate symptoms of acute campylobacteriosis, while minimizing the risk of anti-microbial resistance [23, 24]. Therefore, natural substances with a high safety profile and affordable production costs, possessing anti-inflammatory and anti-microbial properties against Campylobacter species, would be highly desirable candidate molecules for treating human campylobacteriosis [25]. Ideally, the substance should selectively target C. jejuni without disrupting the human gut microbiota.

The scarcity of a suitable small animal model that mimics clinical signs and immunopathological responses of human C. jejuni infections limited the state of the art in research focusing on the interactions between C. jejuni and the vertebrate host in the past [26]. Mice for example, possess a natural colonization resistance against C. jejuni due to their specific gut microbiota [27, 28]. Furthermore, even microbiota-depleted wildtype mice do not develop overt clinical signs upon C. jejuni infection as seen in human campylobacteriosis [26], since mice are around 10,000 times more resistant to enteropathogenic LOS if compared to humans or birds [29]. To overcome these limitations, microbiota-depleted IL-10 −/− mice have been generated and proven suitable as a C. jejuni infection and inflammation model of human campylobacteriosis [16, 30–32]. After antibiotic eradication of the murine gut microbiota, animals can be stably colonized with C. jejuni, whereas the il10 gene deficiency abrogates LOS resistance and facilitates the manifestation of campylobacteriosis symptoms in mice comparably to those seen in infected humans. Within a week post-infection (p.i.) the animals develop an acute enteritis syndrome, accompanied by pronounced intestinal, extra-intestinal, and systemic pro-inflammatory responses [16, 30–32]. This C. jejuni infection and inflammation model has already been used successfully for testing the efficacy of various molecules and compounds in preclinical studies to evaluate their anti-bacterial, as well as anti-inflammatory properties and ultimately therapeutic potential during murine campylobacteriosis [33–50].

Organic acids encompass a diverse group of carboxylic acids, each with unique characteristics such as anti-bacterial properties, immune-modulatory effects, and overall health benefits [51]. Benzoic acid is an odorless aromatic carboxylic acid and a natural metabolite found in cinnamon, for instance [52]. Its name originates from the styx tree, where gum benzoin was first isolated and utilized in traditional Chinese medicine [53, 54]. Due to their acidity and broad anti-microbial properties, benzoic acid and its derivatives are often used as additives in foods, beverages, condiments, cosmetics, and medicinal products [52, 55]. The practical application of benzoic acid and its derivatives as preservatives has spanned decades, rendering it a thoroughly studied molecule with a well-documented safety profile [56]. To date, the only approved use of benzoic acid within the medical context is against non-ketotic hyperglycemia and in combination with phenylacetate for the treatment of urea cycle disorders given that this drug combination was shown to effectively facilitate the elimination of ammonia [57, 58]. In addition, benzoic acid exhibits pronounced anti-microbial effects, primarily against yeasts [59], but also extends to various bacteria such as Escherichia coli, Pseudomonas fluorescens, Salmonella spp., Listeria monocytogenes, and Staphylococcus aureus [60–62]. Its anti-bacterial efficacy against C. jejuni has also been noted, particularly when applied in combination with other molecules such as organic acids [61, 63].

Recently, an increasing number of studies have explored potential disease-attenuating effects of benzoic acid beyond its traditional role as a preservative [64]. Studies have shown potent anti-inflammatory capabilities for sodium benzoate when supplemented through the diet of animals [61, 64–67]. In fact, anti-inflammatory effects were attributed to the inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, resulting in reduced pro-inflammatory cytokine release [61, 64–67]. In addition, neuroprotective effects and anti-inflammatory properties in nervous tissues were shown for neurological diseases such as Alzheimer’s disease [68, 69].

Previously, we have investigated benzoic acid in combination with different organic acids, including butyric acid,
caprylic acid, and sorbic acid, for disease-alleviating properties during murine C. jejuni induced enterocolitis. The organic acid combination exhibited pronounced anti-inflammatory effects and ultimately reduced symptoms of C. jejuni induced disease [49]. Therefore, it was of high interest to investigate benzoic acid as a novel therapeutic strategy, now applied individually, in the combat of acute campylobacteriosis. This prompted us to perform a placebo-controlled preclinical intervention study in which microbiota-depleted IL-10−/− mice were infected with C. jejuni, subjected to a therapeutic oral benzoic acid treatment, and the disease outcomes analyzed.

