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Executive Summary

Campylobacter jejuni is a common cause of bacterial food poisoning in human worldwide. Chickens are the major source of these human infections. Despite the recognized significance of this pathogen, there is still a lack of effective and practical interventions to reduce human infections or to limit *C. jejuni* colonization in chickens. The Twin-arginine translocation (TAT) pathway is a critical virulence determinant of *C. jejuni* and is required for stress responses and commensal colonization in chickens. As mammals and chickens do not have proteins or receptors that are homologous to bacterial TAT proteins; development of selective small-molecule inhibiting the TAT pathway makes an ideal target for the development of specific antimicrobial therapies against *C. jejuni*. Our high-throughput screen (HTS) of over 51,000 compounds identified 679 compounds that seemed to interfere with *C. jejuni* growth in the presence of 1 mM copper sulfate, an indicator of TAT-dependent inhibition. *In silica* studies resulted in the selection of approximately 250 compounds that have high potential for developing into commercially viable products. The combination of two independent TAT-dependent assays [a $CuSO_4$ based screening and formate dehydrogenase (Fdh) inhibition assay] identified 37 potential TAT dependent inhibitors. Based on the minimum inhibitory and bactericidal concentrations against a diverse range of *C. jejuni* strains and the lack of effect on commensal and beneficial gut microbes, we identified 13 compounds with specific effect on *Campylobacter* strains only. Among these compounds, four of them possessed low cytotoxicity to human intestinal epithelial cells (Caco-2 cells) at 6.25 µg of compounds and had an anti-*C. jejuni* effect in Caco-2 cells at 0.312 µg of compounds. Finally, three out of four molecules tested in three- and five-week-old broiler chickens displayed up to 1.2-log reduction in the *C. jejuni* population in ceca. Delivering the compounds in the presence of $CuSO_4$ seemed to enhance the anti- *C. jejuni* effect for some of the compounds. No major modification of the chicken cecal microbiome was observed with the TAT dependent inhibitors. Future studies on compound derivatization and enhancing the water solubility would enable the development of these potential leads for control of *Campylobacter* in a commercial setting.

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Introduction

Campylobacter is a leading cause of bacterial foodborne gastroenteritis worldwide and is a major public health problem (Adak et al, 2005, Allos et al, 2004, Mead et al, 1999). Human infections with *C. jejuni* are characterized by a rapid onset of fever, diarrhea, abdominal pain and vomiting. *C. jejuni* is a zoonotic pathogen that exists as a commensal in the gastrointestinal tract of chickens and mammals (Beery et al., 1988; Young et al., 2007) and is widespread in food-producing animals (Young et al., 2007). Human infections are primarily associated with consumption of contaminated chicken products (Allos 2001). *C. jejuni* is found in extremely high numbers in chicken ceca [10^9 colony forming units (CFU)/gram] (Wassenaar et al., 1993), and colonized chickens shed the organisms in their faeces until slaughter. Despite extensive colonization in the intestinal tract, *Campylobacter* infection produces little or no clinical diseases in poultry (Corry and Atabay, 2001, Lee and Newell, 2006, Newell and Fearnley, 2003). Prevalence studies conducted in Europe and the U.S. have reported *Campylobacter*-positive flocks ranging up to 100% (Gregory et al, 1997, Luangtongkum et al, 2006, Newell and Fearnley, 2003, Stern et al, 2001). This has contributed to the high prevalence of *Campylobacter* on carcasses and in the chicken production chain (Stern and Line, 1992; Wempe et al., 1993), raising concerns over food safety and highlighting the role of poultry in human campylobacteriosis. At present, there are no vaccines available for use in humans or chickens, and the use of antibiotics in poultry production has been implicated in the emergence of highly resistant *Campylobacter* strains (Young et al., 2007).

On-farm *Campylobacter* control efforts have not been successful in mitigating infections as evidenced by the continuous increase in disease incidents in humans. Though improving biosecurity has some beneficial effect on lowering the overall flock prevalence, these measures have not resulted in consistent and predictable outcomes in controlling *Campylobacter* (Arsenault et al., 2007, Food Standards Agency, U.K. 2008, Nather et al., 2009, Ridley et al., 2011). In addition, stringent biosecurity measures are cost-prohibitive, hard to maintain, and their effectiveness seems to vary with production systems (Food Standards Agency, U.K. 2008, Sahin et al., 2003). Although non-biosecurity measures have been evaluated for control of *Campylobacter* in live birds, currently there are no commercially available competitive exclusion products, vaccines, bacteriocins, bacteriophages or feed/water additives for excluding *Campylobacter* from chickens under production conditions although some promising results obtained under laboratory conditions have recently been reported (Buckley et al., 2010, Hermans et al.,

2011, Layton et al., 2011, Lin 2009). Therefore, a critical need exists for the development of novel anti-*Campylobacter* strategies that can amend and/or replace on-going efforts, while specifically targeting pathways and novel mechanisms to control *Campylobacter* in poultry and to combat emergence of antibiotic resistance *Campylobacter*.

The secretion mechanisms of *C. jejuni* and their role in pathogenesis are not well studied. *C. jejuni* lacks the classical type III secretion system that is found in many bacterial pathogens. The TAT secretion system uses proton motive force to direct pre-folded proteins across the cytoplasmic membrane (Berks et al., 2000). The TAT pathway secretes a wide spectrum of proteins, including virulence factors (Berks et al., 2003; Dilks et al., 2005; De Buck et al., 2008). In many bacteria, TAT mutants show a wide range of defects, including motility, outer membrane permeability, biofilm formation, antibiotic resistance, growth under oxygen-limiting conditions, iron acquisition and copper homeostasis (Ding and Christie, 2003; McDonough et al., 2005; Ochsner et al., 2002; Stanley et al., 2001). The *C. jejuni* TAT system is highly conserved between strains and *Campylobacter* species and it is believed to be critical for *C. jejuni* survival because it (a) targets assembly and membrane stability of surface structures necessary for *C. jejuni*-host interactions and (b) mediates key physiological processes and stress responses that promote bacterial survival both *in vivo* and outside the chicken environment. Our study on the *C. jejuni* TAT system has indicated that the deletion of the *tatC* gene significantly compromises the bacterial ability to tolerate various stresses and cause persistent colonization in chickens (Rajashekara et al., 2009). Notably, chickens and mammals do not have proteins or receptors that are homologous to bacterial TAT proteins. The TAT system also shows substrate-specificity (Jongbloed et al., 2000); therefore, it is possible to design drugs that target a single pathogen without affecting the commensal bacteria. Inactivation of TAT export with selective small-molecule inhibitors could lead to new antimicrobial agents with no toxicity to humans or chickens.

The successful use of high-throughput small molecule screens for the discovery of antibacterials has been described previously. In *V. cholerae,* this technique has successfully been used to identify virstatin, a drug candidate that inhibits the transcription of the regulator ToxT in classic *V. cholerae* strains (Hung et al., 2005). In *Salmonella,* the type III secretion system (T3SS) has been screened for a small molecule inhibitor drug candidate (Felise et al., 2008). Like the TAT system, the T3SS is conserved across bacterial genomes, but does not have a eukaryotic homolog. Therefore, both the T3SS and the TAT system seem to be good drug candidates for similar reasons; the specific

inhibition of these transport systems should likely to have no side effects and inhibit pathogenesis without selection for resistant bacteria. The effect of an inhibitor of T3SS that was identified through the phospholipase assay was then further enhanced through structural optimization (Kline et al., 2008). The refined molecule was more soluble, showed a tenfold increase in potency, was not toxic to mammalian tissue culture cells, and protected mammalian cells from infection by *Salmonella* (Felise et al., 2008). Therefore, HTS screening of small molecules for TAT system inhibition could be a practical approach to identify and subsequently develop potential antimicrobial therapies against C. *jejuni* that would be alternatives to conventional control methods.

