Impact of dietary sulfolipid-derived sulfoquinovose on gut microbiota composition and inflammatory status of colitis-prone interleukin-10-deficient mice

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A B S T R A C T

The interplay between diet, intestinal microbiota and host is a major factor impacting health. A diet rich in unsaturated fatty acids has been reported to stimulate the growth of *Bilophila wadsworthia* by increasing the proportion of the sulfonated bile acid taurocholate (TC). The taurine-induced overgrowth of *B. wadsworthia* promoted the development of colitis in interleukin-10-deficient (IL-10$^{-/-}$) mice. This study aimed to investigate whether intake of the sulfonates sulfoquinovosyl diacylglycerols (SQDG) with a dietary supplement or their degradation product sulfoquinovose (SQ), stimulate the growth of *B. wadsworthia* in a similar manner and, thereby, cause intestinal inflammation. Conventional IL-10$^{-/-}$ mice were fed a diet supplemented with the SQDG-rich cyanobacterium *Arthrospira platensis* (Spirulina). SQ or TC were orally applied to conventional IL-10$^{-/-}$ mice and gnotobiotic IL-10$^{-/-}$ mice harboring a simplified human intestinal microbiota with or without *B. wadsworthia*. Analyses of inflammatory parameters revealed that none of the sulfonates induced severe colitis, but both, Spirulina and TC, induced expression of pro-inflammatory cytokines in cecal mucosa. Cell numbers of *B. wadsworthia* decreased almost two orders of magnitude by Spirulina feeding but slightly increased in gnotobiotic SQ and conventional TC mice. Changes in microbiota composition were observed in feces as a result of Spirulina or TC feeding in conventional mice. In conclusion, the dietary sulfonates SQDG and their metabolite SQ did not elicit bacteria-induced intestinal inflammation in IL-10$^{-/-}$ mice and, thus, do not promote colitis.

1. Introduction

The incidence of inflammatory bowel diseases (IBD) is rising worldwide (Ng et al., 2017). Risk factors include genetic predisposition, a Westernized lifestyle and a detrimental bacterial composition in the gut (Chiba et al., 2019; Piovani et al., 2019). To mimic the genetic predisposition for IBD, different animal models of colitis have been generated over the years (Kiesler et al., 2015). The interleukin-10-deficient (IL-10$^{-/-}$) mouse model develops spontaneous chronic colitis under specific pathogen-free (SPF) conditions, which is mediated by a lack of anti-inflammatory IL-10 (Kühn et al., 1993;

Shouval et al., 2014). Since germ-free IL-10$^{-/-}$ mice do not develop colitis, intestinal bacteria were identified as a pivotal factor in disease development (Sellon et al., 1998). The microbial community in the gut, referred to as intestinal microbiota, can be influenced favorably or adversely by the diet ingested by the host (David et al., 2014). For example, it has been shown that a diet rich in fiber increases the abundance of bacteria that produce short chain fatty acids in the colon, which promotes host health (Bishehsari et al., 2018). Conversely, a diet rich in saturated fatty acids fed to mice shifted the bile composition towards a greater proportion of taurocholate (TC) and, thereby, stimulated the growth of the sulfite-reducing pathobiont *Bilophila*

wadsworthia. The overgrowth of *B. wadsworthia* was accompanied by an increased incidence and severity of colitis in IL-10$^{-/-}$ mice. This colitogenic effect was even more pronounced when the sulfonate TC was orally applied to IL-10$^{-/-}$ mice fed a low-fat diet (Devkota et al., 2012) (Fig. 1). Based on these findings, the question arose whether sulfonates ingested with diet may also stimulate the growth of *B. wadsworthia* and, thus, promote intestinal inflammation in a susceptible host.

Widespread sulfonates in nature are sulfolipids present in photosynthetic organisms. Dietary sources rich in sulfolipids are green leafy vegetables and cyanobacteria, some of which are used as functional supplements. Main representatives of sulfolipids in such sources are sulfoquinovosyl diacylglycerols (SQDG, structure in Fig. 1). Consumption of high amounts of green vegetables or cyanobacteria such as *Arthrospira platensis* (commercially known as Spirulina) as dietary supplements may increase the availability of sulfonates in the intestinal tract of humans. The sulfoquinovosidase cleaves SQDG to release the sulfonated glucose derivative sulfoquinovose (SQ, structure in Fig. 1). The enzyme was identified in *Escherichia coli* K-12 and characterized (Denger et al., 2014; Speciale et al., 2016). Moreover, *E. coli* K-12 was shown to degrade SQ *via* sulfofructose-1-phosphate to 2,3-dihydroxypropane-1-sulfonate (DHPS, structure in Fig. 1) and dihydroxyacetone phosphate by the sulfo-Embden-Meyerhof-Parnas (sulfo-EMP) pathway (Denger et al., 2014). The corresponding gene cluster was identified in *E. coli* K-12 MG1655 but is also present in many other *E. coli* strains and a wide range of *Enterobacteriaceae* (Denger et al., 2014; Li et al., 2020). A second sulfoglycolytic pathway was first described in *Pseudomonas putida* SQ1 and seems to be active in other respiring α-, β- and γ-proteobacteria (Felux et al., 2015). This sulfo-Entner-Doudoroff (sulfo-ED) pathway degrades SQ *via* 6-deoxy-6-sulfogluconate to sulfolactate and pyruvate. A third pathway for SQ degradation involving a novel 6-deoxy-6-sulfofructose transaldolase and resulting in formation of fructose-6-phosphate and sulfolactate has recently been reported in *Bacillus* strains (Frommeyer et al., 2020; Liu et al., 2020a). Corresponding gene clusters of this transaldol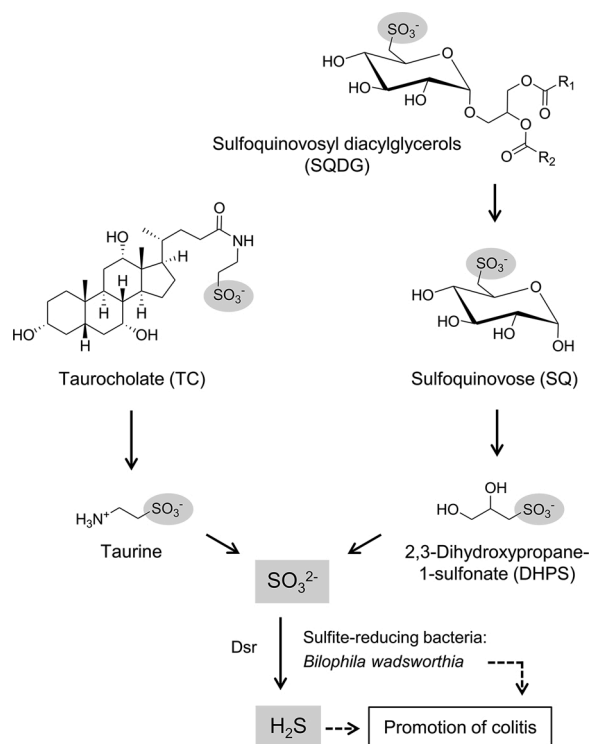ase pathway were identified in a number of environmental and intestinal Firmicutes species. However, strictly anaerobic clostridia among those bacteria seem to produce DHPS instead of sulfolactate by employing a sulfolactaldehyde reductase similar to that found in the sulfo-EMP pathway (Frommeyer et al., 2020). Thus, it is conceivable that DHPS formed from SQ by intestinal *E. coli* or Firmicutes strains is released into the intestinal lumen, where it may serve as a sulfite source for *B. wadsworthia* or other sulfite-reducing bacteria, similar to taurine. While utilization of taurine by *B. wadsworthia* has been known for a long time (Laue et al., 1997), degradation of DHPS by this bacterium and the enzymes involved have been reported only recently (Liu et al., 2020b). Sulfonate-dependent stimulation of growth of these sulfite-reducing bacteria may promote intestinal inflammation, either owing to their cell-based immune-activating properties or the increased production of hydrogen sulfide (H$_2$S) in the colon (Fig. 1). The role of H$_2$S has been discussed controversially, ranging from health-promoting (Faller et al., 2018) to toxic (Leschelle et al., 2005) effects. Recent studies argue in favor of a concentration-dependent mode of action of H$_2$S with beneficial effects at physiological doses and detrimental consequences evoked by high concentrations (Beaumont et al., 2016). *B. wadsworthia* has been linked to various types of inflammation since its discovery by Baron et al. in 1989, as it was isolated from appendicitis, abscesses and other inflamed tissues (Baron et al., 1989; Finegold et al., 1992). The potential of *B. wadsworthia* to induce a T helper 1 (Th1) cell-mediated inflammatory response, metabolic dysfunctions and even systemic inflammation in its host has been described earlier (Devkota et al., 2012; Feng et al., 2017; Natividad et al., 2018), but the underlying mechanisms have not been elucidated.