MATERIAL AND METHODS

Gut microbiota depleted IL-10−/− mice

IL-10−/− mice (C57BL/6j background) were bred and housed at the animal husbandry at Charité – Universitätsmedizin, Berlin, Germany. Cages were equipped with filter tops and mice were kept in a semi-barrier environment under standard experimental conditions (23°C room temperature, 55 ± 15% humidity, 12 h light/12 h dark cycle). Animals had free access to autoclaved water (ad libitum) and standard chow (food pellets: ssniff R/M-H, V1534-300, Sniff, Soest, Germany). To eradicate the commensal gut microbiota, 3-week-old female and male mice were transferred to autoclaved cages (on average 3–4 mice per cage) and received an antibiotic treatment with ampicillin plus sulbactam (2 g L−1 plus 1 g L−1, respectively; Dr. Friedrich Eberth Arzneimittel, Ursensollen, Germany) as reported recently [70]. Two days before the first C. jejuni infection, the antibiotic solution was replaced by autoclaved tap water to achieve proper antibiotic washout.

Campylobacter jejuni infection

C. jejuni strain 81-176 (a human clinical isolate) was cultured on selective Karmali agar plates (Oxoid, Wesel, Germany) at 37°C for 48 h under microaerophilic conditions as described earlier [26]. Following cultivation, the bacteria were harvested and suspended in phosphate-buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA, USA) under aseptic conditions. Cardiac blood was taken to isolate serum and bacterial colonies were cultured on selective Karmali agar plates (Oxoid, Wesel, Germany). The inoculated plates were then incubated at 37°C for at least 48 h under microaerophilic conditions (in a jar containing CampyGas Packs; Oxoid, Wesel, Germany) following previous protocols [26]. The limit of detection for viable C. jejuni cells was 100 CFU per g.

Monitoring of clinical conditions of mice

Upon initiation of respective therapeutic treatment and furthermore, immediately before and every day after infection, we conducted quantitative evaluations of the murine clinical conditions using established clinical scoring methodologies as outlined (Table 1, [71]).

Sampling procedures

Following the sacrifice of mice by carbon dioxide asphyxiation on day 6 p.i., samples were collected under strict aseptic conditions. Cardiac blood was taken to isolate serum samples for the measurement of pro-inflammatory mediators. Ex vivo biopsies from kidneys, lungs, mesenteric lymph nodes (MLN), terminal ileum, and colon were collected in addition to luminal samples from stomach, duodenum, ileum, and colon. The lengths of the small intestines were determined minimum inhibitory concentration (MIC) of 977 mg L−1 [63].

Gastrointestinal pathogen loads

The numbers of viable C. jejuni cells were quantified in daily collected fecal samples (day 2–5 p.i.) and additionally, upon necropsy (day 6 p.i.) from intraluminal gastrointestinal specimens. Samples were homogenized in sterile PBS (Thermo Fisher Scientific, Waltham, MA, USA) using a sterile pestle, and serial dilutions were plated on Karmali agar plates (Oxoid, Wesel, Germany). The inoculated plates were then incubated at 37°C for at least 48 h under microaerophilic conditions (in a jar containing CampyGas Packs; Oxoid, Wesel, Germany) following previous protocols [26]. The limit of detection for viable C. jejuni cells was 100 CFU per g.

Table 1: Scoring system to evaluate clinical aspects of campylobacteriosis (maximum 12 points)

