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Novel narrow spectrum benzyl thiophene sulfonamide derivatives to control *Campylobacter*

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Abstract
Campylobacter is a leading cause of bacterial foodborne gastroenteritis worldwide, and poultry are a major source of human campylobacteriosis. The control of *Campylobacter* from farm to fork is challenging due to emergence of microbial resistance and lack of effective control methods. We identified a benzyl thiophene sulfonamide based small molecule (compound **1**) with a minimal inhibitory concentration (MIC) of 100 μM against *Campylobacter jejuni* 81–176 and *Campylobacter coli* ATCC33559, good drug-like properties, and low toxicity on eukaryotic cells. Compound **1** was used as a lead for the preparation of 13 analogues. Two analogues, compounds **4** and **8** (TH-4 and TH-8), were identified with better antimicrobial properties than compound **1**. TH-4 and TH-8 had a MIC of 12.5 μM and 25 μM for *C. coli* and 50 μM and 100 μM for *C. jejuni*, respectively. Cytological studies revealed that both compounds affected *C. jejuni* envelope integrity. Further, both compounds had no effect on other foodborne pathogens. TH-4 and TH-8 had a minimal impact on the chicken cecal microbiota and were not toxic to colon epithelial cells and chicken macrophages, and red blood cells at 200 μM. Further, TH-4 and TH-8 reduced the *Campylobacter* load in chicken ceca (up to 2-log reduction) when infected chickens were orally treated for 5 days with 0.254 mg kg^{-1}; as well as against internalized *Campylobacter* in Caco-2 cells at 12.5 μM and higher. Our study identified two novel specific and safe benzyl thiophene sulfonamide derivatives having potential for control of *Campylobacter* in chickens and humans.

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Introduction

Campylobacter is a leading cause of bacterial foodborne gastroenteritis worldwide and is a major public health problem [1, 2]. Over 800,000 illnesses and 8,000 hospitalizations are estimated annually due to *Campylobacter* infections in the U.S. [3]. *Campylobacter* infections are also associated with Guillain-Barré syndrome, Miller Fisher syndrome, and reactive arthritis [4]. The treatment of *Campylobacter* infections cost approximately $1.7 billion annually in the United States [5].

Poultry are considered a major source of campylobacteriosis cases in humans. However *Campylobacter* produces little or no clinical disease in poultry despite the extensive colonization of the chicken intestinal tract [6], which represents a high risk of carcass contamination during post-slaughter processes [7]. The majority of human campylobacteriosis cases are associated with poor handling of raw chickens or the consumption of undercooked chickens [8, 9]. It is estimated that a reduction in *Campylobacter* by at least 2-logs in poultry could result in

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up to 90% reduction in human campylobacteriosis cases [10].

Macrolides (erythromycin) and fluoroquinolones (ciprofloxacin) are commonly used antibiotics against *Campylobacter* in humans [6]. However, antibiotic-resistant *Campylobacter* have emerged over the past decades, making antibiotic treatments less effective [11, 12]. The Food and Drug Administration (Guidance for Industry, #213, FDA) has recently restricted the use of antibiotics (virginiamycin, enrofloxacin, and avoparcin) as growth promoters in poultry production to avoid the development of cross antibiotic resistance and reduce the emergence of multidrug-resistant bacteria [11, 13, 14]. Therefore, the discovery of novel antimicrobials specific to *Campylobacter* is needed for the development of safe and sustainable control methods [15].

Recently, several classes of small molecules (SMs) have been shown to be effective against multidrug resistant pathogens such as *Staphylococcus*, *Burkholderia*, *Pseudomonas*, and *Candida*, where conventional antibiotics have failed [16–19]. In most cases, these SMs present characteristic physisco-chemical properties allowing them to enhance their absorption and permeability, and can passively diffuse across the cell membranes and reach their targets at low concentration [20–22].We previously identified 478 SMs cidal to *Campylobacter jejuni in vitro* at 200 μM [20]. One of these growth inhibitors (compound **1**) displayed high druggability properties. In this study, we generated 13 structural derivatives of compound **1**, and

evaluated their antimicrobial efficacy and specificity against *Campylobacter* species. The two most potent analogues [compounds **4** (TH-4) and **8** (TH-8)] significantly reduced *Campylobacter* population in chicken ceca, with minimal impact on its microbiota and also displayed minimal toxicity on eukaryotic cells. Further, cytological studies showed that TH-4 and TH-8 affect *C. jejuni* cell envelope integrity.

Materials and methods

Compounds used for this study

Compound **1** (5-chloro-*N*-(4-chlorobenzyl)-2-thiophene-sulfonamide; JA41; Fig. 1a) synthesized in our laboratory (original compound Chembridge™; ID 2169209) was used to synthesize 13 analogues (compounds **2–14**; Fig. 1b). SM 2169209 was previously identified as a growth inhibitor effective against *C. jejuni* 81–176 at 200 μM [20]. The compounds were suspended into 100% dimethyl sulfoxide (DMSO) to 100 mM concentration and stored at −80 °C.