Objectives

The main goal of the proposed research was to identify and test small molecules affecting the TAT pathways for on-farm control of *Campylobacter*.

Aim1. To develop a reporter assay and screen a library of small molecule drug candidates that interfere with the TAT system transport.

Aim 2. To screen selected compounds in secondary screens and *in-vitro* studies to identify compounds with greatest potency and selectivity against the TAT system.

Aim 3. To test the selected compounds for their effect on *C. jejuni* in chickens.

Methodology

Bacterial strains, plasmids, and culture conditions: *C. jejuni* strain 81-176 (WT), a highly invasive strain originally isolated from an outbreak associated with raw milk, was used to generate the *tatC* deletion mutant (Δ*tatC*), the *phoX* deletion mutant (Δ*phoX*), and the *phoX* mutant complemented with wild type copy of *phoX* with its RBS (*phoX*+) as previously described (Korlath et al., 1985, Rajashekara et al., 2009). Bacterial strains used in this study are described in **Table 1**. *C. jejuni* strains were routinely grown on Mueller-Hinton agar (MH; Oxoid) microaerobically [(85% N_2 (v/v), 10% CO_2 (v/v) and 5% O_2 (v/v)] in a DG250 Microaerophilic Workstation (Microbiology International) at 42°C. MH agar plates were supplemented with kanamycin (30 µg/mL) when necessary for the *tatC* deletion mutant. Strains were validated via PCR for clonal purity as well as for *tatC* deletion as described in Rajashekara et al., 2009.

Small molecule libraries: For this study, 50,917 small molecules coming from 11 diverse libraries were screened. These libraries were from the NSRB-NERC small-molecule collection, which included FDA approved bio-active molecules, two NIH libraries containing molecules used in recent clinical trials, bio-active screens, and diversified compounds synthesized for favorable physico-chemical properties such as solubility, decreased toxicity and increased stability (**Table 2**). Final concentration of small-molecule used was 6.25 μg for most compounds, but some of the libraries contained different final concentrations. For instance Biomol4 and Microsource1 compounds were tested at 2.5 μg, NIH Clinical Collection 1 and 2 (NCC1 and NCC2) compounds were tested at 12.5 μg. Despite variation between libraries, final concentration of small molecules used in the primary screening was comparable and slightly higher than other observed primary screen concentration (Hung et al., 2005). Small molecules were suspended in 100% DMSO.

Alkaline Phosphate Activity Assay in 96-well plates: Constitutive *phoX*+ and Δ*phoX C. jejuni* strains were grown overnight on MH agar plates. The Δ*phoX C. jejuni* strain was used as a negative control. Cells were normalized to an OD_{600} of 0.4, 100 □l of cells was transferred into each well of a 96-well plate and incubated in a micro-aerobic chamber at 42°C, 70 rpm for 2 h. After incubation, the optical density (OD) at 600nm was measured and plates were centrifuged for 15 min at 7000 g at room temperature. Supernatant was removed using a 96 well plate liquid-handling robot (Biomek3000; Beckman Coulter, Inc. Fullerton, CA). One hundred microliters of *p*-Nitrophenyl Phosphate (PNPP**)** buffer (10 mM Tris-HCl pH 8.0, 0.1% SDS, 2 mM PNPP; ddH_20, Tris-HCl 100 mM stock and 10% SDS) was added in each wells and mixed via Biomek 3000 robot. Cells were incubated for 20 min at 37°C. Absorption at 550nm and 420nm were taken and the phosphatase activity was calculated using the following formula: phosphatase activity = 1000 x [OD_{420} - (1.75 x OD_{550}) / (20 min x OD_{600} x 100 µl)]. Z' score was calculated in three independent experiments as measure of assay fitness as described below.

Calculation of Hits: The positive and negative controls on each assay plate were used to calculate a Z' value for that plate. The National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Disease (NSRB-NERC, NIH) suggests specificity optimization of $0.5 < Z < 1.0$ where $Z = 1 - [3 \times SD_{positive} - 3 \times SD_{negative} / (Mean_{positive} - Mean_{negative})]$.

Copper Sulfate Growth Assay: For this experiment, the wild-type (*C. jejuni* strain 81176) and *tatC* deletion mutant were used. Strains were grown in micro-aerobic conditions for 24 h at 42°C on MH agar plates. Cells were harvested and resuspended to an OD_{600} of 0.08 for both strains using fresh MH broth supplemented with and without 1 mM $CuSO_4$. This copper sulfate ($CuSO_4$) concentration was chosen after a dose-response assay with concentration ranging from 2 mM to 0.25 mM $CuSO_4$. The 1 mM concentration was the highest concentration that did not impact the growth of wild-type cells. A first 384-well plate was divided into two equal parts and filled with the normalized Δ*tatC* (columns 1-12) and wild-type (columns 13-24) cultures containing no $CuSO_4$; a second 384-well plate was similarly prepared using cultures containing $CuSO_4$. Both plates were incubated in micro-aerobic conditions at 42°C for 30 h. OD_{600} was read on a Spectramax384 spectrophotometer before and after the incubation period. Z' score was calculated in three independent experiments as measure of assay fitness as described above.

Primary screening

High-throughput screen of small-molecule libraries: The High-throughput small-molecule screens were performed at the NSRB facilities at Harvard Medical School in Cambridge MA. In a 384 well-plate, columns 1 to 24 of assay plates (Corning 3710) were filled with 40 µl of MH broth supplemented with 1 mM $CuSO_4$ using a Matrix WellMate (Thermo-Fisher) automatic plate filler. One hundred nanoliters of compound were pin-transferred to each plate using the ICCB Longwood Screen Facility Seiko D-TRAN XM3106-31 PN 4-axis cartesian robot (V&P Scientific), controlled by SRC-310A Controller/SPEL for Windows. Then *C. jejuni* WT was normalized at 0.16 OD_{600} in MH broth supplemented with 1 mM CuS04 and 40 µl of the suspension was added to columns 1-23 of the assay plates. Wells in column 24 were filled with the *tatC* deletion mutant (positive control) normalized at 0.16 OD_{600} in MH broth plus 1 mM CuS04. Plates were incubated at 42°C for 36 h under microaerophilic. After incubation, OD_{600} was read after 30 sec of shaking on a Biotek Synergy HT spectrophotometer.

Primary *in silica* Counter-screens Using the Screensaver Small-molecule Database: The NSRB Screensaver database was used to eliminate primary hits that were unlikely to target TAT inhibition in two ways. First, commercial and pharmaceutical based libraries with known antibacterial applications were cross-referenced with the primary hit list and

deprioritized. Second, hits that had positive results in eukaryote-based screens were deprioritized on the rationale that as the TAT system does not have a eukaryote homologue, cross-activity with eukaryotic screens was likely an indication of non-TAT inhibition of growth in our screen. Due to the likelihood of false positives in primary screens, molecules that were positive in two or more eukaryotic screens were deprioritized.

Counter-screen Using Medicinal Chemistry Software: In order to select the compounds for further studies, a series of filters was established to examine the hit set. The criteria for selecting compounds used four categories: 1) Physicochemical Descriptors, 2) Potential Liabilities (i.e. predicted toxicity), 3) Structural Diversity, and 4) Novelty. In category 1, the ChemDraw suite (company), in particular ChemDraw for Excel, was used to calculate molecular weight from SMILES notation. From this calculation, molecular weights less than 200 Daltons and more than 550 Daltons were deprioritized from further study via the Golden triangle measurements for drug-like characteristics (Johnson *et al*., 2009). In category 2, previous notations for primary hits in the screensaver program as well as published work literature search were used in ChemBioFinder (CambridgeSoft) to assess toxicity potential from previous studies. In category 3, ChemBioFinder was used to produce ChemDraw structures, which were examined for 'structure families', compounds with both high structural similarity and common structural components. In the fourth category, compounds were assessed for novelty using SciFinder (Chemical Abstracts Service): those compounds with equal or more than 90% similarity to previously investigated drugs or compounds were not included for further screening. From these categories, an initial batch of 250 'cherry pick' hits was chosen for secondary screen testing.