<table>
<thead>
<tr>
<th>Clinical aspect</th>
<th>Scores</th>
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<tbody>
<tr>
<td>Wasting symptoms</td>
<td>0: normal</td>
</tr>
<tr>
<td>Stool consistency</td>
<td>0: formed feces</td>
</tr>
<tr>
<td>Fecal blood</td>
<td>0: no blood</td>
</tr>
<tr>
<td>1: ruffled fur</td>
<td>2: pasty feces</td>
</tr>
<tr>
<td>2: less locomotion</td>
<td>4: liquid feces</td>
</tr>
<tr>
<td>3: isolation</td>
<td>4: severely compromised locomotion, pre-final aspect</td>
</tr>
<tr>
<td>4: liquid feces</td>
<td>2: microscopic detection of blood by the Guajac method (Haemoccult, Beckman Coulter/PCD, Krefeld, Germany)</td>
</tr>
<tr>
<td>4: liquid feces</td>
<td>4: macroscopic blood visible</td>
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</tbody>
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Wasting symptoms
- 0: normal
- 1: ruffled fur
- 2: less locomotion
- 3: isolation
- 4: severely compromised locomotion, pre-final aspect

Stool consistency
- 0: formed feces
- 2: pasty feces
- 4: liquid feces

Fecal blood
- 0: no blood
- 2: microscopic detection of blood by the Guajac method (Haemoccult, Beckman Coulter/PCD, Krefeld, Germany)
- 4: macroscopic blood visible
measured from the gastric-duodenal to the ileo-caecal transitions, and the colonic lengths from the beginning of the ascending colon to the anus, both by a ruler.

**Histopathology**

Histopathological analyses were performed in colonic ex vivo biopsies that had been fixed in 5% formalin and embedded in paraffin. 5-μm-thick sections were stained with hematoxylin and eosin (H&E), examined under light microscopy at 100-times magnification, and histopathological changes in the large intestines quantitatively assessed applying a defined histopathological score [72]: Score 0, intact epithelium, no inflammatory cell infiltrates. Score 1, minimal inflammatory cell infiltrates in the mucosa with intact epithelium. Score 2, mild inflammatory cell infiltrates in the mucosa and submucosa with mild hyperplasia and mild goblet cell loss. Score 3, moderate inflammatory cell infiltrates in the mucosa and submucosa with moderate goblet cell loss. Score 4, marked inflammatory cell infiltration into the mucosa and submucosa with marked goblet cell loss, multiple crypt abscesses, and crypt loss.

**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 [73]. In order to detect apoptotic epithelial cells, macrophages/monocytes, neutrophils, T lymphocytes, regulatory T cells, and B lymphocytes, colonic paraffin sections (5 μm) were stained with primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, Beverly, MA, USA; 1:200), F4/80 (no. 14-4801, clone BM8, eBioscience, San Diego, CA, USA; 1:50), MPO7 (no. A0398, Dako, Glostrup, Denmark; 1:500), CD3 (no. N1580, Dako, Glostrup, Denmark; 1:10), FOXP3 (clone FJK-165, no. 14-5773, eBioscience, San Diego, CA, USA; 1:100), and B220 (no. 14-0452-81, eBioscience, San Diego, CA, USA; 1:200). Positively stained cells were quantitated by a blinded independent investigator using light microscopy (400-times magnification). The average number of respective positively stained cells in each sample was determined within at least six high power fields (HPF, 0.287 mm²).

**Pro-inflammatory mediators**

Intestinal ex vivo biopsies were collected from MLN (3 nodes) as well as from the colon and terminal ileum (longitudinally cut strips of approximately 1 cm²), one kidney (one half after the longitudinal cut), and one lung. Samples were washed with sterile PBS (Thermo Fisher Scientific, Waltham, MA, USA) and transferred to 24-flat-bottom well culture plates (Thermo Fisher Scientific, Waltham, MA, USA) containing 500 μL serum-free RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with penicillin and streptomycin (both 100 μg mL⁻¹; Biochrom, Berlin, Germany). After an 18-h incubation period at 37°C, respective culture supernatants and serum samples were tested for tumor necrosis factor-alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) using the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Heidelberg, Germany) and the BD FACSCanto II flow cytometer (BD Biosciences, Heidelberg, Germany).

**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-way ANOVA with Tukey post-hoc test (for normally distributed data) and the Kruskal-Wallis test with Dunn’s post-hoc test (for not normally distributed data) were performed. Two-sided probability (p) values ≤0.05 were considered statistically significant. Experiments were repeated twice.

**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; registration number G0104/19). The clinical conditions of mice were monitored daily.