Compound derivatization

The desired compounds were synthesized in one step from 5-chlorothiophene-2-sulfonyl chloride upon treatment with the appropriate primary or secondary amines. The crude reaction products were purified using silica gel column chromatography. The compounds were characterized using
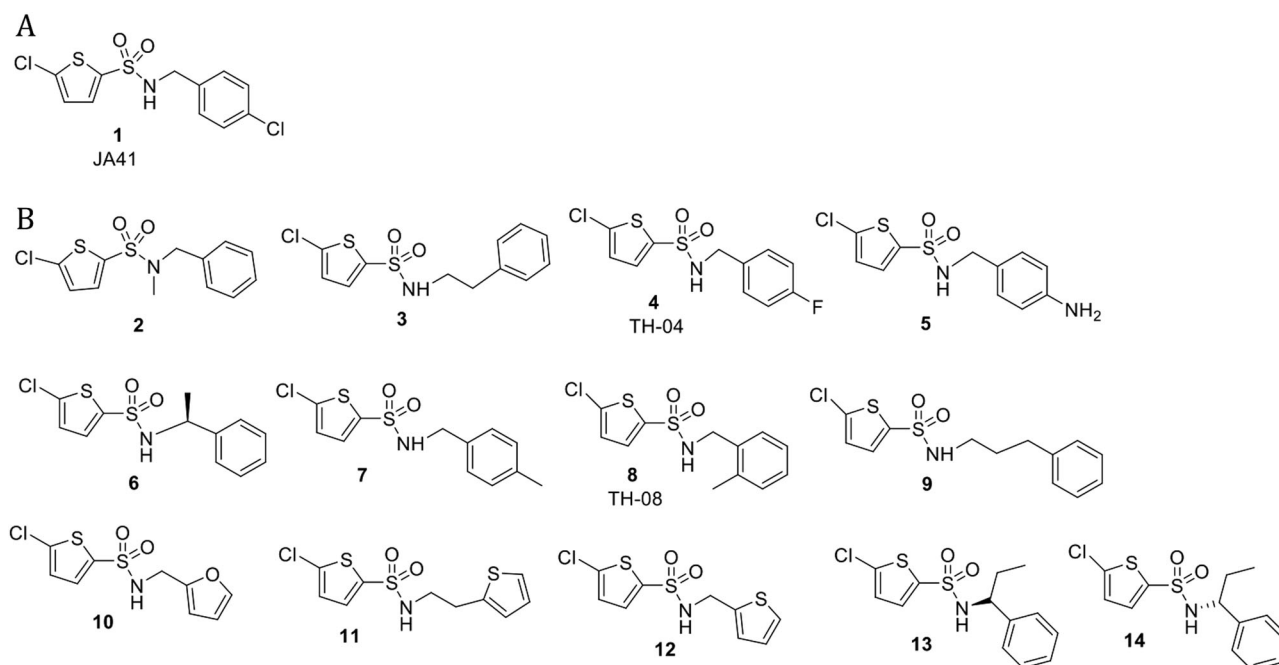
Fig. 1 Chemical structure of the compound library. **a** Chemical structure of compound **1** (also called JA41; PubChemID: 2169209) used for the creation of the 13 analogues. **b** Chemical structure of the 13 analogues of compound **1**. The name of each analogues is located on the bottom of the chemical structure (compounds **2–14**)

nuclear magnetic resonance (NMR), infrared radiation (IR), and mass spectrometry. Detailed reaction conditions and spectroscopic data have been included in the supplemental information (Figure S1).

Chemical structure analysis of the SMs

The physico-chemical properties of the SMs were analyzed using a variety of tools, including PubChem Compounds (National Center for Biotechnology Information; Rockville Pike, MD), the ChemMine website, ChemDraw15.0, and ACD Labs Percepta Suite [23, 24]. Analysis was based on molecular weight, polar surface area, log P, the presence of reactive functionality, synthetic feasibility, and the potential for rapid derivatization.

Bacterial strains

C. jejuni 81–176 and *C. coli* ATCC33559 were used as primary strains in this study. Seven additional *C. jejuni* strains isolated from chicken feces, and environment (crates used in beds, darkling beetles and their larvae) were used to confirm the antimicrobial efficacy of the compounds against diverse *Campylobacter* isolates. Details regarding the isolation and characterization of the isolates were previously described [25]. Further, foodborne (*Salmonella enterica* subsp. *enterica* serotype Typhimurium LT2, *Listeria monocytogenes*, and *Escherichia coli* O157:H7) and poultry (avian pathogenic *E. coli* O78 and *Mycoplasma gallisepticum*) pathogens, and a probiotic (*E. coli* Nissle 1917) were used for the activity spectrum characterization (Table S1).

Eukaryotic models

The cytotoxicity and/or clearance of internalized *C. jejuni* by TH-4 and TH-8 were evaluated using normal colon cells (CCD-112CoN, ATCC CRL-1541), Caco-2 (human colonic carcinoma cells, ATCC-HTB-37) and HD11 (chicken macrophages, CVCL_4685) cells. The hemolytic activity of TH-4 and TH-8 was tested in sheep and chicken red blood cells (RBCs; Table S1).

Dose–response and activity spectrum assay

The dose–response assay was conducted with *C. jejuni* and *C. coli* using sterile, non-treated, and flat bottom 96-well plates, as previously described [20]. The 14 compounds were serial two-fold diluted in 100% DMSO with final concentrations (between 200 μM to 6.25 μM). Plates were incubated for 24 h at 42 °C with 150 rpm shaking in microaerobic condition. The optical density (OD) was measured at 600 nm before and after incubation using a Sunrise Tecan kinetic microplate reader (San Jose, CA,

USA). The viability of *Campylobacter* cells was tested on a MH agar plate after treatment. SMs were considered as bactericidal if they displayed 100% growth inhibition and no bacteria was recovered after plating on agar. The lowest SM concentration with bacteriostatic or bactericidal effects was considered as the minimal inhibitory concentration (MIC) or the minimal bactericidal concentration (MBC), respectively.

The antimicrobial activity of the 14 compounds was tested on other foodborne pathogens (*S.* Typhimurium LT2, *L. monocytogenes*, and *E. coli* O157:H7) and probiotic bacteria (*E. coli* Nissle 1917) at 200 μM, as previously described [20]. Further, TH-4 and TH-8 were also tested at 200 μM on two poultry pathogens (avian pathogenic *E. coli* O78 and *M. gallisepticum*). Bacteria alone (optical density (OD) of 0.05 at 600 nm), treated with 1% DMSO or 20 μg ml^{-1} chloramphenicol, and MH medium only were used as controls. Details of each strain tested and the growth conditions are included in Table S1.

Antimicrobial resistance studies

Single step and sequential passage resistance assays were performed with TH-4 and TH-8 as previously described [20, 26, 27]. The MBC values displayed in Table 1 for *C. jejuni* 81–176 were used as reference for the lethal (2X MBC) and sub-lethal (0.80X MBC) doses.