Secondary screening of selected compounds

Hit confirmation of selected compounds: From ~250 compounds that were initially identified to have potential for future drug development, we purchased 177 molecules that were functionally diverse for further screening. These molecules were obtained from four different vendors: Asinex (n=107), Chembridge (n=28), Life chemicals (n=17), and Maybridge (n=25). In a 96-well plate, 100 µl of an overnight *C. jejuni* suspension normalized at 0.16 OD_{600} in MH broth supplemented with 0.5 mM $CuSO_4$ and the same concentration of small molecules as the one used during the primary screening (**Table 2**).

In parallel, another 96-well plate was prepared with the same condition but no $CuSO_4$. Plates were incubated at 42˚C under micro-aerobic conditions for 24 h. OD_{600} was measured before and after incubation. Plates OD results were compared to determine the specificity of the TAT-dependent inhibition of these small molecules.

Inhibition of Fdh activity: The selected 250 compounds were also screened for inhibition of formate dehydrogenase activity, an enzyme whose secretion is TAT dependent (Kassem and Rajashekara, 2014). *C. jejuni* cultures (0.05 OD_{600}) were incubated with small molecules with appropriate controls for 24 h at micro-aerobic conditions. Subsequently cultures were suspended in an oxygen-restricted solution containing 25 mM sodium phosphate buffer (pH 7) with 1 mM benzyl-viologen and 10 mM sodium formate. The absorbance was measured at 15 min interval at 578nm using an ELISA reader. The percentage of Fdh activity inhibition was calculated using positive (WT) and negative (*tatC* mutant) controls. The compounds that inhibited ≥50% Fdh activity were considered for further analysis.

Based on the $CuSO_4$ and Fdh assays, we prepared a hit plate of 37 compounds, identified positive in both assays, to further screen in various *in vitro* assays. It is necessary to identify a list of compounds (out of 37 compounds) that possess desirable properties like effect on diverse *C. jejuni* strains, no impact on commensals/probiotics bacteria, and have low minimum inhibitory concentration (MIC) and also possess least cytotoxicity to humans cells. Hence, in our *in vitro* assays we focused on prioritizing compounds that had maximum desirable properties by the end of each assay.

Effect on diverse *Campylobacter jejnuni* strains. In order to test whether the small molecules identified in the growth inhibition have effects on diverse strains of *C. jejuni* of different origin, a MTA was executed and several *C. jejuni* strains isolated from poultry in Australia were procured from Ms. Jillian Templeton, Department of Agriculture and Fisheries, Queensland, Australia. To screen for broad-spectrum effect, we selected ten Australian poultry *C. jejuni* isolates based on their SNP type and percentage of prevalence (out of 50 strains procured from Ms. Jillian Templeton). We screened 37 compounds against each of the ten isolates along with *C. jejuni* 81-176 and *C. coli* ATCC 33559 strains in the presence of 0.5 mM CuSO4. We used a cut off of ≥75% *Campylobacter* growth inhibition to consider as effective against that specific strain. The percentage of growth

inhibition for the *C. jejuni* isolates was calculated as mentioned previously (Kumar et al., 2016; De La Fuente et al., 2006) using the following formula: percentage of inhibition = 100 x ($OD_{negative\ control} - OD_{test\ compound}$) / ($OD_{negative\ control} - OD_{positive\ control}$). Where culture + DMSO and culture + antibiotics were positive and negative controls respectively. Compounds were considered desirable if they were effective against at least eight out of twelve screened *C. jejuni* strains.

Screening against probiotic/commensal bacteria. The selected compounds were tested for activity against Gram positive (*Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacteriun lactis, Enterococcus faecalis*, *Lactobacillus brevis*, *Lactobacillus rhamnosus*,) and Gram negative (*Escherichia coli* Nissle 1917) bacteria that were either commonly used probiotics or commensal bacteria. Briefly, the OD adjusted (0.05 OD_{600}) cultures were treated with 6.25 µg of each compound and incubated at specific culture conditions (**Table 3**). A cut off value of ≥50% growth inhibition and affecting at least two strains of commensal or probiotic bacteria was used to determine exclusion criteria for further analysis.

Structure Activity Relationship (SAR) analysis: The 37 compounds were also analysed for SAR in collaboration with Dr. Ewin (Zoetis). These compounds were ranked between one to four based on desired drug like properties using SciFinder, with one being the best and four being the least desired.

Minimum inhibition concentration on selected 22: The selected 22 compounds were diluted (two-fold serial dilutions) from ~6.25 to 0.012 µg in DMSO and MIC was determined against *C. jejuni* 81-176 strain. To determine the MIC, a growth inhibition assay was carried out as described above in the presence of 0.5 mM $CuSO_4$. Plates were incubated at 42˚C under micro-aerobic conditions for 24 h. OD_{600} was measured before and after incubation.

Cytotoxicity of selected compounds to human intestinal epithelial cells. We evaluated 22 selected compounds for cytotoxicity to Caco-2 (human colonic carcinoma) cells as described previously (Kumar et al., 2016). The Caco-2 cells were obtained from the American Type Culture Collection (ATCC Rockville, MD) and maintained in minimal essential medium (MEM) supplemented with 20% foetal bovine serum (FBS), 1% non-

essential amino acid (NEAA; Life Technologies, NY) and with 1 mM sodium pyruvate at 37°C in a humidified 5% CO_2 incubator. A 96-well tissue culture plate was seeded with approximately $1.4x10^5$ cells per well and incubated for 24 h at 37°C in an incubator until a confluent monolayer formed. It was necessary to assess the dose dependent cytotoxicity effect of DMSO as compounds were diluted in DMSO. Therefore, we determined the cytotoxicity effect of varying concentrations of the DMSO vehicle control (1%, 2% and 5%) in Caco-2 cells containing 150 µl of media (without FBS) incubated at 24 h. There was no significant difference among the varying DMSO concentrations and their cytotoxicity values. Therefore, we used 1% DMSO in duplicate wells along with other appropriate controls for cytotoxicity experiment. It is likely that the compounds can bind to serum proteins (FBS) present in the media and thus influence the true cytotoxic effect of the compounds; therefore, we performed the cytotoxicity assay in absence of FBS. The cytotoxicity assay was performed according to manufacturer instructions (Pierce TM; Thermo Scientific, IL) and the percentage of cytotoxicity was calculated by measuring the release of lactate dehydrogenase (LDH) enzyme from the treated cells. Briefly, approximately $1.4x10^5$ cells were grown in a 96-well tissue culture plate containing 150 µl of growth medium. After three washes with medium without supplementation, 6.25 µg of compound was added to duplicate wells and incubated for 4 h at 37°C in a humidified, 5% CO_2 incubator. Subsequently, 50 µl of supernatant was collected for measuring LDH release and the degree of cytotoxicity was determined according to the manufacturer instructions using the following formula: cytotoxicity (%) = (Compound treated LDH activity - Spontaneous LDH activity) / (Maximum LDH activity – Spontaneous LDH activity). Three independent experiments were conducted in duplicate samples in each experiment and the average cytotoxicity values were plotted.

Complementary secondary screening for the selected eight TAT inhibitors:

The number of compounds that qualified our pre-defined criterion from different *in vitro* assays (MIC and cytotoxic) were analysed using SciFinder, a similarity search tool (performed by Dr. Richard Ewin, Zoetis and our laboratory). The eight compounds were prioritized based on their lack of anti-cancer activity (as they are likely to have safety concerns) or a central reactive molecule or patent concerns. The selected eight compounds were further tested (in the presence of 0.5 mM $CuSO_4$) for dose dependent efficacies in; (i) minimum inhibitory concentration (MIC), and (ii) minimum bactericidal concentrations (MBC) using diverse genotypic *Campylobacter* strains including *C. coli*

ATCC strain. We also determined the dose-dependent intracellular *C. jejuni* 81-176 clearance ability and dose-dependent cytotoxicity effect on Caco-2 cell line.