**RESULTS**

**C. jejuni colonization in the gastrointestinal tract following oral benzoic acid treatment of microbiota-depleted infected mice**

First, we addressed whether benzoic acid treatment interfered with the establishment of the enteropathogen in the gastrointestinal tract of C. jejuni infected mice. Our cultural analyses revealed that the fecal bacterial numbers did not differ between benzoic acid and placebo treated mice between day 2 and 5 p.i. (not significant (n.s.); Fig. 1), which also held true for the C. jejuni loads detected in the stomach, duodenum, terminal ileum, and colon lumen on day 6 p.i. (n.s.; Fig. 2). Hence, benzoic acid treatment did not affect C. jejuni colonization in the gastrointestinal tract upon infection as pathogen loads remained stable throughout the experiments.

**Clinical aspects of campylobacteriosis over time following oral benzoic acid treatment of microbiota-depleted C. jejuni infected mice**

Then, we surveyed the clinical outcome upon benzoic acid treatment of C. jejuni infected mice and quantitated the clinical signs of acute campylobacteriosis such as bloody diarrhea and
wasting symptoms with defined clinical scores. As early as 24 h after initiation of the treatment (i.e., day 3 p.i.; Fig. 3A), mice from the benzoic acid cohort displayed lower campylobacteriosis scores as compared to placebo counterparts, which was also the case on days 4 and 5 p.i. \((P < 0.05; \text{Fig. 3B and C}).\) At the end of the observation period on day 6 p.i., benzoic acid treated mice displayed a trend towards lower campylobacteriosis scores as compared to the placebo group \((\text{n.s. due to high standard deviations}; \text{Fig. 3D}).\) Furthermore, we focused on individual parameters contributing to the overall clinical outcome and found that only placebo control mice displayed \(C.\ jejunii\) induced diarrheal symptoms on days 3, 4, and 5 p.i. \((P < 0.001\ \text{versus naive}),\) whereas benzoic acid treated mice exhibited basal values \((\text{n.s. versus naive}; \text{from Fig. 4A–C}).\) On day 6 p.i., however, a trend towards lower diarrheal scores were recorded in benzoic acid treated animals if compared to placebo counterparts \((\text{n.s.}; \text{Fig. 4D}).\) Notably, infected mice from both treatment cohorts exhibited comparably elevated scores for abundance of fecal blood between day 3 and day 6 p.i. \((\text{n.s.; } P < 0.001\ \text{versus naive}; \text{Fig. 5}).\) In case of wasting symptoms \((\text{Fig. 6}),\) however, respective scores were exclusively elevated in placebo control animals on days 4 and 5 p.i. \((P < 0.001\ \text{versus naive}; \text{Fig. 6B and C}),\) whereas on day 6 p.i., a trend towards lower wasting scores were determined in benzoic acid as compared to placebo treated mice \((\text{n.s.; } \text{Fig. 6D}).\) Hence, benzoic acid treatment alleviated clinical signs, particularly diarrheal and wasting symptoms, during acute campylobacteriosis development.

**Effects of oral benzoic acid treatment on macroscopic and microscopic inflammatory responses in the colon of microbiota-depleted \(C.\ jejunii\) infected mice**

Since intestinal inflammation causes a shrinkage of the inflamed intestine \([74–76]),\) we measured both, the small and large intestinal lengths upon necropsy. On day 6 p.i., we found comparable colonic lengths in mice from both the treatment and placebo cohorts \((\text{n.s.}; \text{Fig. 7A}),\) whereas shorter small intestines were determined in placebo control counterparts only \((P < 0.01\ \text{versus naive}; \text{Fig. 7B})\) indicative for less severe macroscopic inflammatory complications of \(C.\ jejunii\) infection in the small intestinal tract due to benzoic acid treatment. Then, we surveyed inflammatory signs of \(C.\ jejunii\) infection on a macroscopic level and found comparable histopathological changes in the colon on day 6 p.i. irrespective of the treatment regimen \((P < 0.001\ \text{versus naive}; \text{Fig. 8A}).\) Given that apoptosis constitutes a valuable parameter to grade intestinal inflammatory changes, we enumerated apoptotic colonic epithelial cells following immunohistochemical staining of large intestinal paraffin sections with antibodies against cleaved caspase-3. \(C.\ jejunii\) infection was accompanied with multi-fold increased numbers of apoptotic colonic epithelial cell numbers in both treatment groups \((P < 0.01–0.001),\) but to a lesser extent in benzoic acid as compared to placebo challenged mice as shown on day 6 p.i. \((P < 0.05; \text{Fig. 8B}).\) Hence, benzoic acid treatment mitigated apoptotic cell responses in the colonic epithelia following \(C.\ jejunii\) infection.