Evaluation of the impact of specific diets or dietary components on gut microbiota composition is challenging, because of this ecosystem's complexity, inter-individual differences and changes over time. Therefore, minimal consortia of representative gut bacterial species have been used as standardized models for mechanistic studies (Basic and Bleich, 2019). We previously established a simplified human intestinal microbiota (SIHUMI) in rodents that mimics metabolic key functions of the human gut microbiota (Becker et al., 2011). The SIHUMI consortium consists of eight bacterial species, which are prominent members of the human intestinal microbiota and are stably transferred from one generation of mice to another. This minimal consortium may be further expanded by additional bacterial species to assess their functional role in a more complex but defined intestinal setting.

In the present study, we investigated the effects of diet-derived sulfonates, SQDG, and their degradation product SQ on gut microbiota composition and consequences for the host health. Therefore, we fed conventional IL-10$^{-/-}$ mice an SQDG-rich Spirulina diet and orally treated both conventional and gnotobiotic IL-10$^{-/-}$ mice with SQ. Subsequent analyses focused on the assessment of intestinal inflammation and abundance of *B. wadsworthia*.

2. Materials and methods

2.1. Bacterial growth conditions

Bilophila wadsworthia DSM 11045 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *B. wadsworthia* was grown under anoxic conditions at 37 °C in a liquid culture medium adapted from da Silva et al. (da Silva et al., 2008) (DS medium), which contained 19 mM NH$_4$Cl, 17 mM NaCl, 2 mM MgCl$_2$, 7 mM KCl, 0.3 mM CaCl$_2$, 1 mM K$_2$HPO$_4$, 40 mM sodium DL-lactate, 40 mM sodium formate, yeast extract (3.5 mg/L), selenite-tungstate solution (1 mL/L, see DSMZ medium 385, www.dsmz.de), trace element solution (1 mL/L, see DSMZ medium 320), 1,4-naphthoquinone (200 µg/L) and 2 µM resazurin. The medium was adjusted to pH 7.4, gas flashed with N$_2$/CO$_2$ (80/20, v/v) and autoclaved in gas-tight Hungate tubes. Directly before use, Ti(III) nitrilotriacetate (20 mL/L, Moench and Zeikus, 1983), seven-vitamin solution (1 mL/L, see

Fig. 1. Conversion of sulfonates by gut bacteria and potentially associated colitogenic effects. R$_1$ and R$_2$ indicate fatty acids. Dsr, dissimilatory sulfite reductase.

DSMZ medium 503), 30 mM $NaHCO_3$ and 20 mM taurine were sterile filtered and added to the medium. Members of the SIHUMI consortium from the strain collection of the German Institute of Human Nutrition Potsdam-Rehbruecke (DIfE) were cultivated anoxically in Brain Heart Infusion (BHI) broth (Roth), supplemented with yeast extract (5 g/l, Roth) and 5 hemin (mg/l, Serva) (YH-BHI medium). To grow *Clostridium ramosum,* YH-BHI medium was mixed in equal parts with Yeast Casitone Fatty Acid (YCFA) medium (DSMZ medium 1611).

2.2. Fecal incubation experiments

Fresh fecal samples from four IL-10$^{-/-}$ mice each were collected, transferred into an anaerobic workstation (M85, Don Whitley Scientific, Shipley, UK) and individually 10-fold diluted (w/v) in DS medium without taurine. Following low-speed centrifugation (300 x g, 4 °C, 5 min), 1 mL fecal suspension was injected into a Hungate tube containing 9 mL DS medium supplemented with 4 mM SQ (MCAT, Donaueschingen, Germany), 20 mM sodium TC (Roth) or without sulfonates. Incubations of SQ and TC in the absence of fecal suspensions were included as controls. All incubations were performed in duplicates. The tubes were incubated at 37 °C for 168 h. Samples (250 μL) were withdrawn for sulfide quantification using the methylene blue method (Strocchi et al., 1992) at 0 h and 168 h and immediately transferred into reaction tubes containing 25 μL 327 mM ZnAc and 5 μL 100 mM NaOH. To these mixtures, 20 μL detection reagent (per liter, 3.45 g N,N-dimethyl-1, 4-phenylenediamine x 2 HCl, 6 g Fe (III)-chloride x 6 H_2O dissolved in 18.5 % HCl) was added, incubated for 20 min and centrifuged (12 000 x g, 23 °C, 3 min). Supernatants were transferred to 96-well plates and analyzed at 670 nm (Infinite M200 PRO, Tecan). Sulfide concentrations were calculated based on a calibration curve prepared from Na_2S solutions (40–200 mM) treated in the same way as the samples. If necessary, samples were diluted with distilled water.

2.3. Animal experiments

Animal experiments were approved by the State Office for Occupational Safety, Consumer Protection and Health of the State of Brandenburg, Germany (approval number: 2347−10-2016). The experiments were conducted with 10- to 12-week-old conventional and gnotobiotic C57BL/6.129P2-Il10^{tm1Cgn} (IL-10$^{-/-}$) mice. The IL-10 gene disruption was confirmed for each mouse by genotyping using DNA extracts of the tail tip and performing specific PCR runs targeting wild type and mutant gene variants. Conventional male mice were bred under SPF conditions in the animal facility of the DIfE. Mice were housed in individually ventilated cages and kept in a 12 h light-dark cycle at 22 ± 2 °C and 55 ± 5% air humidity. Feed (Ssniff standard chow A153 F0300 Am R/M−H; Ssniff, Soest, Germany) and water were applied *ad libitum.*

Male and female gnotobiotic mice harbored the SIHUMI consortium from birth on. This community consists of eight bacterial species: *Anaerostipes caccae* DSM 14662^{T}, *Bacteroides thetaiotaomicron* DSM 2079^{T}, *Bifidobacterium longum* NCC 2705, *Blautia producta* DSM 2950^{T}, *Clostridium butyricum* DSM 10702^{T}, *Clostridium ramosum* DSM 1402^{T}, *Escherichia coli* K-12 MG1655 and *Lactobacillus plantarum* DSM 20174^{T}. Mice were kept under the same environmental conditions as the conventional animals but in sterile positive-pressure isolators (Metall & Plastik, Radolfzell, Germany). Wood chips and feed (Altromin fortified type 1310; Altromin, Lage, Germany) were irradiated with 50 kGy (Synergy Health Radeberg, Radeberg, Germany). Drinking water was autoclaved twice and applied *ad libitum.*

For the feeding experiments, 24 conventional mice were adapted to an irradiated semisynthetic diet (control diet, Supplementary Table 1) for two weeks. Subsequently, 12 mice were switched to an irradiated diet containing 20 % Spirulina powder (Spirulina diet, Supplementary Table 1). The remaining 12 mice kept on consuming the control diet. The two diets were prepared to contain comparable amounts of macronutrients and minerals by considering their levels present in the Spirulina supplement (Supplementary Table 1). The similar caloric content of CD and SD was subsequently confirmed by bomb calorimetry (C5003 calorimeter, IKA, Staufen, Germany).

For experiments involving conventional mice gavaged with pure compounds, 34 animals were fed the control diet and allocated to three groups. Following a two-week adaptation phase, mice were orally gavaged with equimolar doses of SQ (0.45 g/kg body weight, n = 12) or sodium TC (1 g/kg body weight, n = 10) or with water as vehicle control (n = 12). SQ and water were applied daily, whereas TC was given twice per week to avoid toxic effects.

For experiments with gnotobiotic mice and pure compounds, 48 SIHUMI mice were adapted to the control diet for two weeks. Thereafter, 24 SIHUMI mice were transferred to a separate isolator and additionally colonized with *B. wadsworthia* by orally gavaging 10^8 cells in 150–200 μl culture medium. Sterile medium was orally applied to the remaining 24 mice. The successful colonization with *B. wadsworthia* was confirmed one day after inoculation by qPCR with DNA extracted from fecal samples. Cell numbers ranged between 9.4 and 9.9 log/g feces. Both the SIHUMI and SIHUMI + *B. wadsworthia* (SIHUMI + Bw) groups were divided into three subgroups that were orally gavaged with SQ (0.45 g/kg body weight, n = 8), sodium TC (1 g/kg body weight, n = 8) or water (n = 8) twice per week.