Minimum inhibition concentration for selected 8 compounds: The selected eight compounds were diluted (two-fold serial dilutions) from their original amount of 5 to 0.008 µg in DMSO and MIC was determined against *C. jejuni* 81-176 strain. To determine the MIC, growth inhibition assay was carried out as described above in the presence of 0.5 mM $CuSO_4$. Plates were incubated at 42˚C under micro-aerobic conditions for 24 h. OD_{600} was measured before and after incubation.

Minimum bactericidal concentrations (MBC) for selected eight compounds: MBC is defined as the concentration at which 99.9% (below detection limit) of *Campylobacter* are killed. The MBC assay was performed as described previously with slight modifications (Ling et al., 2015). At the end of the MIC assay, plates were briefly centrifuged at 2,000 *g* for 10 min to collect the pellet. The supernatant from the wells were removed and suspended with 100 µl of fresh MH broth. The suspended culture was plated on MH agar and incubated for 48 h at 42ºC in microaerophilic conditions. To determine the MBC three dilutions prior to the MIC cut off point were plated.

Dose-dependent cytotoxicity on selected eight compounds: We determined the cytotoxicity of selected eight compounds using 5 µg, 25 µg and 50 µg of each compounds on Caco-2 cell line. Cytotoxicity was assessed as described above.

Effect of selected compounds on the intracellular survival of *C. jejuni*: To test the effect of the selected eight compounds on *C. jejuni* intracellular clearance, Caco-2 cells were infected with *C. jejuni* 81-176 strain and a 96 well intracellular survival assay was performed as described previously (Kumar et al., 2016). Briefly, a mid-log phase *C. jejuni* 81-176 culture was pelleted by centrifuging at 9500 *g* for 10 min and washed three times with Dulbecco Phosphate Buffer Saline (DPBS, Gibco) containing 1% (v/v) FBS and adjusted to the desired OD_{600}. Approximately $1.4x10^5$ Caco-2 cells/well were seeded in 100 µl of media containing varying amount of compounds into each well of a 96 well plate and subsequently infected with *C. jejuni* at multiplicity of infection (MOI) of 100 in duplicate wells. To determine clearance, Caco-2 cells were incubated with bacteria for 3 h and treated with gentamicin (150 µg/ml) and incubated for additional 2 h. Cells were washed

three times with DPBS with no calcium or magnesium and incubated with 1 µl of diluted compounds (~5.0 to 0.08 µg) for 24 h. Infected cells were washed twice with MEM, lysed with 0.1% (v/v) Triton-X100. Subsequently, 100 µl aliquot from each well was 10-fold serially diluted in MEM and plated on MH agar in duplicate to determine CFUs. The appropriate controls included; (i) infected Caco-2 cell treated with kanamycin or chloramphenicol, (ii) infected Caco-2 cells treated with 1 µl of 100% Trition-X, (iii) infected Caco-2 treated with 1 µl of 100% DMSO. Two independent experiments were performed with duplicate treatments in each experiment and the average CFU value was used to plot the graph.

Effect of selected compounds on the survival of *C. jejuni* in three- and five-weeks-old SFP chickens: Three-week-old *C. jejuni*-free chickens were inoculated orally with 10^4 CFU of *C. jejuni* cocktail (mixture of 5 chicken isolates). At 24 h post inoculation, a rectal swab was taken to confirm the *C. jejuni* colonization. At 48 h of inoculation, chickens were administrated orally twice a day for five days with different treatments as described in **Table 4**. Treatments were given orally in 200 µl of 30% DMSO. Following the last treatment, the chickens were sacrificed and ceca removed aseptically. Each treatment had between 5 to 7 chickens. Body weight was recorded before and after treatment, as well as weights for the collected ceca. One cecum was processed for CFU enumeration, while other cecum was stored at -80°C for microbiota studies. The ceca used for bacterial quantification were homogenized with two 2 ml of 1X PBS, serial ten-fold dilutions performed and 0.1 ml plated onto MH-CSS agar plate, and incubated for at least 48 h at 42°C in a microaerobic chamber.

The same experiment was performed with five-weeks-old chickens. The same protocol was followed; chickens were administrated orally twice a day for five days with different treatments as described in **Table 5**. This experiment was duplicated with 5 chickens per treatment.

Microbiome analysis

Samples used for the microbiome study were from the same chicken experiment described above. Bacterial diversity and abundance were investigated using the ceca stored in -80°C. Genomic DNA was extracted using the PureLink Microbiome DNA *Purification* Kit (Life Technologies, Invitrogen Corp.), combined with RNAse treatment. About 0.15 to 0.20 g of cecum content was processed according to

manufacturer's instructions. After quality control with electrophoresis and spectrophotometric examination, DNA samples were submitted for sequencing at the MCIC (OARDC, Wooster OH) using Illumina sequencer targeting the V4 –V5 region of the 16S ribosome gene.

Bioinformatics analyses: The estimation of the bacterial diversity and abundance in each sample was based on sequencing of the V4 –V5 region of the 16S ribosome gene. All analyses were performed with QIIME 1.9 software package. Trimmomatic was used for trimming of FASTQ data and removal of Nextera adapter sequences. For the quality filtering steps, reads with a length inferior than 40 nucleotides, or a minimum quality threshold lower than 20% were removed from the library. Pandaseq was used for removal of spacer sequences as well as the stitching of the forward sequences with the matching reverse sequences. The maximum length used for this step was 575 nucleotides. After quality filtering, an open reference operational taxonomic unit (OTU) picking strategy (pick_open_reference_otus.py) was performed using the Greengenes reference database (http://greengenes.secondgenome.com/downloads/database/13_5; minimum OTU size picked was 10). Finally, microbiome diversity and abundance was analyzed using total amount of OTUs, rarefaction curves (alpha_rarefaction.py), principal coordinate analysis (principal_coordinates.py), jackknife test (jackknifed_beta_diversity.py), and relative abundance studies (summarize_taxa_through_plots.py). All the bioinformatics analyses were performed using the Terminal interface combined with Trimmaseq, Pandaseq, Qiime, Empior and Figtree softwares.

Statistical analysis. Data were analyzed using one-way analysis of variance with mean separation by a least significant difference test at 5% level of significance in GraphPad Prism version 6 software and JMP PRO 12 software.

Results

Alkaline phosphatase assay development and validation: In order to test the effectiveness of small molecule compounds, we developed an assay that measures the ability of *C. jejuni* to secrete alkaline phosphatase, PhoA(cj), also referred to as PhoX. *C. jejuni* has only one alkaline phosphatase, PhoX, and is a substrate for the TAT system translocation. Wild-type PhoX expression is controlled by the PhosS/PhosR two-component system, which normally changes PhoX expression in response to

environmental conditions. To prevent different environmental conditions from affecting PhoX secretion, we developed a constitutively PhoX expressing strain by deleting *phoX* from the *C. jejuni* chromosome and re-inserting the *phoX* gene into a rRNA locus under the control of a constitutive *metK* promoter.

Using a p-nitrophenyl phosphate (PNPP) based assay that measures the enzymatic activity of the TAT substrate PhoX, we confirmed that *phoX* deletion mutant, as expected, has almost no phosphatase activity (**Figure 1A**). The PhoX+ strain possessed alkaline phosphatase activity similar to wild type. Further, we adopted the assay to a 96-well microassay format using robotic liquid handling system, Biomek 3000, for high throughput screening of small molecules. Using The National Screening Laboratory for the Regional Center for Excellence in Biodefense and Emerging Infectious Disease (NSRB, NIH) guidelines, we calculated a Z' score of 0.61 (Zhang et al., 1999). However, since this assay was found to be labor intensive, particularly when screening large number of compounds, we developed another TAT-dependent assay to facilitate our primary screening of large libraries of small molecules.