**Pro-inflammatory immune responses in the intestinal tract following oral benzoic acid treatment of microbiota-depleted \(C.\ jejunii\) infected mice**

Next, we tested whether benzoic acid treatment had immune-modulatory effects and quantitated innate and
adaptive immune cell responses upon *C. jejuni* infection in immunohistochemically stained colonic paraffin sections. Our analyses revealed that increased numbers of innate immune cell populations such as macrophages, monocytes, and neutrophils as well as of adaptive immune cell subsets including T lymphocytes, regulatory T cells, and B lymphocytes could be detected in the colonic mucosa and lamina propria of mice from both treatment groups on day 6 p.i. (*P* < 0.01–0.001 versus naive; Fig. 9). The *C. jejuni* induced increases in T cell numbers were, however, slightly less pronounced following benzoic acid treatment as compared to the control group (*P* < 0.05; Fig. 9C). Furthermore, we measured

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**Fig. 3.** Clinical outcome of campylobacteriosis over time following oral benzoic acid treatment of *C. jejuni* infected microbiota-depleted IL-10−/− mice. Microbiota-depleted IL-10−/− mice were orally infected with *C. jejuni* strain 81-176 on day (d) 0 and d1 and treated with benzoic acid (BEN) via the drinking water (*ad libitum*) starting on d2 post-infection or received placebo (PLC) instead. Naïve mice were used as untreated and uninfected controls. Clinical outcome of campylobacteriosis comprising wasting and bloody diarrhea were quantitated with defined clinical scores (see methods) on (A) d3, (B) d4, (C) d5, and (D) d6 post-infection. Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (red bar in boxes), significance levels (*P* values, calculated by the Kruskal-Wallis test with Dunn's post-hoc test), and mouse numbers (in parentheses) are shown. Data were pooled from three independent experiments.
pro-inflammatory mediators in explants from distinct intestinal compartments and found lower TNF-α concentrations in MLN taken from benzoic acid as compared to placebo treated mice on day 6 p.i. (P < 0.05; Fig. 10A). In addition, increases in ileal MCP-1 concentrations induced by C. jejuni were observed in infected mice from the placebo control group, whereas benzoic acid treated mice displayed basal values on day 6 p.i. (n.s. versus naive; Fig. 10B). Hence, benzoic acid treatment resulted in less distinct C. jejuni induced pro-inflammatory immune responses.

Fig. 4. Diarrheal symptoms over time following oral benzoic acid treatment of C. jejuni infected microbiota-depleted IL-10<sup>−/−</sup> mice. Microbiota-depleted IL-10<sup>−/−</sup> mice were orally infected with C. jejuni strain 81-176 on day (d) 0 and d1 and treated with benzoic acid (BEN) via the drinking water (ad libitum) starting on d2 post-infection or received placebo (PLC) instead. Naive mice were used as untreated and uninfected controls. Diarrheal symptoms were quantitated with defined clinical scores (see methods) on (A) d3, (B) d4, (C) d5, and (D) d6 post-infection. Box plots (25th and 75th percentiles), whiskers (minimum and maximum values), medians (red bar in boxes), significance levels (P values, calculated by the Kruskal-Wallis test with Dunn’s post-hoc test), and mouse numbers (in parentheses) are shown. Data were pooled from three independent experiments.
Effects of oral benzoic acid treatment on extra-intestinal including systemic pro-inflammatory mediator secretion of microbiota-depleted *C. jejuni* infected mice