For the single step resistance assay, 10^9 *C. jejuni* bacteria were plated in a well of a 24-well plate containing MH agar supplemented with 2X MBC of SMs and incubated for 15 days at 42 °C in microaerobic conditions. Then 100 μl of MH broth was added to each well to resuspend any surviving bacteria, transferred into a tube containing 5 ml of MH medium, and incubated for 24 h at 42 °C with 150 rpm shaking in microaerobic conditions.

Table 1 Minimal bacteriostatic (MIC) and bactericidal (MBC) concentrations of TH-4 and TH-8 on several *Campylobacter* strains

Strains	TH-4		TH-8	
	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)
C. jejuni Au-12	25	25	12.5	200
C. jejuni Au-13	50	50	100	200
C. jejuni Au-18	25	25	50	200
C. jejuni Au-21	25	50	100	200
C. jejuni Au-35	12.5	25	50	100
C. jejuni NCTC 11168	25	25	50	200
C. jejuni 81–116	25	50	50	200
C. jejuni 81–176	50	50	100	100
C. coli ATCC33559	12.5	12.5	25	25

For the sequential passage resistance assay: *C. jejuni* (10^6 CFU/well) was challenged in a 96-well plate containing MH medium supplemented with 0.8X MBC of SMs (concentration allowing at least 70% growth inhibition) as described in the primary screening. The 96-well plate was incubated in the dark at 42 °C with 150 rpm shaking for 18 h in microaerobic conditions. After the first passage, the plate was centrifuged for seven min at $4700 \times g$, supernatant was replaced with a fresh MH broth medium amended with 0.8X MBC of the corresponding SM and grown for 12 h in microaerobic conditions. This procedure was repeated fourteen times. Following the 15th passage, MIC and MBC were determined as described previously. Tubes showing an increase in OD_{600} after the sequential passage or any colonies on agar after the single step resistance assays were tested for MIC and MBC. For both experiments, *C. jejuni* growth in 1% DMSO, 20 µg ml^{-1} chloramphenicol, or 50 µg ml^{-1} kanamycin, and MH medium only were used as controls.

Scanning Electron Microscopy (SEM)

An overnight suspension of *C. jejuni* 81–176 grown in MH broth (approximately 0.5 OD_{600}) was washed in 1X phosphate-buffered saline (PBS), resuspended in fresh MH medium containing a lethal dose of SMs (5X MBC) and grown for 3 h. *C. jejuni* treated with 1% DMSO was used as control. Processing of the samples was performed as previously described [26, 27]. Briefly, one volume of bacterial suspension was mixed with one volume of fixative (3% glutaraldehyde, 1% paraformaldehyde in 0.1 M potassium phosphate buffer, pH 7.2), and incubated for 2 h at 4 °C. Fixed cells were centrifuged for 5 min at $1,200 \times g$, washed twice with PBS, and resuspended in 1% osmium tetroxide and incubated for 1 h at room temperature in the dark, followed by serial dehydration of the sample in ethanol and platinum splatter-coating. Visualization and imaging of the samples was performed using a Hitachi S-4700 scanning electron microscope at the Molecular Cellular Imaging Center (OARDC, Ohio). At least 30 *C. jejuni* cells per treatment were observed under the microscope.

Cytotoxicity assay using normal colon cells and Sulforhodamine B–Protocol

Cytotoxic activity was measured using Sulforhodamine B (SRB) as indicator of cell proliferation according to previously published methods [28]. SRB binds stoichiometrically to proteins under mild acidic conditions and then can be extracted using basic conditions; thus, the amount of bound dye can be used as a proxy for cell mass, which can then be extrapolated to measure cell proliferation. Briefly, a 96-well plate was seeded with 190 µl of normal colon cells (approximately 7.6×10^3 cells/well) in the appropriate cell culture medium and treated with a determined concentrations of SMs ranging between 0.00256 µM and 200 µM (Table S1). The plate was incubated for 72 h at 37 °C in a humidified 5% CO_2 incubator. After incubation, cells were fixed with 20% trichloroacetic acid (TCA 100 µl) and incubated at 4 °C for 30 min. Cells were washed three times with sterile water. The plate was dried at room temperature, followed by a staining with 0.4% SRB. After 30 min, excess stain was removed using 10% (v/v) acetic acid and the plate was dried. Two hundred microliters of 10 mM Tris base solution was added to each well and homogenized for 5 min before measuring the absorbance at 515 nm using Floustar Optima plate reader (BMG Labtech Inc., Durham, NC). Results were calculated from triplicate samples in two separate experiments. Paclitaxel was used as control.

Cytotoxicity of TH-4 and TH-8 on eukaryotic cell lines

Cytotoxicity of TH-4 and TH-8 was tested on Caco-2 and HD11 cells at 200 µM by measuring the abundance of lactate dehydrogenase released from damaged cells in to growth medium as previously described [20, 26, 27, 29]. LDH released is quantified by a coupled enzymatic reaction, which results in reduction of tetrazolium salt to a red formazan product that can be measured at 490 nm. The level of formazan formation is directly proportional to the amount of LDH released into the medium thus indicative of cytotoxicity. Briefly, a 96-well plate was seeded with 150 µl Caco-2 or HD11 cells (approximately 1.4×10^5 cells/well) in the appropriate cell culture medium and incubated at 37 °C in a humidified 5% CO_2 incubator (Table S1). Once a confluent monolayer was formed, cells were washed three times with PBS and 150 µl of growth medium supplemented with 200 µM of SMs was added. After 24 h of incubation, cytotoxicity was determined using the Pierce™ Lactate Dehydrogenase Cytotoxicity Assay Kit (ThermoFisher Scientific). One percent DMSO and 10X lysis buffer were used as control. The cytotoxicity was calculated according to manufacturer instructions.