Copper sulfate sensitivity assay development and validation: We also developed a growth inhibition assay based on the susceptibility of the *tatC* mutant to $CuSO_4$ (**Figure 1B**). The TAT substrate, *cue*A, confers protection against $CuSO_4$ (Hall et al., 2008). Therefore, if a small molecule inhibits the TAT system, *Campylobacter* will have an increased sensitivity to $CuSO_4$. This assay was adapted to 384-well plates. Because of the labor intensiveness of alkaline phosphatase assay, we screened for $CuSO_4$ growth inhibition in primary screening instead of alkaline phosphatase activity.

In this experiment each strain (wild type *C. jejuni* and *tatC* mutant) was grown on a 384-well plate. We confirmed that, because of inhibition of the TAT system, that the *tatC* deletion mutant was not able to grow in the presence of even 0.5 mM CuSO4 and that the difference between the mutant and parent was highly significant ($P<0.001$).

By comparison, wild-type *Campylobacter* grows normally in the presence of 0.5 or 1 mM $CuSO_4$. Non-specific growth inhibition due to small molecule toxicity was investigated with a parallel survival assay in the absence $CuSO_4$. In the absence of $CuSO_4$, *tatC* deletion mutants had a partial growth defects compared wild-type cells. Our assay met the screening standards suggested by the NSRB-NERC (NIH) (Z' = 0.52), and this assay was used in the preliminary screening.

Primary screen resulted in 781 hit compounds: A total of approximately 51,000 small molecules were screened in the primary screening against *C. jejuni* 81176 for TAT system dependent inhibitors in 384-well plate format. We identified close to 781 small molecules, which met our criteria for strong hits (**Figure 2**). OD_{600} values were compared to the positive control from the control plate (= z' prime) to determine an average OD_{600} for 'positive' cells. Average OD_{600} for the negative control = 0.18. Based on these OD_{600} values, a substance was considered active with and OD_{600}<0.201 for the experimental strains (**Figure 3**) and having a value at least 3xSD lower than the mean (**Figure 4**).

Primary in silica counter-screens using small molecule database: From these molecules, we performed an *in silico* counter-screen based on bioactivity in eukaryotic targets. Those that were concurrently a positive hit in a eukaryotic screen were deprioritized from our results. From our primary screen, 679 small molecules met our criteria for an active compound **(Figure 2).** These molecules were considered as a 'hit'. The hit ratio varied considerably by library, with the highest hit rates found in NCC2 (5.34%) and BioMol4 (4.06%) compound libraries. The lowest primary screen hits occurred in the LifeChemicals1 and Maybridge5 library, which had hit rates of 0.98% and 0.80% respectively. The average hit rate across all libraries was 1.33%, which is significantly higher than the optimal hit rate proposed by NSRB screening guidelines (0.3% or approximately one hit/plate) (**Table 2**).

These counter-screens reduced the number of small-molecule hits to 476 compounds (0.94% hit rate). Perhaps unsurprisingly, the small molecules in bioactive libraries; NCC1, NCC2, Microsource1, MSDiscovery1, and Biomol4 had the greatest percentage of primary molecules disqualified by the secondary screens. The non-bioactive screens had a more modest reduction in small-molecule candidates (a 13-20% reduction in potential drugs) although the Enamine2 library had a 30% reduction in small-molecule candidates. This may be, in part, because there is less information available about the chemicals in these libraries.

Counter-screen using medicinal chemistry software: Across all five trials, 27% (127/476 compounds) were eliminated via physiochemical descriptors. Additionally, 95 compounds were deprioritized due to general physiological concerns: potentially reactive groups, compounds that lacked "drug-like" functionality (i.e. only had one potentially bioactive group), or compounds that did not lend themselves to structural modification.

Trial 3 results are presented graphically as examples of how the other trials were analysed. We found that in trial 3, 28% of primary hits (14/48 compounds) were eliminated as unsuitable using these methods. Ten of these compounds were eliminated due to low molecule weight (**Figure 5**), and four had a logD outside of the acceptable range (indicating higher solubility in hydrophilic solutions than aqueous ones at neutral pH). Trial 3 revealed 14 compounds that have a common structural moiety: thiourea. These compounds can then be further subdivided based on structure as shown to determine the degree of structural similarity (**Figure 6**). This technique is graphically depicted as representative of results across all trials.

Across all trials there were 150 compounds that had significant similarities: 66 compounds with thiourea groups (19% of the compounds, out of 349), 46 benzimidazoles (13%), and 38 acylhydrazones (11%). By looking at the structures and eliminating very similar compounds, we chose compounds 22, 19, and 13 as representative of those groups. Also, there was a group of oxadiazoles, which had 9 of 11 compounds that were very structurally similar.

Secondary screening of selected compounds: From approximately 250 compounds that were initially identified to have potential for future drug development (based on the *in silico* medicinal chemistry analysis) from preliminary screening of 51,000 compounds, we purchased 177 molecules that were functionally diverse for further screening. These molecules were obtained from four different vendors: Asinex (n=107), Chembridge (n=28), Life chemicals (n=17), and Maybridge (n=25).

To confirm our preliminary screening results, these compounds were first screened for $CuSO_4$ dependent (0.5 mM) or independent inhibition of *C. jejuni*. Based on these studies, we identified 59 compounds having varying inhibitory activity only in the presence of $CuSO_4$ and were non-inhibitory in the absence of $CuSO_4$ to *C. jejuni* 81-176 (TAT dependent) (**Figure 7**). There were 53 compounds that non-specifically affected the *C. jejuni* growth irrespective of the presence or absence of 0.5 mM $CuSO_4$ (TAT independent) (**Figure 7**). However, 65 compounds failed to show any inhibitory activity (≥30 percentage growth inhibition). This could be due either inter assay variations; factors like robotic vs manual, nanoliter vs microliter scale or batch to batch variation in compounds synthesis and/ or storage.

To further confirm that these compounds are working through TAT pathway, we screened all the 177 compounds using another TAT dependent assay, formate

dehydrogenase (Fdh) assay. The Fdh is translocated through TAT system and TAT mutant has very minimum Fdh activity compared to WT. *C. jejuni* (Kassem and Rajashekara, 2014). The compounds that inhibit Fdh activity in the WT (activity similar to that of *tatC* mutant or lower) but not affecting the growth of the WT were selected. We identified 100 compounds that have varying (30 to 100%) Fdh inhibitory activity (**Figure 7**). We further compared the $CuSO_4$ dependent inhibitors (59) with Fdh inhibitors (100) and observed that 37 common inhibitors that were TAT dependent. This further suggests that these compounds are working through a common pathway, more likely TAT pathway.

We prepared a hit plate of 37 compounds to further screen in various *in vitro* assays. It is necessary to identify a list of compounds (out of 37 compounds) that possess desirable properties like effect on diverse *C. jejuni* strains, no impact on commensals/probiotics bacteria, and have low minimum inhibitory concentration (MIC) and also possess least cytotoxicity to eukaryotic cells. Hence, in our *in vitro* assays we focused on prioritizing compounds that has maximum desirable properties at the end of each assay.

Effect of selected compounds on multiple *C. jejuni* strains: In order to test whether the small molecules identified in the TAT- dependent growth inhibition screen have effects on diverse strains of *C. jejuni* of different origin, we tested all 37 compounds against diverse *C. jejuni* strains of poultry origin. With this approach we identified 21 compounds (out of 37) that have effect against a pool of ten diverse Australian poultry *C. jejuni* isolates (**Figure 8**).

Effect of selected compounds on probiotic/commensal bacteria: Traditional antibiotics have limitations as they impact normal gut microflora. Hence, we screened the 37 compounds against known commensals or probiotic strains *in vitro*. We identified 13 compounds (out of 37) having adverse effect on the probiotics/commensal tested and nine compounds among these 13 compounds were also represented in the 21 compounds shown to have an effect on multiple *C. jejuni* strains (**Figure 9**).