Furthermore, we addressed whether oral benzoic acid treatment also exerted an anti-inflammatory effect in extra-intestinal including systemic compartments during acute campylobacteriosis. Therefore, we measured TNF-α concentrations in kidney and lung explants taken on day 6 p.i. We found that *C. jejuni* induced increases in placebo control mice only ($P < 0.05–0.01$), whereas mice from the benzoic acid cohort exhibited basal values (n.s. versus naive; Fig. 11). In addition, benzoic acid treated mice
displayed approximately 50% lower median TNF-α serum concentrations if compared to placebo counterparts on day 6 p.i. (n.s. due to high standard deviations; Fig. 12A), whereas MCP-1 levels were exclusively increased in sera taken from infected placebo controls ($P < 0.001$ versus naive; Fig. 12B) with basal values measured in the benzoic acid cohort (n.s. versus naive; Fig. 12B). Hence, benzoic acid exerts its anti-inflammatory effects during acute campylobacteriosis also in extra-intestinal and even systemic compartments.

**DISCUSSION**

Here, we investigated the potential of benzoic acid as an antibiotic-independent therapeutic disease-alleviating strategy...
in acute campylobacteriosis applying microbiota-depleted IL-10−/− mice. The fact that the 4-day treatment did not reduce C. jejuni bacterial loads alongside the gastrointestinal tract indicated that our treatment regimen was not selective for C. jejuni variants resistant to benzoic acid. However, improved clinical conditions of mice, accompanied by alleviated macroscopic and microscopic inflammatory responses, as well as lessened intestinal and extra-intestinal including systemic pro-inflammatory mediator secretion were observed. Even though the concentrations of the here administered benzoic acid solution (i.e., 3,900 mg L−1) exceeded the previously determined MIC (i.e., 977 mg L−1) by 4-fold, the gastrointestinal pathogen loads of C. jejuni infected mice could not be reduced. Possible explanations for the lack of...
overt anti-

C. jejuni effects may include dilution effects by the intestinal secretions or premature metabolization of the molecule before reaching its target organs. Benzoic acid undergoes rapid metabolism in the liver and kidneys, where it is conjugated with glycine and glucuronic acid, ultimately being eliminated through the urine within 24 h [55]. One might want to take into consideration that a longer duration of treatment starting prior infection, for instance, may have impacted gastrointestinal enteropathogen numbers. It is hence possible that inadequate dosage or insufficient treatment duration might explain the absence of anti-bacterial effects against intestinal C. jejuni in our present study.

Despite comparably high intestinal pathogen loads, benzoic acid treated mice were clinically less compromised upon C. jejuni infection within 24 h after initiating the treatment regimen if compared to placebo controls as indicated by lower overall campylobacteriosis scores in the former versus the latter. It is tempting to speculate that benzoic acid could have influenced the expression of certain pathogenicity factors of C. jejuni, such as those coding for bacterial motility, adhesion, and invasion. Previously, sodium benzoate was shown to impact the transcription of the phosphate-specific transport system in E. coli, which is also a virulence factor for C. jejuni [77]. Therefore, oral benzoic acid could potentially lead to a reduced ability of the pathogens to produce lipo-polysaccharides and phospholipids in the cell membrane [78]. Furthermore, benzoic acid treatment particularly alleviated diarrheal and wasting symptoms but did not affect the abundance of fecal blood in infected mice, whereas a trend towards reduced fecal blood scores could be observed as early as day 3 p.i. The improved clinical outcome observed here aligns with previous studies that reported beneficial effects on gut motility, gastrointestinal functionality, intestinal morphology, and overall productivity after supplementing benzoic acid in the diet of farm animals [79–81].

The improved clinical symptoms observed in our study corresponded with less severe microscopic inflammatory complications of infection. Whereas the C. jejuni induced histopathological changes did not differ between
the two infected cohorts, apoptotic cell responses in colonic epithelia were significantly less pronounced upon benzoic acid treatment. This is particularly noteworthy, as the compromised intestinal epithelial barrier upon C. jejuni infection leads to the so-called “leaky gut disease” characterized by diarrhea and malabsorption during campylobacteriosis [52, 82] that could be alleviated by a 4-day course of oral benzoic acid application, however. In addition, recent reports revealed enhanced/preserved gut epithelial barrier function upon benzoic acid application due to an up-regulated expression of tight junction proteins thereby stabilizing the intestinal epithelial integrity and restoring gut epithelial function [52, 83].