Body weight and feed intake were recorded twice per week. Fresh fecal samples were collected before start of intervention (0 d) and after 18 days of intervention (18 d) and stored at – 20 °C until being processed for 16S rRNA gene sequencing (see below). At day 14 of the intervention, a colon permeability test (see below) was performed. After three weeks of intervention, mice were euthanized with isoflurane and blood was withdrawn by cardiac puncture into sterile EDTA-coated monovettes (Sarstedt). The tip of the cecum including its content was fixed in Carnoy's solution and embedded in paraffin for later histological assessment. Contents of the remaining part of the cecum were collected and the mucosa scraped. Mucosa samples were snap-frozen in liquid nitrogen and stored at - 80 °C. Intestinal contents were kept on ice until storage at - 20 °C. Blood was centrifuged (2 000 x g, 4 °C, 10 min), and the plasma stored at - 80 °C.

2.4. Colon permeability test

Colonic permeability was assessed with the fluorescein isothiocyanate (FITC)-dextran assay (Volynets et al., 2016) after two weeks of intervention. Briefly, mice were fasted for three hours and subsequently received an oral dose of 4 kDa FITC-dextran (600 mg/kg body weight, Sigma) in phosphate-buffered saline (PBS) or PBS for blank measurements. Four hours later, blood samples were withdrawn from the retrobulbar capillary plexus into heparinized microvettes (Sarstedt). Plasma was gained after centrifugation (2 000 x g, 4 °C, 5 min). The fluorescence measurements were performed as described previously (Woting and Blaut, 2018). Mean fluorescent values of mice receiving PBS were subtracted from those determined for FITC-dextran-treated mice.

2.5. Histopathological assessment

Slices of paraffin-embedded cecal and colonic tissues were cut, stained with hematoxylin and eosin and examined in a standardized and blinded fashion. The histopathology score included evaluation of the lumen, surface epithelium, lamina propria and submucosa and reached from zero (no inflammation in any region) to 28 (severe inflammation in all four regions) (Ring et al., 2019).

2.6. Gene expression analysis

Total RNA was extracted from cecal and colonic mucosa samples using the peqGOLD TriFast™ reagent (Peqlab, Erlangen, Germany)

according to the manufacturer's instructions. Genomic DNA was degraded by treatment with DNAse I (Thermo Fisher Scientific), and its removal controlled by qPCR using the TaqMan Universal PCR master mix (Thermo Fisher Scientific) with primers (Eurofins Genomics) targeting the 18S rRNA gene (Katterle et al., 2008). RNA integrity was assessed by performing a denaturing gel electrophoresis in the presence of ethidium bromide. Subsequent synthesis of complementary DNA (cDNA) was done using 1 μg of RNA and the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific). Each qPCR reaction mix contained 2.5 μL Power SYBR Green master mix (Thermo Fisher Scientific), 0.5 μL each forward and reverse primer (3 μM each, Supplementary Table 2; Eurofins Genomics), 0.5 μL diethyl pyrocarbonate (DEPC)-treated water and 1 μL cDNA solution corresponding to 5 ng of original RNA. The Viia™ 7–Real-Time PCR System (Applied Biosystems) was employed with the QuantStudio Real-Time PCR System software version 1.3 (Thermo Fisher Scientific). Thermal cycling conditions of the qPCR reactions were: polymerase activation at 94 °C for 4 min, 40 cycles of denaturation at 94 °C for 15 s and annealing/elongation at 60 °C for 30 s. Specificity of the amplification was confirmed by melting curve analysis. Gene expression levels were calculated by the $\Delta\Delta CT$ method (Pfaffl, 2001) with the gene encoding hypoxanthine guanine phosphoribosyl transferase (*Hprt*) as the housekeeping gene and relative to the respective control group normalized to the value one.

2.7. Cytokine analysis

Enzyme-linked immunosorbent assays (ELISA) were performed with mouse-specific kits quantifying tumor necrosis factor (TNF)-α (BMS607−3), interferon (IFN)-γ (KMC4021), serum amyloid (SA) A (KMA0021) (all from Thermo Fisher Scientific) or lipocalin (LCN) 2 (MLCN20; R & D Systems, Minneapolis, MN, USA) in plasma samples. Procedures and quantification were performed in accordance with the manufacturer's instructions.

2.8. Quantification of bacterial cells

DNA was extracted from cecal and colonic contents and fecal samples using the PSP Spin Stool DNA kit (Invitek, Berlin, Germany) by following the manufacturer's instructions. For each qPCR reaction, 2.5 μL QuantiNova SYBR Green PCR master mix, 0.025 μL ROX reference dye (both from Qiagen, Hilden, Germany), 0.5 μL each forward and reverse primer (3 μM each, Supplementary Table 3; Eurofins Genomics) and 0.475 μL water were combined with 1 μL extracted DNA. The thermal conditions for the qPCR reactions were as described for the gene expression analysis, with individual adjustments for the annealing and elongation temperature (Supplementary Table 3). Reactions were run using the same real-time PCR system as described above for gene expression analysis. Bacterial counts were determined according to standard curves based on samples with known cell numbers. Therefore, cells in fresh liquid cultures were counted microscopically using a Thoma counting chamber. Volumes containing 10^8 (for *B. wadsworthia*) or 10^9 (for SIHUMI bacteria) cells were pelleted (14 000 x g, 4 °C, 5 min). Sterile feces from germ-free mice were spiked with the cell pellet, and DNA was extracted as described above. Serial dilutions ranging from 10^9 or 10^8 to 10^3 cells were prepared and included in each qPCR run. All samples and standards were analyzed in triplicates. Specificity of amplification was tested by melting curve analysis, and samples showing unspecific amplification were excluded. Cell numbers were calculated per sample wet weight. As low cell numbers of *L. plantarum* were not detectable by qPCR, the presence of this bacterium was verified by plating fecal suspensions on Rogosa agar (Oxoid).

2.9. Analysis of microbiota composition

DNA was extracted from cecal content and feces followed by sequencing of the V3 and V4 regions of the 16S rRNA gene (ZIEL Core Facility Microbiome/NGS, Technical University Munich, Germany) using the Illumina technology described previously (Lagkouvardos et al., 2015). Sequences were further processed using the Integrated Microbial Next Generation Sequencing (IMNGS) platform (Lagkouvardos et al., 2016) and the Rhea software (Lagkouvardos et al., 2017). Non-metric Multidimensional Scaling (NMDS) plots visualize β-diversity.

2.10. Statistical analyses

The distribution of data was tested for normality using the Kolmogorov-Smirnov test. If a data set passed the normality test, it is presented as mean ± SEM and analyzed for differences using the Student's *t*-test. If not, the data set is shown as median ± 95 % CI and the Mann-Whitney *U* test was applied. Outliers were identified using the ROUT method (Q = 0.1 %) and excluded. Sets of data were considered significantly different at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and $****P < 0.0001$. The software used for statistical analysis and visualization was Graph Pad Prism 8.2.1 (GraphPad Software, La Jolla, CA, USA). For analysis of β-diversity of 16S rRNA gene sequencing data, generalized UniFrac was applied with *P* values from the PERMANOVA test, and the Bonferroni-Hochberg test was used to correct for multiple testing.

3. Results

3.1. Intestinal microbiota of conventional mice converted SQ and TC to sulfide

Whether the intestinal microbiota of conventional IL-10$^{-/-}$ mice is capable of liberating sulfide from SQ or TC was investigated by incubating fecal suspensions individually with either sulfonate under anoxic conditions for 168 h. The fecal microbiotas of all four donor mice catalyzed the formation of sulfide from both SQ and TC. The mean estimated concentrations of sulfide formed from SQ and TC in fecal incubations were 6.6 ± 0.6 mM and 5.2 ± 1.6 mM, respectively (Supplementary Fig. 1). No sulfide was observed in fecal incubations without sulfonates or *vice versa*.