Hemolytic activity of TH-4 and TH-8 on RBCs

The hemolytic activity of TH-4 and TH-8 was performed as previously described [20, 26, 27, 29]. Briefly, 200 µl of 10% sheep or chicken RBC suspension was incubated with 200 µM of SMs for 1 h at 37 °C in a 96-well plate. After incubation, the plate was centrifuged at $3700 \times g$ for 5 min at 4 °C and then placed on ice for 5 min. One hundred microliters of the supernatant were transferred into a fresh 96-well plate, and the OD was measured at 540 nm. One percent DMSO and 0.1% TritonX-100 were used as controls,

respectively. Percentage hemolysis was calculated as: $[(OD_{540\ SM} - OD_{540\ DMSO}) / (OD_{540\ 0.1\%\ tritonX\text{-}100} - OD_{540\ PBS})] \times 100$.

Effect of TH-4 and TH-8 on *C. jejuni* intracellular survival in Caco-2 cells

The effect of TH-4 and TH-8 on intracellular *C. jejuni* 81–176 was evaluated using Caco-2 cells as previously described [20, 30]. A multiplicity of infection (MOI) of 100 was used. Infected cells were treated with a final concentration ranging between 200 μM and 3.125 μM and incubated at 37 °C for 24 h in humidified, 5% CO_2 incubator. Following incubation, cells were washed once with PBS, lysed with 0.1% TritonX-100, serial ten-fold diluted in PBS, and plated on MH agar plate. Plates were incubated at 37 °C for 24 h to determine the intracellular bacteria. Cells not infected and not treated, and cells infected and treated with 1% DMSO were used as controls.

Effect of TH-4 and TH-8 on the survival of *C. jejuni* in the ceca of three-week-old chickens

Animal experiments were approved and performed in accordance to the Institutional Animal Care and Use Committee of The Ohio State University (Protocol # 2010A00000149-R1). Three-week-old *Campylobacter*-free chickens were inoculated orally with a cocktail of *Campylobacter* strains (10^5 CFU of each strain per chicken; Table S2). Rectal swabs were collected at 2 days post-inoculation (DPI) to confirm the intestinal colonization by *Campylobacter*. At 3DPI, infected chickens were treated orally twice a day for 5 days with 100 μg of SMs dissolved in water (approximately 0.254 mg of SM per kg body weight). Details of the treatment groups are described in Table S2. Following treatment, chickens were euthanized and both ceca aseptically collected. One cecum per pair was immediately stored at −80 °C for microbiota studies. The second cecum was homogenized into PBS, serial ten-fold diluted, plated on MH CSS agar plate, and plates were incubated for at least 48 h at 42 °C in microaerophilic conditions.

DNA extraction and 16S sequencing

Genomic DNA was extracted from ceca using the *PureLink Microbiome DNA Purification Kit* (Life Technologies, Invitrogen Corp.), combined with RNAse treatment (10 units/hr). About 0.20 g of cecal content was used for DNA extraction. After quality control with electrophoresis and nanodrop, extracted DNA samples were subjected to 16 S rRNA V4-V5 variable region sequencing. Amplicon libraries were prepared by using Phusion® High-Fidelity PCR Kit (New England Biolabs Inc, Ipswich, MA) as previously described [26]. PCR products were cleaned using AMPure XP PCR (Beckman Coulter Inc, Beverly MA) and sequenced using Illumina MiSeq 300-base, paired-end kit at the Molecular and Cellular Imaging Center (https://mcic.osu.edu/).

Bioinformatics analyses

Quality control of the raw reads was performed using FastQC (Babraham Bioinformatics, Cambridge, USA). Only nucleotides with a base sequence quality whose median quality score was above 25 and whose lower quartile median quality score was above 10 were used for further analysis. Trimmomatic was used for trimming and removal of NexteraPE-PE adapter sequences [31] (http://mcbl.readthedocs.io/en/latest/mcbl-tutorials-AD-clean.html). The resulting forward and reverse sequences were merged using Pandaseq (https://github.com/neufeld/pandaseq). Any sequence with less than 0.7 threshold overlap was removed and spacers used for amplification were trimmed. Samples were processed using Quantitative Insights Into Microbial Ecology (QIIME) software version 1.9 [32]. Operational Taxonomy Units (OTUs) were determined by clustering reads against Greengenes 16S reference dataset (2013–08 release) at a 97% identity using an open-reference OTU picking (pick_open_reference_otus.py) method using default parameters, except setting minimum OTU size to 10. Microbial diversity was studied after rarefaction of the sequences based on the lowest number of sequences among the samples tested ($n = 14,000$). Alpha and beta diversities were analyzed using the core analysis package (core_diveristy_analyses.py), which included the comparison of the phylogenetic diversity and richness, PCoA, and relative abundance studies. A weighted UniFrac distance matrix was generated from the open OTU picking results and was visualized in a PCoA plot using the EMPeror program. The identification of microbial relative abundance differences between treatments was performed using linear discriminant analysis (LDA) in the Galaxy|Hutlab website (https://huttenhower.sph.harvard.edu/galaxy/).

Statistical analysis

C. jejuni growth inhibition data were compared to the DMSO control using one-way analysis of variance, followed by Tukey test with JMP PRO 12 software (SAS Institute, Cary, NC). A linear discriminant analysis effective size (LefSe) combined with A Kruskall-Wallis test and a pairwise Wilcoxon test were performed to identify statistical differences in relative abundance [33]. *P* value ≤ 0.01 was considered as statistically significant [34].

Results

Benzyl thiophene sulfonamide derivatives library

Compound **1** (5-chloro-*N*-(4-chlorobenzyl)-2-thiophenesulfonamide; PubChem ID: 2169209; Fig. 1a), was selected as a lead compound and used to synthesize 13 analogues (Fig. 1b) tested in this study. Compond **1** belongs to a pool of 478 SMs previously identified as complete growth inhibitors effective against *C. jejuni* 81–176 at 200 μM (Fig. 1 and Table S1) [20]. The selection of compound **1** was based on analysis of physico-chemical property data generated using ChemDraw 15.0 and ACD Labs Percepta Suite, including acceptable molecular weight (322 Da), LogP (3.79), and polar surface area (46.17). In general, the compound followed Lipinski's rule of five and lacked reactive functional groups [35]. The 13 analogues were composed of a 5-chloro-thiophene-2-sulfonic acid amide backbone with an aromatic or heteroaromatic moiety connected through a carbon linker (Fig. 1b).