Based on the SAR analysis a total of 22 compounds out of 37 (twelve from above analysis and ten from SAR analysis) were selected for further analysis.

Minimum inhibition concentration of selected 22 compounds: Six compounds were effective at 0.195 µg, four were effective even at 0.0488 µg and three were effective at

0.024 µg **(Figure 10).** Growth inhibition concentrations for the 22 compounds were equivalent between the two independent experiments.

Cytotoxicity of selected 22 compounds to Caco-2 cells: All compounds had cytotoxicity value ≤15%, except for four compounds. Overall, all selected compounds had least cytotoxicity value when compared to standard positive control supplied in the kit (**Figure 11**).

From the 22 compounds eight compounds were prioritized (**Figure 12**) by further in silico analysis (Dr. Richard Ewin, Zoetis). These compounds did not possess anti-cancer activity (as they are likely to have safety concerns), a central reactive molecule or patent concerns. These selected eight compounds were further tested (in the presence of 0.5 mM $CuSO_4$) for dose dependent efficacies in; (i) minimum inhibitory concentration (MIC), and (ii) minimum bactericidal concentrations (MBC) using diverse genotypic *Campylobacter* strains including *C. coli* ATCC strain. Further they were tested for dose-dependent intracellular *C. jejuni* 81-176 clearance ability and dose-dependent cytotoxic effect on Caco-2 cell line. These eight compounds were also tested for the effect on Fdh activity.

MIC and MBC selected eight compounds: The most of compounds were effective at least two-fold dilution (2.5 µg) or lower. The compd #7 and #8 were effective at the final concentration of 0.31 µg) against diverse *Campylobacter* strains (**Table 6**). However compd #3 and #4 did not completely inhibit *Campylobacter* strains at 5.0 µg. As MIC cut off concentrations not necessarily indicate the complete absence of culturable *Campylobacter*, we also determined the minimum bactericidal concentrations (MBC) for these top-eight compounds. The MBCs of majority of the compounds (6 out of 8) were two times their MIC value (**Table 7**).

Inhibition of Fdh activity: All the selected 8 compounds at least inhibited three or more *C. jejuni* strains however compd #8 inhibited as many as eight *C. jejuni* strains at the tested quantity of small molecules (**Table 8**).

Dose-dependent cytotoxicity of selected 8 compounds: At 5 µg of small molecules, except compd #1 and #8, rest of the six compounds had cytotoxicity <10%. Similar trend was observed when when tested at 25 µg and 50 µg (**Figure 13**). Although compd #8 had >30% cytotoxicity, it had the least MIC of all the compounds (0.08 µg), this implied that

cytotoxicity was assessed at least 625-fold higher than its MIC value suggesting that this compound can be a useful candidate for in vivo studies.

Dose-dependent intracellular clearance ability: Caco-2 cells infected with *C. jejuni* 81-176 strain were used to determine the dose-dependent intracellular clearance ability of selected eight compounds. All the compounds were effective in clearing (limit of detection 10 CFU/ml) intracellular *C. jejuni* at 5 µg. Four of the compounds (Compd #1, #2, #7 and #8) were effective in reducing the *C. jejuni* load by at least one log even at 0.312 µg (**Table 8**).

Evaluation of TAT dependent inhibitors in three-week-old broiler chickens: There were no significant differences between treatment groups in body weights before or after five days of treatment (T-test; $P>0.05$) (**Figure 14**). A significant reduction in the *C. jejuni* population after five days of treatment was observed with T5 (compd#7) (T-test; $P<0.003$). The T5 treated group displayed a ~1-log reduction in the *C. jejuni* population compared to the DMSO control (**Figure 14**). However, treatment with T4 (compd#8) and T6 (compd#1) did not show any significant difference in the CFU numbers compared to control group (**Figure 14**).

Evaluation of TAT dependent inhibitors in five-week-old broiler chickens: There were no significant differences between treatment groups in body weights before or after five days of treatment (T-test; $P>0.05$) (**Figure 15**). A significant reduction in the *C. jejuni* population after five days of treatment was observed with T7 (compd#2) and T6/C (T-test; $P<0.001$ and $P<0.05$ respectively). T7 and T6/C treated groups displayed a 1.2-log and 0.7-log reduction of the *C. jejuni* population respectively compared to the DMSO control (**Figure 15**). Addition of $CuSO_4$ to the small molecule T6 enhanced the anti-*Campylobacter* effect *in vivo*. However, while treatment with T5 reduced the number of *C. jejuni*, the reduction was not significant (T-test; $P<0.06$) in this trial (**Figure 15**).

Microbiome study of three-weeks-old and five-weeks-old chickens treated with anti-*Campylobacter* compounds (TAT compounds):

To complement the bacterial quantification results, microbiome studies were performed to understand the relationship behind the anti-*C. jejuni* effect and the impact on the gut bacteria. Briefly, using Illumina sequencing combined with bioinformatics tools,

bacterial populations in chicken ceca were compared between the treated and the controls groups. Samples were examined via rarefaction curves, principal coordinate analysis, jackknife test, and relative abundance.

Impact of TAT compounds on three-week-old chicken ceca microbiome: For this experiment, chickens treated with the compounds T4, T5, and T6 (**Table 4**). Only T5 (n=6) and T6 (n=6) were selected for further microbiome studies due to the promising reduction of *C. jejuni* abundance in the cecum. Also three controls, untreated and un-challenged chickens (NC; n=3); *C. jejuni* challenged and untreated (P; n=5); and *C. jejuni* challenged and treated with DMSO (DMSO; n=6), were used to identify the precise effects of the *C. jejuni,* DMSO, and each molecule on the cecal microbiome.

Following the OTU picking, between 13,813 and 24,449 OTUs were found among the samples studied (minimum threshold for OTU picking was 10). No difference in the total OTUs was detected between treatments (Student T test; P>0.05; **Figure 16A**).

The first component of our microbiome analysis was to study the alpha microbial diversity (**Figure 16B**) and the richness (**Figure 16C**) for each treatment using the rarefaction technique. Each rarefaction curves had the same trend, no differences were observed (Student T test; P>0.05). Treatment did not affect the OTU richness of the microbiome in the cecum.

To determine whether the TAT compounds affected the bacterial diversity and abundance of the cecal microbiota, two robust and complementary analytic methods were applied. The principal coordinate analysis (PCoA) identified dissimilarities between treatments (also called eigenvectors and labeled PC1, PC2, PC3, up to PC10). Each eigenvectors were associated with a corresponding percentage of variation (called eigenvalue). The study of the microbial diversity using unweighted uniFrac values (alpha diversity only) identified eigenvectors with an eigenvalue ranging between 0% and 13% (data not shown). The three eigenvectors with the highest dissimilarities values were selected to create a 3D plot (PC1, PC2, and PC3; **Figure 17A**). The 3D representation showed a homogeneous and clustered distribution of the samples. Even though PCoA identified 13% variation between the tested samples, it didn't seem to separate T5 and T6 (in green and purple) from the DMSO control (in red), however P and NC controls (in orange and blue) tend to shift a little on the left side of the 3D plot, suggesting that the presence of DMSO might affected the bacterial population. This hypothesis was further confirmed using the jackknife test (**Figure 17B**). The P and NC controls were mainly

clustered on the top side of the polar tree while the DMSO control was mostly clustered on the bottom side of the tree. While T5 and T6 were distributed throughout the tree but there was still a higher proportion close to the DMSO control. These observations strongly suggest that DMSO affected the bacterial diversity in the cecum and these variations might not be caused by the compounds T5 and T6.