3.2. Spirulina feeding modulated immune-related factors and bacterial abundance

To investigate whether SQDG stimulates the growth of colitogenic *B. wadsworthia*, conventional IL-10$^{-/-}$ mice were fed a diet supplemented with 20 % Spirulina (SD), which is particularly rich in SQDG compared to other sources, or an isocaloric control diet (CD) for 3 weeks. Within the first three days, body weight gain and feed consumption of SD-fed mice were increased compared to CD-fed mice (Fig. 2A, B). Thereafter, the body weight of the SD group remained at an elevated level until day 18, but did not differ significantly from the body weight of the CD group at the end of the experiment on day 21 (Fig. 2A). From day seven on until the end of the intervention phase, the feed consumption of SD-fed mice decreased to amounts similar to those observed for CD-fed mice (Fig. 2B). Histopathological scores of cecal tissue obtained after 21 d of intervention were slightly elevated in SD-fed mice (mean score 2.9 ± 0.4) compared to CD-fed mice (mean score 0.8 ± 0.5) (Fig. 2C). The increased histopathology score was mainly due to mild to moderate immune-cell infiltration into the lamina propria of the cecum. In addition, expression of *Tnf-α* in the cecal mucosa of SD-fed animals was 2.4-fold increased, while gene expression of other cytokines did not differ between the SD and CD groups (Fig. 2D). Systemic inflammation was not observed as plasma concentrations of the inflammatory markers TNF-α, IFN-γ, SAA, and LCN 2 were not significantly increased in the SD compared to the CD group (Fig. 2E). Colon permeability was not affected by Spirulina feeding as determined with the FITC-dextran assay (Fig. 2F).

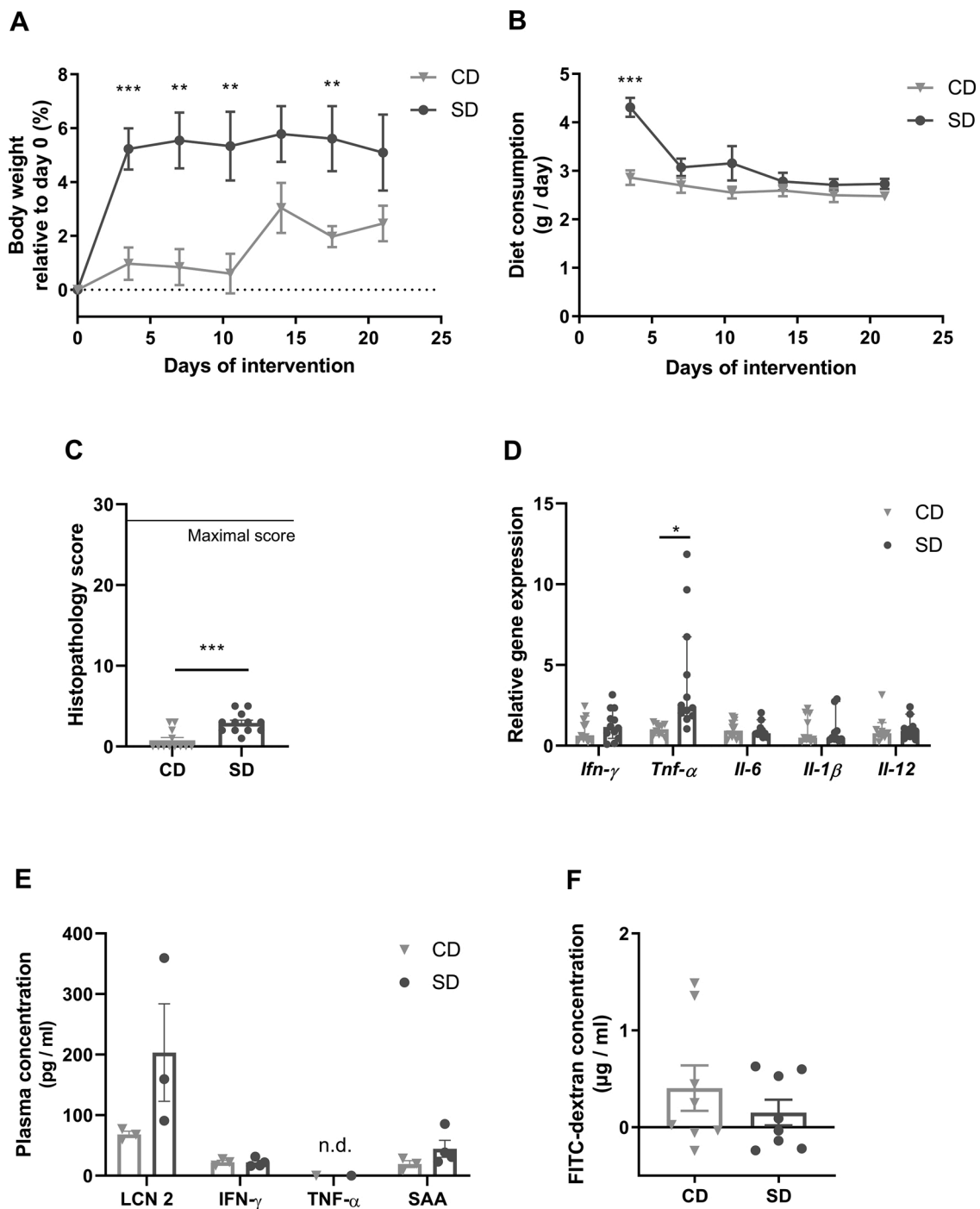Fecal cell numbers of *B. wadsworthia* determined before diet change

Fig. 2. Inflammation-associated parameters in conventional IL-10$^{-/-}$ mice fed Spirulina diet (SD) or control diet (CD). (A) Body weight development relative to the first day of intervention. Dashed line at 0 % body weight change (mean ± SEM, **$P < 0.01$, ***$P < 0.001$, n = 12 per group). (B) Diet consumed per mouse and day (mean ± SEM, ***$P < 0.001$, n = 12 per group). (C) Histopathological scoring of cecal tissue (mean ± SEM, ***$P < 0.001$, n = 12 per group). Straight line indicates the maximal score of 28. (D) Gene expression analysis of cytokines in cecal mucosa (median + 95 % CI, *$P < 0.05$, n = 12 per group). (E) Plasma concentrations of inflammatory markers (mean ± SEM, n = 3 – 4 per group). (F) FITC-dextran concentrations in plasma following the colon permeability test conducted after two weeks of intervention (mean ± SEM, n = 8 per FITC-dextran group, n = 4 per PBS group). Ifn-γ, interferon γ; Il, interleukin; LCN 2, lipocalin 2; SAA, serum amyloid A; Tnf-α, tumor necrosis factor α; n.d. = not detected.

were approximately 8 log/g and did not differ between SD and CD mice (Supplementary Fig. 2A). No difference in microbiota composition was observed between the groups at baseline (Supplementary Fig. 3A). After 21 d of intervention, the cell number of *B. wadsworthia* was significantly decreased by 1.9 log/g in cecal contents of SD-fed compared to CD-fed mice (Fig. 3A). Overall, a difference in β-diversity of cecal microbiota between SD and CD mice was observed (Fig. 3B), but none in α-diversity