TH-4 and TH-8, best SMs inhibiting *C. jejuni* and *C. coli* growth at low concentration

A dose–response assay of the 14 SMs (compound **1** and 13 analogues) was performed on *C. coli* ATCC33559 and *C. jejuni* 81–176 strains (Fig. 2). Compound **1**, with the *p*-chlorobenzyl substituent, had a MIC of 100 μM for both *C. coli* and *C. jejuni*. Encouragingly, seven out of the 13 synthetic analogues possessing various substituents on the N atom of the sulfonamide displayed lower MIC values than compound **1** against *C. coli* and/or *C. jejuni*. For example, lower MIC values were obtained with analogues possessing *p*-fluoro- or *p*-methyl-, or *o*-methylbenzyl (**4**, **7**, and **8**, respectively), phenethyl (**3**), phenpropyl (**9**), and 2-thienyl (**11** and **12**) substituents. On the other hand, analogues containing the unsubstituted benzyl ring (**2**), furyl (**10**), aminophenyl (**5**), or enatiomeric ethyl-substituted benzyl (**13** and **14**) substituents displayed higher MIC values than compound **1** against *C. coli* and/or *C. jejuni*.

The 14 SMs were tested at 200 μM against three foodborne pathogens (*S.* Typhimurium LT2, *L. monocytogenes*, and enterohemorrhagic *E. coli* O157:H7), two poultry pathogens (avian pathogenic *E. coli* O78 and *M. gallisepticum*), and a probiotic (*E. coli* Nissle 1917). This probiotic was previously identified to have anti-*Campylobacter* effect [30]. Only TH-4 (**4**) and TH-8 (**8**) had no effect on the other strains tested and were only lethal to *Campylobacter* spp., suggesting that their bacterial target might be restricted to the *Campylobacter* genus. Based on the efficacy and activity spectrum data, the two most potent SMs (TH-4 and TH-8) were selected for further studies. These two SMs were also cidal to seven additional *C. jejuni* strains at 12.5 μM and higher (Table 1).

C#	Substituents	Molecular weight (g/mol)	MIC (μM)	
			C. coli	*C. jejuni*
2		301.80	>200	>200
3		301.80	50	100
4		305.78	12.5	50
5		302.80	100	>200
6		301.80	100	200
7		301.81	50	100
8		301.80	25	100
9		315.84	50	50
10		275.75	200	>200
11		307.84	50	100
12		293.81	50	100
13		315.84	>200	>200
14		315.84	>200	>200
1		322.23	100	100

Fig. 2 Minimal inhibitory concentration (MIC) of the 14 compounds (C#) against *Campylobacter coli* ATCC33559 and *Campylobacter jejuni* 81–176. Substituents: variation of the groups attached to the sulfonamide of compound 1. > 200: MIC higher than 200 μM. The experiment was performed two times with three replicates

A dose–response assay with TH-4 and TH-8 was also performed on nine *Campylobacter* strains (Table 1 and Table S1). TH-4 consistently displayed lower MICs and MBCs than TH-8. Depending on the strain tested, MICs ranged between 12.5 μM and 50 μM for TH-4 and between 12.5 μM and 100 μM for TH-8. MBC values were either equal or up to eight times higher than their MIC.

No resistant bacteria were detected when *C. jejuni* was treated with a sub-lethal or lethal dose of TH-4 and TH-8

When *C. jejuni* 81–176 was challenged with a lethal dose of SMs (2X MBC) on a solid medium or with repeated exposure to a sub-lethal dose of SMs (0.8X MBC) in a

liquid medium for 18 days, no resistant bacteria were detected for either TH-4 or TH-8.

TH-4 and TH-8 affected *C. jejuni* cell envelope integrity

The cell morphology of *C. jejuni* 81–176 treated with a lethal dose of SM (5X MBC) was analyzed using SEM (Fig. 3). After 3 h of treatment with TH-4 or TH-8, *C. jejuni* displayed alterations of the cell morphology compared to the DMSO treated control. DMSO treated cells displayed a normal spiral cell-shaped phenotype (approximately 3 μm long) with a smooth surface (Fig. 3a). On the other hand, at least 80% of the *C. jejuni* cells treated with TH4 and TH8 showed an important reduction of their cell length (approximately 1–2 μm long) (Fig. 3b, c, respectively). In addition, TH4 and TH8 treated cells displayed a rod shape and deformation of the cell surface with bulb-like structures.

TH-4 and TH-8 reduced *Campylobacter* population in infected eukaryotic models at low concentrations, with minimal impact on the epithelial cells

A toxicity dose–response assay was performed to assess the effect of TH-4 and TH-8 on the survival of normal colon cells when treated for 72 h with a concentration of SMs ranging between 0.00256 and 200 μM (Fig. 4a). TH-4 and TH-8 treated cells displayed higher survival rate than the paclitaxel-treated cells. The survival rate of paclitaxel-treated cells ranged between 79.1% at 0.00256 μM and 56.5% at 200 μM; while the survival rate ranged between 83% at 0.00256 μM and 60.4% at 200 μM for TH-4 treated cells, and between 100% at 0.00256 μM and 87.5% at 200 μM for TH-8 treated cells. Further, TH-4 and TH-8

displayed low toxicity on epithelial cells (Caco-2) and chicken macrophages (HD11) after 24 h of treatment with 200 μM of SMs. Cytotoxicity was below 12% for Caco-2 cells and below 15% for HD11 cells for TH-4 and TH-8 (Fig. 4b). In addition, no hemolytic activity was detected on chicken and sheep RBCs after 1 h of treatment with 200 μM of TH-4 or TH-8 (Fig. 4b), suggesting that SMs have little or no adverse effect on the eukaryotic cells tested.