The same analyses were performed using weighted uniFrac values, allowing a determination ofwhether the TAT compounds did affect the diversity and abundance of the bacterial population inside the cecum. The PCoA identified eigenvectors with an eigenvalue ranging between 0% and 44% (data not shown). The three eigenvectors with the highest dissimilarities values were selected to create a 3D plot (PC1, PC2, and PC3; **Figure 17C**). The 3D representation showed a better dissimilarity between samples with and without DMSO, confirming that DMSO affected the diversity and abundance of the bacterial population in the cecum. T5 was still closely clustered with the DMSO control while T6 tended to shift more on the right side of the 3D plot, suggesting that T6 might not affect the diversity but it did alter the bacterial abundance. These observations were confirmed with the jackknife test (**Figure 17D**). Again NC and P controls were clustered in the top left side of the tree and the DMSO control was clustered at the bottom side of the tree. On the other hand T6 shifted more on the top right side of the tree while T5 stayed closely distributed around the DMSO control. More precise qualitative and quantitative analyses were performed at the genus level to confirm and explain these variations between treatments.

The second component of our microbiome analysis was to identify the bacterial populations affected at the phylum/genus level by the selected compounds using the relative abundance of each OTU identified during the sequencing (**Figure 18A and 18B**). At the phyla level, *Proteobacteria* were significantly increased and *Firmicutes* significantly decreased by the presence of DMSO compared to the P and NC controls, $P<0.001$ and $P<0.004$ respectively. There was no significant difference in these phyla in T5 and T6 treated chickens compared to DMSO control. Only bacteria belonging to unassigned group during the open reference OTU picking showed a significant increase with T5 compared to the DMSO control. Unassigned bacteria were similar between T5 and the NC and P controls; however, they were significantly higher compared to T6 and DMSO control ($P<0.05$). As expected at the genus level, chicken infected with *Campylobacter* (P) and the ones treated with DMSO (DMSO) did affect the microbial diversity and abundance compared to the *Campylobacter* free chickens (NC). For better accuracy in statistical

analysis, we compared T5 and T6 with the DMSO control first and then observed the differences with the P and NC controls (**Table 10**). T5 significantly decreased *Proteus* ($P<0.02$) and *Oscillospira* ($P<0.02$) abundance compared to the DMSO; however these two genus represented less than 5% of the total relative abundance described in the cecum microbiome. T6 significantly decreased (*Ruminococcus*) ($P<0.04$), *Enterobacteriaceae* (other) ($P<0.05$), and *Oscillospira* ($P<0.02$) abundance compared to the DMSO control; however these two genus represented less than 8% of the total relative abundance described in the cecum microbiome. Also, T6 significantly increased *Streptococcus* ($P<0.007$) abundance compared to the DMSO control. *Streptococcus* represented at least 17% of the total bacterial population which might explain the differences observed with the PCoA and jackknife test.

Impact of TAT compounds on five-week-old chicken cecal microbiome: For this experiment, chickens were treated with the compounds T4, T5, T6, T7, T4C, T5C, T6C (**Table 5**). In this Table, C indicates compounds mixed with 0.5 mM $CuSO_4$ before administering to birds. The compounds T5 (n=5), T7 (n=5), T5C (n=5), and T6C (n=5) were selected for microbiome studies due to a promising reduction of *C. jejuni* abundance in the cecum. Three controls, *C. jejuni* challenged and untreated (P; n=5); *C. jejuni* challenged and treated with DMSO (DMSO; n=5), and *C. jejuni* challenged and treated with $CuSO_4$ ($CuSO_4$; n=4) were used to characterize the microbiome fluctuations caused by the solvents and by the small molecules. Also, compounds showing low anti-*C jejuni* effect such as T4 (n=5) and T6 (n=5) were used in this study to increase the understanding of the microbiome data by comparing them to the effective compounds.

Following the OTU picking, between 9,293 and 23,179 OTUs were found among the samples studied (minimum threshold for OTU picking was 10). No difference in the total OTUs was detected between treatments (Student T test; $P>0.05$; **Figure 19A**).

As above, the rarefaction study showed no significant differences in the diversity and richness of OTUs depending on the treatment (**Figure 19B and 19C**). The PCoA of the unweighted uniFrac values identified eigenvectors with an eigenvalue ranging between 0% and 9% (data not shown). The three eigenvectors with the highest dissimilarities values were selected to create a 3D plot (PC1, PC2, and PC3; **Figure 20A**). Once again, chicken treated with DMSO (in blue) showed dissimilarity in the bacterial diversity compared chickens not treated with DMSO (P; in orange). The presence of $CuSO_4$ (in red) didn't seem to affect the microbiome diversity compared to the DMSO control (in blue). The TAT

compounds, T5 and T6 (in green and light blue) showed the similar pattern as observed with the 3 week-old chickens with microbiota composition closely resembling the DMSO control group. On the other hand, T5 and T6 combined with $CuSO_4$ (T5C and T6C; in yellow and pink) showed a different distribution compared to the original T5 and T6. T5C also shifted to the far left side of the 3D plot, away from the DMSO and $CuSO_4$ controls. Finally, T4 (in green) showed the same dissimilarity than T5C and T7 (in green/grey) clustered between the $CuSO_4$ and P controls (in red and orange). These observations were confirmed with the jackknife test (**Figure 20B**). P and DMSO controls had an opposite distribution in the tree while $CuSO_4$ control was distributed everywhere. T7 was principally clustered around the P control while T5 and T6 were clustered around the DMSO control, and T5C was closely associated with T4.

The same analyses were performed using weighted uniFrac values, to identify potential differences of abundance and diversity related to the treatment. The PCoA identified eigenvectors with an eigenvalue ranging between 0% and 38% (data not shown). The three eigenvectors with the highest dissimilarities values were selected to create a 3D plot (PC1, PC2, and PC3; **Figure 20C**). Once again a clear dissimilarity was observed between the treatment with and without DMSO. On the other hand, the differences detected in the Figures 21A and 21B were reduced in Figure 21C; suggesting that T4, T7, and T5C induced family or genus specific alterations in the bacterial diversity. However at the whole microbiome level, the OTU quantities were the same between treatments (**Figure 19A**). Jackknife results (**Figure 20D**) supported PCoA observations (**Figure 20C**).

In order to explain this variation between treatments, the relative OTU abundance was studied to identify the bacterial populations affected at the phyla/genus level by the selected compounds using the relative abundance of each OTUs identified during the sequencing (**Figure 21A and 21B**). At the phyla level, T6C significantly increased the abundance of bacteria population belonging to unassigned group compared to the $CuSO_4$ control ($P<0.004$), following the open reference OTU picking. T4 and T7 significantly decreased *Tenericutes* abundance compared to the DMSO control ($P<0.03$ and $P<0.003$ respectively). At the genus level, some contradictory results were observed compared to the experiment with three-week-old chickens. It is possible that the differences in chicken ages may explain these contradictions. Table 11 shows the list of the genera identified as significantly different from their respective control (Student t test). For a better accuracy of the statistical analysis, we compared T4, T5, T6, and T7 with the DMSO control, and T5C and T6C with the $CuSO_4$ control. Despite all these microbial fluctuation between

treatments, most of these genera had a relative abundance lower than 1%. Only *Lactobacillus* and *Ruminococcaceae* represented each about 15 to 20% of total microbiome population.

Discussion

Outcomes presented in this study were the results of an HTS screening for the identification of TAT system inhibitors, aiming at the development of potential antimicrobial therapies against *C. jejuni* as an alternative to conventional control methods on-farm. Among the 51,000 molecules used in the study, we identified eight potential TAT inhibitors with suitable range of characteristics with three effective in reducing *C. jejuni* population in broilers (**Figure 12**).