(data not shown). The microbiota composition determined in feces of SD and CD mice after 18 d of the study differed accordingly ($P = 0.018$) (Supplementary Fig. 3B). Microbiota changes occurred in the course of 0 d to 18 d in the SD mice ($P = 0.008$) (Supplementary Fig. 3C). Such changes were not observed in CD mice (Supplementary Fig. 3D). After 18 d, *Porphyromonadaceae* were more abundant in feces of the mice fed SD compared to the mice fed CD (31.4 *versus* 12.6 %, $P = 0.008$). The
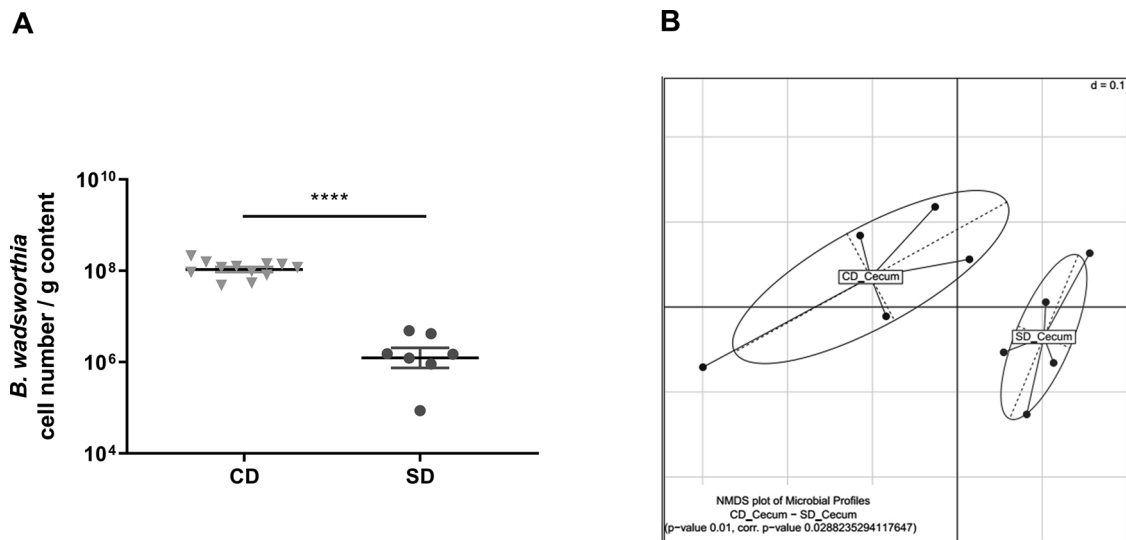
A   **B**

Fig. 3. Bacterial abundance in cecal contents of conventional IL-10$^{-/-}$ mice fed control diet (CD) or Spirulina diet (SD). (A) Cell counts of *B. wadsworthia* determined by qPCR (mean ± SEM, ****$P < 0.0001$, n = 12 per group, note that 5 values had to be excluded in the SD group, as no specific amplification was detectable). (B) β-Diversity of microbial profiles as assessed by 16S rRNA gene sequencing (n = 5 per group).

same applied to cecal content after 21 d (27.4 *versus* 15.9 %, $P = 0.008$) of intervention. After 21 d, the relative abundance of members of *Desulfovibrionaceae* in cecal content was lower in SD mice than in CD mice (3.1 *versus* 1.5 %, $P = 0.008$). These data correspond to lower cecal *B. wadsworthia* counts in SD mice compared to CD mice detected by qPCR (Fig. 3A).

3.3. Sulfoquinovose did not promote intestinal inflammation or growth of B. wadsworthia in conventional mice

To assess the effects of the SQDG metabolite SQ, the latter was orally applied to conventional IL-10$^{-/-}$ mice for three weeks. TC was administered to a second group of mice as a positive control, whereas water was applied as vehicle control to a third mouse group. The applied single TC doses correspond to those reported in the previous study (Devkota et al., 2012), and SQ was used at equimolar doses. No changes in body weight
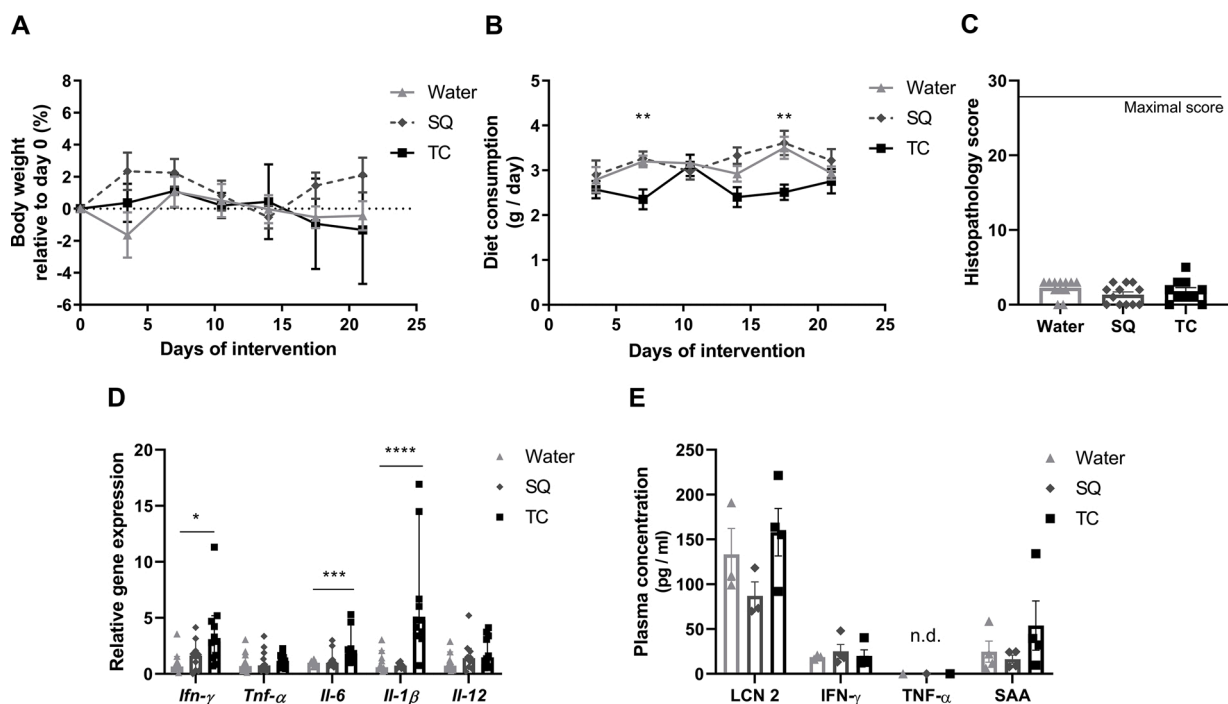

Fig. 4. Inflammation-associated parameters in conventional IL-10$^{-/-}$ mice gavaged with water, sulfoquinovose (SQ) or taurocholate (TC). (A) Body weight development relative to the first day of intervention. Dashed line at 0 % body weight change (mean ± SEM, n = 10 - 12 per group). (B) Consumed diet per mouse and day (mean ± SEM, **$P < 0.01$, n = 10 - 12 per group). (C) Histopathologic scoring of cecal tissue (mean ± SEM, n = 10 – 12 per group). Straight line indicates the maximal score of 28. (D) Gene expression analysis of cytokines in cecal mucosa (median + 95 % CI, *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, n = 10 - 12 per group). (C) Plasma concentrations of inflammatory markers (mean ± SEM, n = 3 – 4 per group). Ifn-γ, interferon γ; Il, interleukin; LCN 2, lipocalin 2; SAA, serum amyloid A; Tnf-α, tumor necrosis factor α; n.d. = not detected.

were observed during the intervention for all groups (Fig. 4A). Feed consumption was reduced in TC mice compared to SQ- and water-treated mice on days seven and 18 by 0.8–1.0 g per mouse on average (Fig. 4B). Pathologic changes in cecal tissues of all groups were not observed, as assessed by histopathological scoring (Fig. 4C). Gene expression of cytokines in cecal mucosa of SQ-treated mice was unaffected. In contrast, expression levels of *Ifn-γ, Il-6* and *Il-1β* in cecal mucosa of TC-treated mice were 2.8-fold, 1.8-fold and 4.8-fold, respectively, higher relative to water-treated mice (Fig. 4D). Plasma concentrations of inflammatory markers of mice gavaged with SQ or TC did not differ from those of the control mice (Fig. 4E).

Fecal cell numbers of *B. wadsworthia* before SQ or TC application were 7.8 ± 0.3 log/g and 7.9 ± 0.4 log/g, respectively, and did not differ from controls (Supplementary Fig. 2B). Cecal abundance of *B. wadsworthia* was not affected by SQ application, whereas TC gavage led to a 0.3 log/g higher cell number of this bacterium compared to the vehicle control (Fig. 5A). Differences in β-diversity of cecal microbiota were observed for TC-gavaged but not SQ-treated mice, each compared to the control group (Fig. 5B, C). No changes in α-diversity were detected (data not shown). The β-diversity of the fecal microbiota of SQ mice did not differ from that of control mice at 0 d and 18 d, and no changes were observed over 18 d in the SQ mice or the control mice (Supplementary Fig. 4). The β-diversity of the fecal microbiota of TC mice differed to some extent from that of control mice at both 0 d ($P = 0.005$) and 18 d ($P = 0.038$) (Supplementary Fig. 5A, B). However, major changes in microbiota composition occurred in response to TC treatment as obvious from comparing the β-diversity in feces at 0 d and 18 d ($P = 0.006$) (Supplementary Fig. 5C). Application of TC to mice resulted in a reduction of the relative abundance of *Porphyromonadaceae* OTUs as observed in feces from 0 d to 18 d (28.2 *versus* 14.1 %, $P = 0.008$), while OTUs of *Desulfovibrionaceae* increased during this time period (1.1 *versus* 2.5 %, $P = 0.008$). *Desulfovibrionaceae* members were also more abundant after 21 d in cecal content of TC-gavaged mice as compared to the control group (5.0 *versus* 2.8 %, $P = 0.008$).