The ability of TH-4 and TH-8 to clear intracellular *C. jejuni* 81–176 was tested in infected Caco-2 cells. Both TH-4 and TH-8 completely cleared internalized *C. jejuni* 81–176 (below the detection level) in Caco-2 cells after 24 h of treatment with 50 μM of SMs (Fig. 4c). Further, infected cells displayed $25 \pm 1.3\%$ and $27 \pm 11.2\%$ reduction in internalized *C. jejuni* when treated for 24 h with 12.5 μM of TH-4 and 25 μM of TH-8 compared to the DMSO control, which harbored 5×10^5 CFU/ml.

TH-4 and TH-8 reduced *Campylobacter* population in infected chickens, with minimal impact on the chicken cecal microbiota

As a proof of concept, TH-4 and TH-8 were tested on three-week-old chickens infected with a cocktail of *C. jejuni* strains (Table S1). Following 5 days of treatment with 100 μg of SMs twice per day, chickens treated with TH-4 or TH-8 displayed a 1.1-log and 2.0-log reduction per gram of cecum respectively compared to the DMSO treated control group, which harbored $\sim 5 \times 10^9$ CFU per gram of cecum ($P < 0.01$; Fig. 5a). Further, no body weight differences were detected between the chicken groups after treatment (data not shown).

The impact of TH-4 and TH-8 treatments on the chicken cecal microbiota was studied using the 16S metagenomic

Fig. 3 Scanning electron microscopy analyses of *Campylobacter jejuni* 81–176 treated for 3 h with 5X the lethal dose of antimicrobial growth inhibitors (TH-4 or TH-8). **a** 1% DMSO treated *C. jejuni* 81–176. **b** TH-4 (100 μM) treated *C. jejuni* 81–176. **c** TH-8 (200 μM) treated *C. jejuni* 81–176; Bar: 1 μm. At least 80% ($n = 24/30$ cells) of the treated *C. jejuni* 81–176 cells displayed the phenotype presented in the corresponding pictures
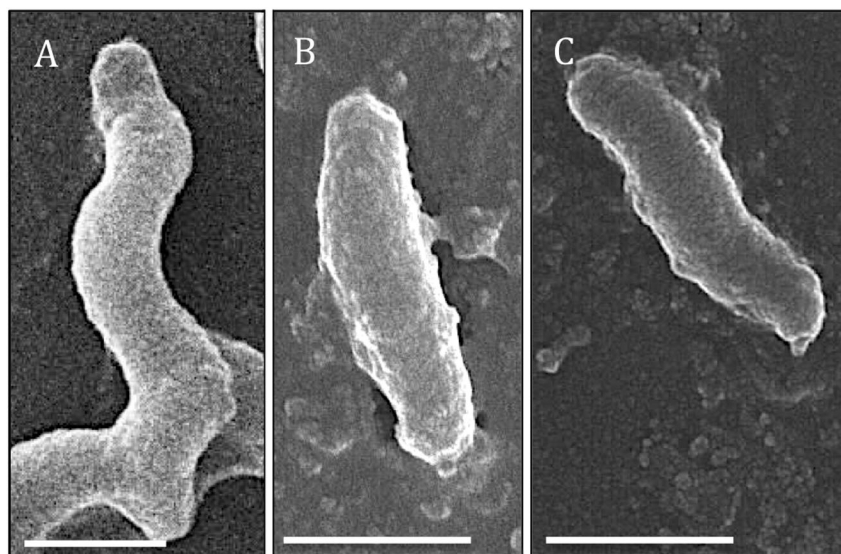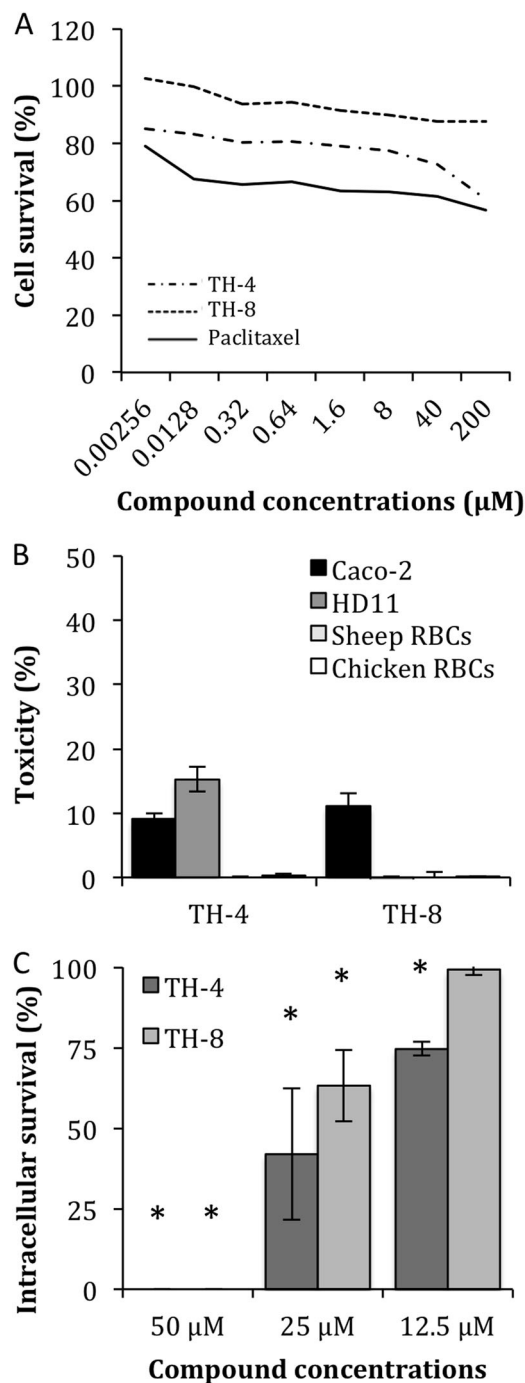
Fig. 4 Impact of TH-4 and TH-8 on eukaryotic cell lines and internalized *Campylobacter jejuni* 81–176. **a** Toxicity dose–response assay of the compounds on normal colon cells. Cells were treated for 72 h at a determined concentration of compounds ranging between 0.00256 and 200 μM. Paclitaxel was used as control. **b** Toxicity of the compounds on Caco-2 colon epithelial cells, HD11 chicken macrophages, chicken and sheep red blood cells (RBCs) at 200 μM. The cytotoxicity on Caco-2 and HD11 cell lines was estimated after 24 h incubation, while the hemolytic activity on RBCs was estimated after 1 h incubation. Dimethyl sulfoxide (1% DMSO) and 0.1% Triton X-100 were used as controls. **c** Clearance assay with *C. jejuni*-infected Caco-2 cells. Infected cells were treated for 24 h. 1% DMSO was used as control. DMSO treated cells were used as 100% survival reference to determine the intracellular survival percentage of the TH-4 and TH-8 treated infected cells. Infected cells treated with DMSO displayed approximately 5.2×10^4 CFU/ml. *: significant reduction of *C. jejuni* population in infected cells at a given concentration of SMs compared to the DMSO control ($P < 0.01$). Experiments performed twice with three replicates