The first steps of this screening were accomplished based on turbidimetric inhibitions and *in silica* studies (Xu et al., 2015; Kumar et al., 2016). The combination of non-lethal concentration of $CuSO_4$ with a specific concentration of small molecule allowed the identification of potential TAT pathway inhibitors, which increased the sensitivity of *C. jejuni* to $CuSO_4$ (Drozd et al., 2011; Hall et al., **2008**). This approach also identified TAT independent *C. jejuni* growth inhibitors. Compounds with an OD_{600} lower than 3xSD compared to the positive control were selected for *in silica* studies (**Figure 4**). A total of 781 compounds affected *C. jejuni* growth and might be related to the TAT pathway. To narrow down the number of compounds before secondary screens, both supplementary *in silico* comparison and medicinal chemistry analysis were performed in order to eliminate eukaryotic drug targets, artefacts, and compounds improper for commercial use. At the end, hits were reduced to 250 bioactive small molecules with promising drug properties.

To confirm whether *C. jejuni* growth defects were TAT dependent, the results from the primary screen in presence of $CuSO_4$ were compared to a second screen done without $CuSO_4$. The capacity of the compounds to inhibit the Fdh activity was also determined due to previous studies showing the relationship of Fdh activity to the TAT system (Kassem and Rajashekara, 2014). A total of 177 compounds from 250 compounds were analysed in secondary assays. Of the 177 candidates, 100 compounds were Fdh inhibitors, 53 inhibited *C. jejuni* growth only in presence of $CuSO_4$ and 37 of them filled both criteria, suggesting that these 37 compounds were working through a common pathway, more likely TAT pathway (**Figure 7**). After spectrum of activity (effect on multiple *C. jejuni* strains) assays, 13 compounds showed a broad activity against twelve *Campylobacter*

strains and no effect on commensal gut bacteria at 6.25 µg (**Figure 8 and 9**), and eight out of these 13 were effective against *C. jejuni* at low quantity (<0.2 µg) (**Figure 10**).

The selected eight compounds (compd #1 to #8; **Figure 12**) were used for cytotoxicity and intracellular clearance studies using Caco-2 cells. Six out of the eight compounds displayed a cytoxicity below 10% and four of the six compounds effectively reduced at least 1-log *C. jejuni* 81-176 population inside Caco-2 cells at 0.312 µg of compounds (**Figure 11 and 13**). These results suggested that these four compounds (compd #1, #2, #7, and #8) did not target any eukaryotic pathways, yet they were still internalized into chicken epithelial cells and effective against *C. jejuni*.

Finally, the selected four compounds were tested on three- and five-week-old broiler chickens. T5 (Compd #7) was effective on three-week-old chickens, reducing the *C. jejuni* population in the caecum by 1 log, and showed a similar trend in 5 week-old birds (**Figure 14 and 15**). T6 (Compd #1) was ineffective on three-week-old chickens and on five-week-old chickens without $CuSO_4$ but it was effective on five-week-old chickens when combined with $CuSO_4$ (1.2-log reduction) (**Figure 14 and 15**). T7 (Compd #2) was effective on five-week-old chickens, reducing by 0.7-log *C. jejuni* population in ceca. On the other hand, T4 (compd #8) did not affect *C. jejuni* population in either three- and five-week-old chickens. These observations highlight the possibility that the addition of $CuSO_4$ into the small molecule treatment could increase the anti-*C. jejuni* effects for some of our candidates. This effect suggests that the addition of $CuSO_4$ before treatment could change the conformation of the small molecules, which could make it more reactive against *C. jejuni*.

The microbiome studies showed that most of the fluctuations were explained by the presence of DMSO, which was used as solvent (30%) to suspend the compounds. TAT-targeting compounds didn't affect the global diversity and abundance of bacteria inside the ceca in both experiments. However at the genus or family level, they increased or decreased the abundance of some bacteria belonging to the *Enterobacteriales* and *Clostridiales* orders in both three-week- and five-week-old birds, which have been reported previously as indicators of the chicken health (Oakley et al., 2014; Sergeant et al., 2014). In conclusion, TAT compounds did not induce major modification of the chicken microbiome and were still effective in reducing *C. jejuni* population on chicken ceca. We successfully identified three small molecules effective against *C. jejuni* in poultry. The compounds evaluated in this study were not, however, readily soluble in water and were therefore administered to chicken in a vehicle containing 30% DMSO. For mass application in poultry production the compounds need to be water soluble. Therefore,

future studies are required to 1) improve the water solubility of these compounds, 2) enhance the bioavailability of these compounds by administering with suitable solubilizers or drug vehicles, 3) understand the impact of $CuSO_4$ on the small molecule by itself, and 4) identify the specific drug target in *C. jejuni*.

Implications

C. jejuni is a leading cause of bacterial foodborne gastroenteritis worldwide *Campylobacter* have been associated with the Inflammatory Bowel Diseases (e.g. Crohn's disease and ulcerative colitis). Infection can also result in fatal reactive arthritis and Guillain-Barr´e syndrome. The majority of human *Campylobacter* infections are predominantly associated with poor handling of raw chicken or consumption of undercooked chicken. The role of contaminated poultry in human infections is supported by the ubiquity of *Campylobacter* in live birds and on carcasses, combined with the fact that identical genotypes are found in poultry and human infections. The increasing prevalence of antibiotic resistant infections is a serious public health concern, and result in additional healthcare costs (national action plan). Furthermore, metagenomics studies show that broad spectrum antibiotics use affects gut microbiota resulting in dysbiosis which contributes to infectious, inflammatory, and/or malnourished conditions and reduce both health and productivity. Campylobacteriosis is a self-limiting disease; however, antimicrobial therapy is warranted in severe disease manifestations and in immune-compromised individuals. The most commonly used antibiotics are macrolides (eg. erythromycin) and fluoroquinolones (eg. ciprofloxacin). Aminoglycosides are recommended in serious cases. As their use increases, more *Campylobacter* isolates have developed resistance to these antimicrobials; for example, *Campylobacter* drug resistance increased from 13% in 1997 to 25% in 2011 (National action plan). Campylobacteriosis is projected to remain one of the top ten bacterial conditions globally, and several antibiotics are no longer effective. Therefore, new and effective narrow spectrum antimicrobials that are less likely to induce resistance in bacteria are critically needed. This can be achieved through high throughput screening (HTS) to identify inhibitors of specific virulence mechanisms. Therefore, TAT-dependent narrow spectrum inhibitors identified in this study through HTS screening offers excellent opportunities for the development of practical intervention to reduce/limit *Campylobacter* colonization of poultry and thus enhance food safety.

Recommendations

The compounds evaluated in this study were not, readily soluble in water and were therefore administered to chicken in a vehicle containing 30% DMSO. For mass application in poultry production the compounds need to be water soluble. Therefore, future studies are required to:

1) Improve the antibacterial activity and aqueous solubility of lead compounds through Structure-Activity-Relationship (SAR) modifications.

2) Enhance the bioavailability of these compounds by administering with appropriate solubilizers or drug vehicles

3) Identify target and biological pathways affected by these compounds and their derivatives to understand the mechanisms of action of most potent small molecule inhibitors.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Small molecule inhibitors as Anti-*Campylobacter jejuni* agents
Poultry CRC Sub-Project No.:	3.1.3 - Rajashekara
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Sub-Project Overview	
Background	
Research	
Sub-Project Progress	
Implications	
Publications	- Kumar A, Drozd M, Pina-Mimbela R, Xu X, Helmy YA, Antwi J, Fuchs JR, and Nislow C. 2016. Anti-Campylobacter Compounds Identified Using High Throughput Screening of a Pre-selected Enriched Small Molecules Library. Front Microbiol. Apr 6;7:405. doi: 10.3389/fmicb.2016.00405. eCollection 2016. - Rajashekara G, Blackall P, Deblais L, Kumar A, et al. Novel *C. jejuni* Novel narrow spectrum growth Inhibitors for the control Campylobacter colonization of poultry. Rajashekara G, Blackall P, Deblais L, Kumar A, et al. Identification of Twin Arginine Transport dependent Inhibitors of *C. jejuni* for control of Campylobacter colonization of poultry (Manuscript in preparation).