3.4. Sulfoquinovose did not elicit an inflammatory response or major alterations in gut bacterial composition in SIHUMI mice with or without *B. wadsworthia*

To investigate the influence of the selected sulfonates on the growth of *B. wadsworthia* in a defined microbial community, gnotobiotic mice harboring a simplified human intestinal microbiota in the presence of *B. wadsworthia* (SIHUMI + Bw) or its absence (SIHUMI) were gavaged with water, SQ or TC over three weeks. After an initial 2.2–3.2 % drop in body weight in the three SIHUMI + Bw groups, mice of all experimental groups gained some weight. No statistically significant differences in body weight gain and feed consumption were detected among SIHUMI or SIHUMI + Bw groups (Supplementary Fig. 6). There were no signs of inflammation in the cecum of mice of any of the groups as assessed by histopathological scoring (Fig. 6A), colon permeability (Fig. 6B) and gene expression of mucosal cytokines (Fig. 6C, D), with exception of the TC-gavaged SIHUMI + Bw animals (Fig. 6B, D). The latter showed elevated expression levels of *Ifn-γ* (3.5-fold) and *Tnf-α* (2.1-fold) (Fig. 6D) and a slightly increased colonic permeability (Fig. 6B) compared to the control mice. Levels of inflammatory markers determined in plasma were not indicative of a systemic inflammation in SQ-
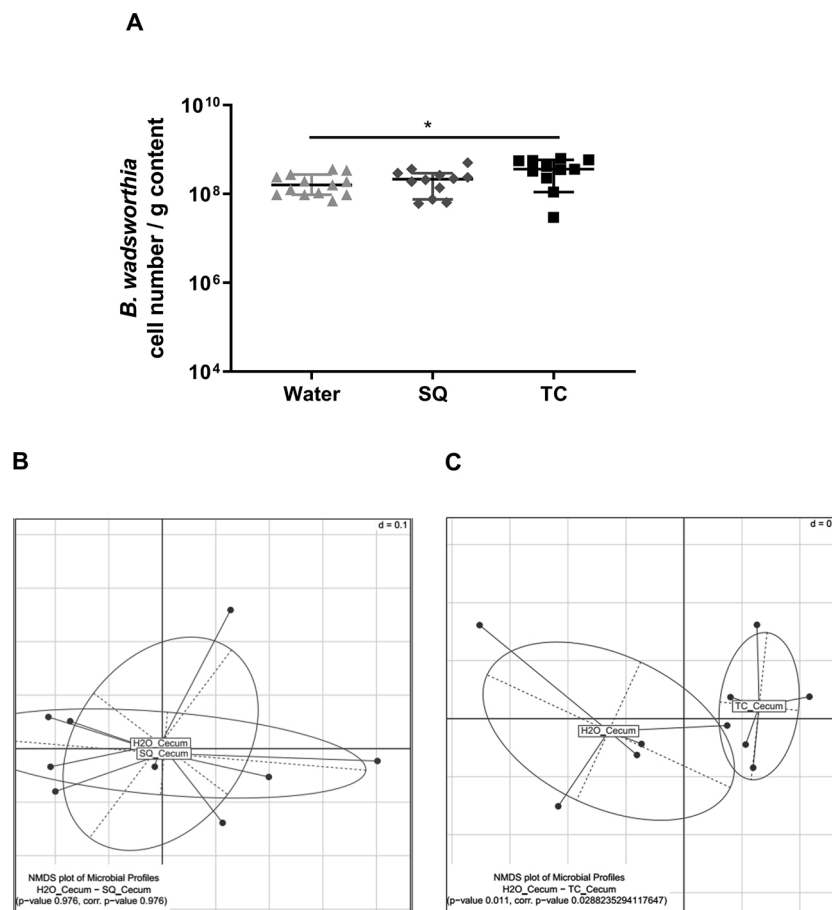

Fig. 5. Bacterial abundance in cecal contents of conventional IL-10$^{-/-}$ mice orally treated with water (H$_2$O), sulfoquinovose (SQ) or taurocholate (TC). (A) Abundance of *B. wadsworthia* determined by qPCR (median $\pm$ 95 % CI, *$P < 0.05$, n = 12 per group). β-Diversity of microbial profiles of (B) SQ *versus* H$_2$O group and (C) TC *versus* H$_2$O group as assessed by 16S rRNA gene sequencing (n = 5 per group).
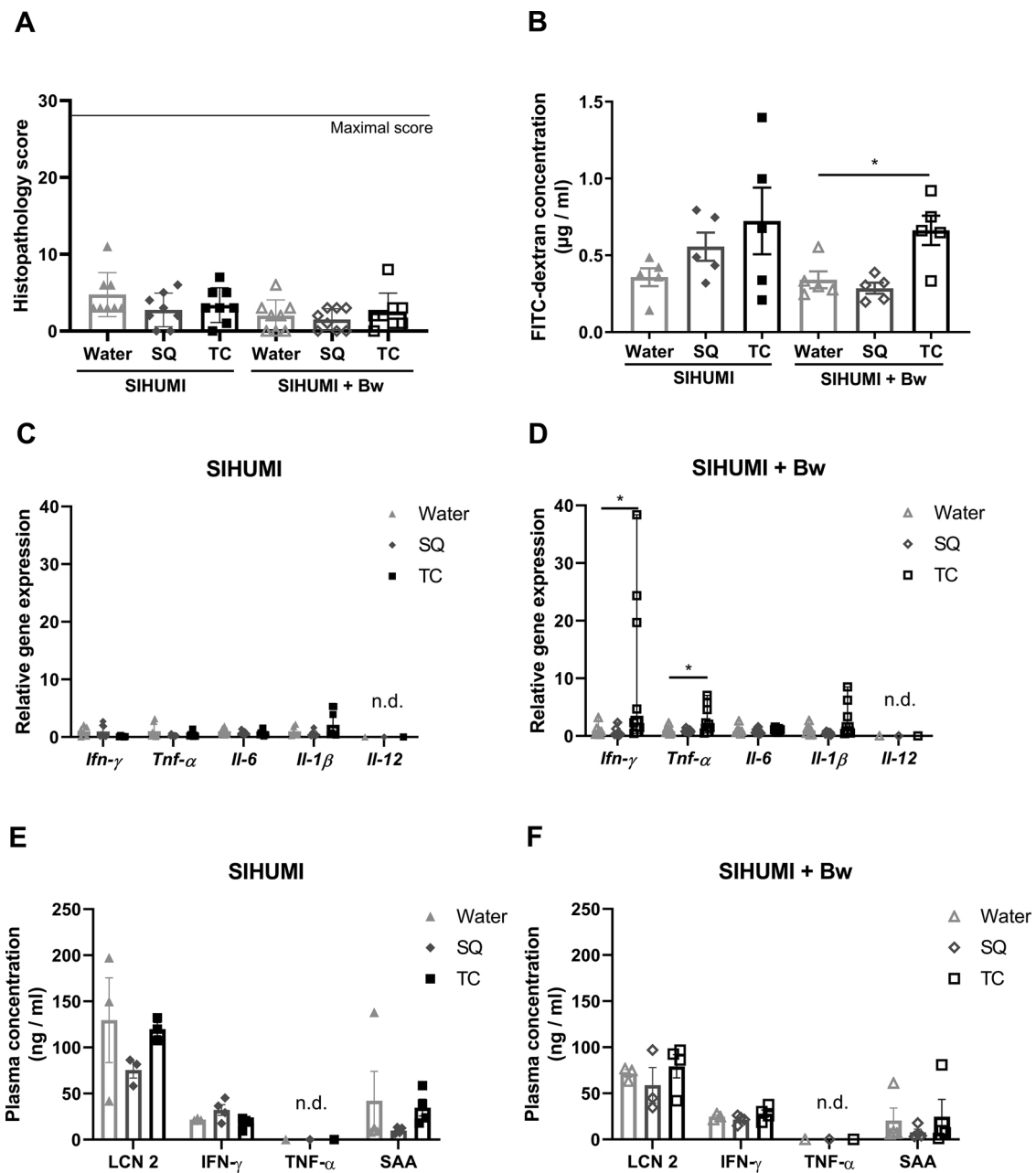
Fig. 6. Inflammation-associated parameters in gnotobiotic IL-10$^{-/-}$ mice treated with water, sulfoquinovose (SQ) or taurocholate (TC). (A) Histopathologic scoring of cecal tissue (mean $\pm$ SEM, n = 8 per group). Straight line indicates the maximal score of 28. (B) FITC-dextran concentrations in plasma following the colon permeability test conducted after two weeks of intervention (mean $\pm$ SEM, *P < 0.05, n = 5 per FITC-dextran group, n = 3 per PBS group). (C) Gene expression analysis of cytokines in cecal mucosa of SIHUMI mice and (D) SIHUMI + *B. wadsworthia* (SIHUMI + Bw) mice (median + 95 % CI, *P < 0.05, n = 8 per group). (E) Concentrations of inflammatory markers in plasma samples of SIHUMI mice and (F) SIHUMI + Bw mice (mean $\pm$ SEM, n = 3 – 4 per group). Ifn-γ, interferon γ; Il, interleukin; LCN 2, lipocalin 2; SAA, serum amyloid A; Tnf-α, tumor necrosis factor α; n.d. = not detected.