treated groups were compared to the infected chickens treated or not with DMSO (DMSO and P groups, respectively). On the other hand, the principal component analysis of the unweighted uniFrac showed that the presence of DMSO affected the microbiota composition in ceca while the presence of *C. jejuni* did not (Figure S2C). Principal component **2** (PC2, which explained 22.29% variation between samples) separated groups based on the presence or absence of DMSO; however, TH-8 and TH-4 samples displayed similar or closely related spatial distribution profiles with the DMSO group.

The analysis of relative abundance data showed that the chicken cecal microbiota was principally composed of *Firmicutes* and *Proteobacteria* (Fig. 5b). The neither treated nor infected chickens (NC) and infected not treated chicken (P) groups displayed similar microbiota profile but they were significantly different from the other groups containing DMSO ($P < 0.01$). NC and P groups had lower relative abundance in *Proteobacteria* ($14.5 \pm 9.2\%$ to $18.9 \pm 10.2\%$, respectively) and higher abundance in *Firmicutes* ($85.2 \pm 9.2\%$ to $80.7 \pm 10.3\%$, respectively) compared to the groups containing DMSO, TH-4, and TH-8, which harbored between $69.4 \pm 5.9\%$ and $63.7 \pm 10\%$ of *Firmicutes* and between $30.2 \pm 5.8\%$ and $35.9 \pm 10\%$ of *Proteobacteria* depending on the groups. The analysis of the sub-taxonomic level showed that the presence of DMSO increased the relative abundance of *Enterococcus* (2.3-fold), *Enterobacteriaceae* (2.8-fold), and *Proteus* (2.1-fold) while decreasing the *Lactobacillus* (5.6-fold) relative abundance compared the P group ($P < 0.01$). TH-4 and TH-8 displayed very similar microbiota profile as the DMSO group at the phylum level. Both TH-4 and TH-8 displayed a reduction in *Campylobacter* population in ceca (2.0- and 4.4-fold, respectively), corroborating bacterial counts data (Fig. 5a). It was also observed that TH-8 did not significantly alter the cecal microbiota compared to the DMSO group, even at the

sequencing. After processing and taxonomic assignment with the Greengene reference database, 478,151 sequences were obtained for a total of 26 samples. Sequencing depth varied between 13,860 and 244,890 reads per sample (mean = 15,371 reads per sample). Cecal samples were normalized to 13,860 sequences per sample.

Analysis of the alpha diversity displayed no significant differences in the phylogenetic diversity and richness (Figure S2A and S2B, respectively) when TH-4 and TH-8
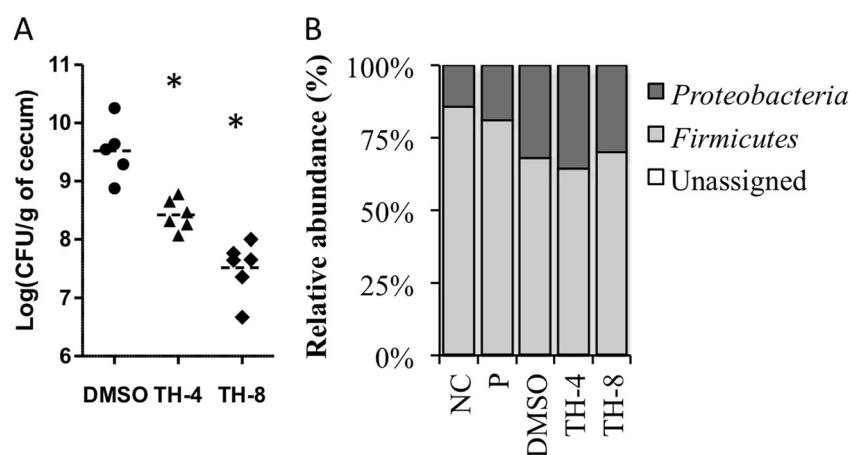
Fig. 5 Impact of TH-4 and TH-8 on *Campylobacter jejuni* 81–176 in chicken ceca and its microbiota. Chicken ceca were collected from experimentally infected birds at 3 weeks of age following 5 days of treatments with 0.254 mg of compounds per kg body weight. Infected chickens treated with dimethyl sulfoxide (DMSO) were used as control. **a** *C. jejuni* counts in chicken ceca after treatments. Each dot represents the bacterial count for one chicken. *: significant reduction of *C. jejuni* population in ceca compared to the DMSO control group ($P < 0.01$). **b** Relative abundance of the chicken cecal microbiota at the phylum level. NC: non-infected chickens ($n = 5$); P: infected chickens not treated ($n = 5$); DMSO: infected chickens treated with DMSO ($n = 6$); TH-4 and TH-8: infected chickens treated with TH-4 or TH-8 ($n = 6$)

genus level ($P > 0.01$); while TH-4 treated chicken ceca had a significant increase in *Coprococcus* (2.57-fold), and a reduction in *Peptostreptococcaceae* (5.2-fold), *Erysipelotrichaceae* cc115 (9.6-fold), and *Eubacterium* (3.0-fold) compared to the DMSO group ($P > 0.01$). Nevertheless, most of bacteria mentioned above represented only a small proportion of the chicken cecal microbiota (below 0.1% all combined). Only *Coprococcus* represented 5.1% ± 1.6% of the chicken cecal microbiota.