Furthermore, oral benzoic acid application had an immune-modulatory effect within the intestinal tract given lower T cell numbers in the infected colonic mucosa and lamina propria that was accompanied by less distinct secretion of pro-inflammatory mediators including TNF-α and MCP-1 in the MLN and ileum, respectively, as measured in mice from the verum versus placebo cohort on day 6 p.i. Our findings align with prior research demonstrating that sodium benzoate treatment of rats suffering...
from rheumatoid arthritis shifts the balance of T cell subsets towards protective responses, suggesting its therapeutic potential for Th1- and Th17-mediated diseases due to dampened pro-inflammatory cytokine secretion [84]. In addition, a study investigating the effects of oral sodium benzoate on pro-inflammatory markers in the hearts tissues of rats found significant reductions in TNF-α and IL-6 levels suggesting a potential modulation of the pro-inflammatory signaling pathways [85].

Remarkably, the disease-alleviating effects of oral benzoic acid during acute campylobacteriosis were not restricted to the intestinal tract but could also be observed in extra-intestinal and even systemic organs as indicated by TNF-α concentrations in the kidneys and lungs that were only increased in C. jejuni infected of mice from the placebo, but not the benzoic acid treatment group. This aligns with a recent study showing kidney protective effects for benzoate, as the oral substitution of sodium benzoate was able to mitigate nephropathy in mice [86]. Remarkably, our study revealed even systemic anti-inflammatory effects of oral benzoic acid application as MCP-1 only increased in the sera of placebo control mice, whereas naive values were obtained upon organic acid treatment on day 6 p.i.

It is well known that C. jejuni-LOS induced TLR-4 mediated signaling pathways play critical roles in the immunopathogenesis of acute campylobacteriosis mounting in an hyper-activation of the innate immune system upon enteropathogenic infection [87]. A recent study suggests, however, that benzoate exerts its anti-inflammatory effects primarily independently from TLR-4, and instead directly targets NF-κB signaling [86].

The optimal therapeutic benzoic acid dosage for the oral treatment of campylobacteriosis needs careful consideration, given that excessive administration increases the risk of toxicity and adverse events. For example, one study revealed beneficial effects in piglets following supplementation of the diet with 0.5% benzoic acid, whereas growth retardation, hematological abnormalities, and organ injury were noticed at higher benzoic acid concentrations of 2.5% and 5.0% indicating potential hazards associated with excessive benzoic acid intake [87]. The lethal dosage for orally ingested benzoic acid has been reported with LD50 values exceeding 2,000 mg kg⁻¹ body weight [88]. In comparison, the daily dosage we administered per mouse for 4 days (i.e., 781.5 mg kg⁻¹ body weight) was much lower and might be considered as safe. Future research is needed, however, to address the appropriate dosage of benzoic acid when planning clinical trials, particularly considering age, renal functions, and indirect intake via processed food products, for instance.

CONCLUSIONS

Our actual preclinical placebo-controlled intervention trial provides evidence that benzoic acid constitutes a promising antibiotics-independent and immune-modulatory therapeutic option to alleviate acute campylobacteriosis and to reduce the risk for the development of post-infectious autoimmune morbidities.

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.

Funding: This work was supported by grants from the German Federal Ministries of Education and Research (BMBF) in frame of the zoonoses research consortium PAC-
Campylobacter to MMH and SB (IP7/01KI2007/D) and from the Federal Ministry for Economic Affairs and Energy following a resolution of the German National Parliament, Deutscher Bundestag to MMH and SB (ZIM, ZF4117908 AJ8). The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Authors’ contributions: KD: Performed experiments, analyzed data, critically discussed results, co-wrote the paper.
SM: Performed experiments, analyzed data, critically discussed results, co-wrote the paper.
MSF: Performed experiments.
SB: Provided advice in experimental design, critically discussed results, co-wrote the paper.
MMH: Designed and performed experiments, analyzed data, critically discussed results, wrote the paper.

ACKNOWLEDGEMENTS

We thank Alexandra Bittroff-Leben, Ines Puschendorf, Ulrike Fiebiger, Sumaya Abdul-Rahman, Gernot Reifenberger, and the staff of the animal research facility at the FEM of Charité - Universitätsmedizin Berlin for excellent technical assistance and animal breeding.

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