or TC-treated gnotobiotic mice (Fig. 6E, F).

Fecal cell numbers of *B. wadsworthia* did not differ among the groups at the beginning of the study (Supplementary Fig. 2C). The growth of *B. wadsworthia* in the cecum was stimulated by application of SQ (0.4 log/g), but not by TC (Fig. 7). The overall composition of the SIHUMI or SIHUMI + Bw microbial communities was neither affected by SQ nor TC gavage (Supplementary Fig. 7).

3.5. The cecal expression of Sqr and Hif-1α was not stimulated in sulfonate-treated mice

Degradation of the sulfonates by gut microbiota, as demonstrated for

SQ and TC in the present study (see above), may result in an increase of the intestinal H$_2$S concentration. This may induce the synthesis of host enzymes involved in H$_2$S detoxification such as sulfide:quinone oxido-reductase (SQR). Highly increased concentrations of H$_2$S could impair respiration in mitochondria triggering the induction of the hypoxia-inducible factor 1α (HIF-1α). To monitor those effects of H$_2$S following sulfonate intake in our study, the expression of *Sqr* and *Hif-1α* was assessed in cecal mucosa of the mice. However, neither Spirulina feeding nor oral treatment with SQ or TC induced the expression of *Sqr* or *Hif-1α* relative to the respective control groups (Supplementary Fig. 8). This result was similar for conventional and gnotobiotic mice.
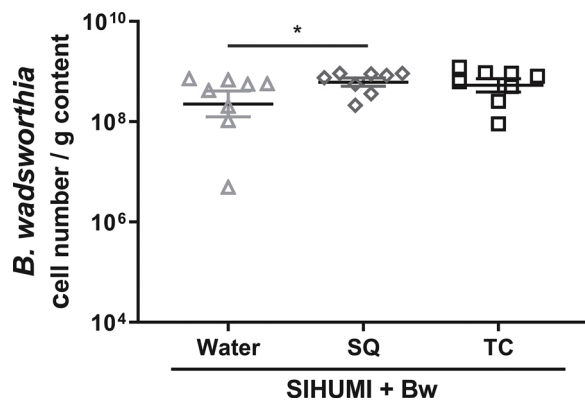
Fig. 7. Abundance of *B. wadsworthia* in cecal contents of SIHUMI + *B. wadsworthia* (SIHUMI + Bw) IL-10$^{-/-}$ mice orally treated with water, sulfoquinovose (SQ) or taurocholate (TC). Data presented as mean ± SEM (*$P < 0.05$, n = 8 per group).

4. Discussion

The onset and severity of IBD are, among others, strongly affected by the diet and ensuing effects on the intestinal microbiota (Devkota and Chang, 2015). Therefore, it is important to identify dietary components that contribute to the gut microbiota-mediated promotion of chronic intestinal inflammation in humans. Based on a report of detrimental, *B. wadsworthia*-mediated effects of the endogenous sulfonated bile acid TC in IL-10$^{-/-}$ mice (Devkota et al., 2012), we studied the microbiota-dependent pro-inflammatory potential of the sulfonates SQDG enriched in diet and their bacterial metabolite SQ in the identical colitis-prone mouse model.

The intestinal microbiota of the conventional IL-10$^{-/-}$ mice used in our study was demonstrated to metabolize both SQ and TC with subsequent formation of H$_2$S. The degradation of SQ to H$_2$S was reported to involve at least two bacterial species, the first of which converts SQ to DHPS (*e.g.*, *E. coli*) and the second degrades DHPS to H$_2$S (*e.g.*, *B. wadsworthia, Desulfovibrio* sp. strain DF1) (Burrichter et al., 2018; Denger et al., 2014; Liu et al., 2020b). TC can be deconjugated by bile salt hydrolase (BSH) present in several gut bacteria (Urdaneta and Casadesús, 2017) and the resulting taurine degraded to H$_2$S by *B. wadsworthia* (Laue et al., 1997; Xing et al., 2019). The latter was a member of the microbial community in the intestines of our experimental mice. In gnotobiotic IL-10$^{-/-}$ mice harboring the SIHUMI + *B. wadsworthia* consortium, SQ is expected to be degraded to H$_2$S by the combined action of *E. coli* and *B. wadsworthia* strains in agreement with previous *in vitro* studies (Haange et al., 2020). Other members of the SIHUMI consortium have not been described to carry genes encoding SQ-converting enzymes so far known (Denger et al., 2014; Frommeyer et al., 2020). The conversion of DHPS to H$_2$S by *B. wadsworthia* is catalyzed by the DHPS-sulfolyase (HpsG) (Liu et al., 2020b). A TC-deconjugating BSH might be active in the *Lactobacillus, Bifidobacterium, Bacteroides* or *Clostridium* strains of the SIHUMI consortium (Urdaneta and Casadesús, 2017; Dong and Lee, 2018). Further taurine degradation is catalyzed by the added *B. wadsworthia* only, as demonstrated previously (Haange et al., 2020).

The results regarding effects of the sulfonates presented here focus on the cecum, as the cell density of *B. wadsworthia* was higher in cecal content and the observed effects were stronger than in the colon (data not shown). Feeding IL-10$^{-/-}$ mice the diet supplemented with 20 % Spirulina led to a consumption of approximately 23 g of this cyanobacterium per kg body weight. This corresponds to an estimated daily intake of 200 mg SQDG per kg body weight, based on the given lipid content of the Spirulina supplement and literature data on the percentage of SQDG in the lipid fraction of *A. platensis* (Xue et al., 2002). The consumption of the SQDG-rich Spirulina diet by mice neither

decreased intestinal permeability nor increased gene expression of major pro-inflammatory cytokines in cecal mucosa or the concentration of inflammatory markers in plasma. However, expression of *Tnf-α* in the cecal mucosa and the cecal histopathology score were moderately increased following Spirulina feeding, but remained overall at a low level compared with previous studies (Ganesh et al., 2013; Ring et al., 2019). Immunomodulatory effects of Spirulina, in particular stimulation of cytokine production, have been described previously (Wu et al., 2016). A moderate increase of TNF-α has been observed in healthy wild type mice in response to the administration of a low Spirulina dose (800 mg/kg body weight) (Shokri et al., 2014). However, most *in vitro* and *in vivo* studies on inflammation-associated diseases such as colitis or arthritis showed a Spirulina-induced reduction of pro-inflammatory cytokines (Wu et al., 2016). Consistently, Spirulina has been reported to have a wide range of health benefits, including anti-oxidant, hepatoprotective and antihyperglycemic effects (Buono et al., 2014) and, therefore, is marketed as functional food. Most of these beneficial effects have been attributed to its richness in trace elements, minerals, vitamins (*e.g.* carotenoids), pigments (*e.g.* C-phycocyanin) and essential fatty acids (Furmaniak et al., 2017). Moreover, Spirulina has long been appreciated as a valuable source of protein with contents of up to 77 % of dry weight (Furmaniak et al., 2017).