Discussion

The control of campylobacteriosis cases in humans is challenging due to the high prevalence of *Campylobacter* in poultry. Further, the emergence of antibiotic-resistant isolates reduce the efficacy of the antimicrobial control methods in human [36]. Therefore, a set of 13 novel compounds was investigated in this study to develop safe and sustainable control method against *Campylobacter*. Compound **1** was previously identified in our laboratory with good drug-like properties, no *in silica* predicted toxicity on eukaryotic cells, and with the ability to completely inhibit *C. jejuni* 81–176 growth at 200 μM [20]. Compound **1** is composed of a thiophene-2-sulfonamide group, which was used as the backbone for the creation of the 13 analogues in this study. Thiophene derivatives are well known for their antimicrobial potential against bacterial pathogens such as *Salmonella*, *Shigella*, *Pseudomonas*, *Bacillus*, and *Staphylococcus* [37–40]; while sulfonic acid derivatives are known to be non-toxic and can enhance the water-solubility of

molecules without altering their properties [40]. Our study showed that some of the thiophene-2-sulfonic acid analogues showed better antimicrobial activity than compound **1** and were able to completely inhibit *C. jejuni* and/or *C. coli* growth at 12.5 μM and higher; however, MBC levels were higher for *C. jejuni* against most of the analogues compared to *C. coli*, suggesting that *C. jejuni* might have better defense mechanisms than *C. coli* for these compounds. We observed that the aromatic or heteroaromatic substituents introduced onto the sulfonamide influenced the antimicrobial efficacy and specificity of the analogues. Most of the analogues containing a substituted phenyl ring (**2**, **4**, **7**, **8**, and **9**) or 2-thienyl (**11** and **12**) group displayed better antibacterial activity against *C. jejuni* and/or *C. coli* than compound **1**; while the addition of an unsubstituted benzene ring (**2**), furan (**10**), or substitution at the benzylic position (**13** and **14**) reduced the antibacterial activity of the compounds (MIC equal to 200 μM or higher) toward *Campylobacter* (Fig. 2).

TH-4 and TH-8, possessing *p*-fluorobenzyl and *p*-methylbenzyl groups respectively, were only effective against *Campylobacter* spp. and did not alter the growth of other foodborne pathogens (*S.* Typhimurium, *L. monocytogenes*, and EHEC O157:H7), avian pathogens (APEC O78 and *M. gallisepticum*) or probiotic (*E. coli* Nissle 1917). Our previous study showed that *E. coli* Nissle 1917 possess anti-*Campylobacter* effect [30, 41]. Since TH-4 and TH-8 have no effect on *E. coli* Nissle 1917, they could be combined with this probiotic to enhance the control of *Campylobacter* on farm. However, additional studies are needed to confirm the synergistic or potentiation effect of

both probiotic and antibacterial agents against *Campylobacter* in vitro and in vivo. Further, based on these results, we propose that TH-4 and TH-8 might target a protein or biological pathway specific to *Campylobacter* spp. This hypothesis was supported with a minimal impact of TH-4 and TH-8 on the chicken cecal microbiota, as well as minimal toxicity of the molecules on eukaryotic cell lines. In addition, cytological studies showed that both TH-4 and TH-8 compromised the cell envelope integrity of *C. jejuni* treated with the compounds (at least 80% of the cells) by reducing its size and causing deformation of the cell shape [42]. Therefore, TH-4 and TH-8 might disrupt the cell membrane and affect the integrity of the cell wall [42, 43]. Further, differences in antimicrobial efficacy observed between *C. jejuni* and *C. coli* might be due to the composition of the cell envelope or the different stress-related pathways involved in *C. jejuni* and *C. coli* envelop integrity [44].

Our study confirmed that TH-4 and TH-8 reduced intracellular *C. jejuni* in infected colon epithelial cells, which confirmed the permeability of TH-4 and TH-8 through eukaryotic cell membranes and their antimicrobial activity inside the infected cells. Similar results were obtained in infected chickens, which also confirmed that TH-4 and TH-8 antimicrobial activity was stable in chickens despite the fluctuations in pH and temperature, the presence of other organic maters and a complex microbial community. Up to 2.0-log reductions were observed in *Campylobacter*-infected chickens orally treated for 5 days with 0.254 mg of compounds per kg body weight. These results remain very promising given the concentration of compounds used in this study was 1000 times and 137 times lower compared to previous studies testing the anti-*Campylobacter* effect of bacteriocins or antibiotic, respectively [13, 45]. The anti-*Campylobacter* effect of TH-8 in ceca was similar or higher compared to chickens treated for 5 days with enrofloxacin (50 mg kg^{-1} body weight) or neomycin (70 mg kg^{-1} body weight) [13].

Despite the common knowledge about the broad activity spectrum of the thiophene derivatives, our study identified two narrow spectrum SMs (TH-4 and TH8), safe to use, and effective in controlling *Campylobacter* in chickens. Though, both TH-4 and TH-8 have sulfonamide group, unlike sulfonamides, they possess narrow spectrum activity specific to *Campylobacter* suggesting likely a different mechanism of action. Further, based on previous studies, we hypothesize that TH-4 and TH8 could be combined with *E. coli* Nissle 1917 to enhance the control of *Campylobacter* in vivo [30, 41]; however, this hypothesis is yet to be confirmed [30]. TH-4 and TH-8 represent promising lead compounds for the development of potential therapeutic agents via subsequent structural optimization studies.

Specifically, our future studies will focus on the development of water-soluble TH-4 and TH-8 derivatives, determining the bioavailability of these molecules in host tissues after treatment, and identification of the molecular target of TH-4 and TH-8 to facilitate their use in humans.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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