In our study, Spirulina-fed mice harbored two orders of magnitude lower cecal cell numbers of *B. wadsworthia* than control mice, which corresponds to the lower relative abundance of *Desulfovibrionaceae* OTUs observed in ceal contents. Such a strong depletion of this pathobiont has not been described in the literature so far and adds to the reported beneficial effects of Spirulina. A general impact of Spirulina feeding on β-diversity of gut microbiota as observed in the present study was already described for mice previously (Hu et al., 2019; Rasmussen et al., 2009). However, the observed effects appear to be caused by bioactive compounds other than SQDG contained in Spirulina, which is also in line with the outcome of the SQ application experiment.

The impact of the SQDG metabolite SQ on the host and its intestinal microbiota composition has not been studied until now. Previous work on SQ focused on its conversion to H$_2$S by environmental bacteria and the distribution of the involved enzymes in α-, β- and γ-proteobacteria (Burrichter et al., 2018; Denger et al., 2014). The formation of high amounts of H$_2$S from SQ by bacteria in the gut may elicit cytotoxic effects (see discussion below), leading to intestinal inflammation. However, SQ did not evoke any inflammatory response when applied at doses equimolar to those of TC for three weeks to conventional or gnotobiotic IL-10$^{-/-}$ mice in our studies. In line with this, the intestinal microbiota profile was not affected by SQ gavage and only a slight increase in *B. wadsworthia* numbers were observed in gnotobiotic mice. The amount of SQ applied daily as pure compound (1.37 mmol/kg body weight) was somewhat higher than its estimated intake with the Spirulina-supplemented diet (*ca.* 0.38 mmol/kg body weight).

The application of TC was included as a positive control in our study, as it was previously reported that TC evoked a higher abundance of *B. wadsworthia* and induced severe colitis in conventional and mono-associated IL-10$^{-/-}$ mice, characterized by elevated histopathology scores and the production of IFN-γ in mesenteric lymph nodes (Devkota et al., 2012). In the present study, TC application to conventional mice stimulated the expression of *Ifn-γ, Il-6* and *Il-1β*. In SIHUMI + Bw mice, TC induced the expression of *Ifn-γ* and *Tnf-α* and increased colonic permeability. However, the histopathology scores and inflammatory markers in plasma were neither elevated in conventional nor in gnotobiotic mice. The present results are in conflict with those previously published for TC (Devkota et al., 2012). Also, these authors reported a 2000-fold higher abundance of *B. wadsworthia* in response to TC treatment (Devkota et al., 2012), whereas in our study the cell number of this bacterium was only two-fold higher in cecal contents of TC mice compared to control mice harboring a complex microbiota. Possible reasons for this discrepancy in conventional mice are differences in the animal facility-specific gut microbiota composition of mice, which may

be crucial for the effects of TC in this mouse model. Environmental factors such as feed composition, water treatment or animal handling may further affect colitis development, even though the same animal model was used (Mähler and Leiter, 2002).

Bile acids have generally been reported to favor the growth of *B. wadsworthia* and impact the overall gut microbiota composition profoundly (Zheng et al., 2017). The fecal microbiota composition of conventional IL-10$^{-/-}$ mice changed in the course of our experiments in response to TC treatment. However, differences in β-diversity between TC and control groups were observed in feces and cecal content not only at the end of the experiment but in feces unexpectedly also at the beginning of the study. A high-fat diet-like phenotype, which is characterized by increased body weight, fat mass and liver weight and can be evoked by bile acid supplementation (Zheng et al., 2017) was not observed in the present study. The TC dose used in our study (1.86 mmol/kg body weight) corresponds to the amount of TC excreted daily with bile into the intestinal tract of approximately 1.73 mmol/kg body weight as estimated according to data from chow-fed C57BL/6 mice of the same age (Lickteig et al., 2019). Regarding transferability of results from mice to humans, it has to be pointed out that the murine and human bile acid composition differs considerably, which also includes the ratio of taurine- and glycine-conjugated bile acids (Ridlon et al., 2016).

In addition to the experiments with conventional IL-10$^{-/-}$ mice, we investigated the influence of sulfonates on the growth of *B. wadsworthia* and ensuing consequences for the host in mice harboring the well-defined microbial SIHUMI consortium, as described above. This experimental setup allows to study the behavior and effects of an individual bacterium *in vivo* as part of an intestinal microbiota of reduced complexity but avoiding the largely artificial mono-association with this species. Colonization of SIHUMI mice with *B. wadsworthia* had no influence on their feed consumption or overall body weight gain. Major differences in the ratio of SIHUMI consortium members did not occur in response to the presence of *B. wadsworthia*. Even in the SIHUMI + Bw control group receiving no TC, colonization of *B. wadsworthia* was stable. Contrary to this, Devkota et al. reported that mono-association of mice with *B. wadsworthia* could only be maintained when TC was gavaged simultaneously (Devkota et al., 2012). This discrepancy illustrates how model selection may impact the outcome of studies on *in vivo* effects of certain gut bacteria.

A moderate immune response towards *B. wadsworthia* was observed after TC application with an elevated gene expression of *Ifn-γ* and *Tnf-α* in the cecal mucosa of gnotobiotic mice and an increased colonic permeability. However, this *B. wadsworthia*-dependent inflammatory response in the presence of TC was much less severe than described in previous studies (Devkota et al., 2012; Feng et al., 2017). A reason for this discrepancy may be the application of different *B. wadsworthia* strains in the individual studies. Feng et al. utilized an isolate from a human patient suffering from latent autoimmune diabetes in adults and Devkota et al. applied strain ATCC 49260^T originating from a patient's perforated appendiceal abscess (Devkota et al., 2012; Feng et al., 2017). In the present study, *B. wadsworthia* DSM 11045 was used, a strain isolated from mud of an anaerobic sewage plant indicating a possible human fecal source. The *B. wadsworthia* strains may differ in their colitogenic potential as has been described for other gut bacterial species (*e. g.* probiotic *E. coli* Nissle 1917 *versus* pathogenic adherent-invasive *E. coli* LF82, Clavel et al., 2017). However, this needs to be clarified in future studies.

Utilization of the applied sulfonates by gut bacteria could have caused an increase of the intestinal H$_2$S concentration. The effects of H$_2$S in the gut are concentration-dependent and controversially discussed. Recent findings indicate that H$_2$S at low physiological concentrations (nM) is effectively oxidized by enzymes such as SQR, resulting in an increased oxygen consumption and ATP production in mitochondria of epithelial cells. Higher levels of H$_2$S (lower μM) induce stronger *Sqr* expression, and oxygen consumption in mitochondria is further

increased. However, when the H$_2$S concentration exceeds physiological levels (middle μM to mM), the mitochondrial cytochrome *c* oxidase is inhibited and the cells enter a hypoxic state. As a consequence, HIF-1α is produced together with pro-inflammatory cytokines (Beaumont et al., 2016; Blachier et al., 2019). In the present study, application of various sulfonates did not stimulate the expression of *Sqr* or *Hif-1α* in cecal mucosa of mice indicating no elevated H$_2$S concentrations and associated hypoxia or inflammation.

5. Conclusions

In recent years, the gut bacterial sulfur metabolism has been discussed intensively in the context of potential clinically relevant consequences related to chronic intestinal inflammation. Sulfonates that may serve as substrates for sulfite-reducing bacteria include taurine-conjugated bile acids and dietary plant components. In the present study, the pro-inflammatory potential of the dietary sulfolipids SQDG and their degradation product SQ were investigated in a murine model of chronic colitis harboring a complex or minimal intestinal microbiota. The SQDG-rich cyanobacterium Spirulina fed to conventional IL-10$^{-/-}$ mice elicited a mild immune response and reduced the abundance of sulfite-reducing *B. wadsworthia* in the cecum. SQ orally applied to conventional IL-10$^{-/-}$ mice had no effects on cecal immune regulation or gut microbiota composition. In IL-10$^{-/-}$ mice colonized with the SIHUMI consortium and *B. wadsworthia*, the cell number of *B. wadsworthia* increased slightly following SQ application. Thus, the effects observed with Spirulina supplementation are most likely mediated by constituents other than SQDG, as its metabolite SQ induced none of these responses. Based on our findings, there are no indications that the dietary sulfonates studied cause bacteria-induced intestinal inflammation in susceptible hosts.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2021.151494.

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