**Microbiome-derived metabolites influence the communication between Enteropathogenic *E. coli* and *Vibrio cholerae***

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Running title: Indole intercepts pathogen communication

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**Abstract**

Reported numbers of diarrheal samples exhibiting co-infections or multiple infections caused by two or more infectious agents are rising, likely due to advances in bacterial diagnostic techniques. Bacterial species detected in these samples include *Vibrio cholerae* (*V. cholerae*) and enteropathogenic *Escherichia coli* (EPEC), which infect the small intestine and are associated with high mortality rates. It has previously been reported that EPEC cells exhibit enhanced virulence in the presence of *V. cholerae* owing to their ability to sense and respond to elevated concentrations of cholera autoinducer 1 (CAI-1), which is the primary quorum-sensing (QS) molecule produced by *V. cholerae*. In this study, we examined this interspecies bacterial communication in the presence of indole, a major microbiome-derived metabolite found at high concentrations in the human gut. Interestingly, we discovered that although indole did not affect bacterial growth or CAI-1 production, it impaired the ability of EPEC to enhance its virulence activity in response to the presence of *V. cholerae*. Furthermore, the co-culture of EPEC and *V. cholerae* in the presence of *B. thetaiotaomicron*, an indole-producing commensal bacteria, ablated the enhancement of EPEC virulence. Together, these results suggest that microbiome compositions or diets that influence indole gut concentrations may differentially impact the virulence of pathogens and their ability to sense and respond to competing bacteria.

**Importance**

The ability of bacteria to determine their population size and the population size of competing bacterial species can enable the more successful colonization of a given target niche. In this study, we found that a pathogenic *Escherichia coli* strain could detect and respond to the presence of *Vibrio cholerae* by enhancing its virulence. Interestingly, we found that indole, a major metabolite produced by commensal bacteria, was able to impair this form of bacterial communication, thereby interfering with the ability of these pathogens to colonize the host. Our results suggest that the disruption of pathogen communication through the production of microbiome-derived metabolites may represent a broader phenomenon, potentially highlighting an exciting new research space with the potential to enable future therapeutic breakthroughs.

**Introduction**

According to the World Health Organization, diarrheal diseases remain one of the leading causes of death among children under the age of five in developing countries, causing more than 500,000 deaths annually. These infections have traditionally been attributed to a single infectious agent. However, improvements in diagnostic techniques have revealed that samples from diarrheal disease patients are often consistent with co-infection by two or more infectious agents (1-3). These multi-pathogen infections can appear in up to 60% of all tested samples, with *Escherichia coli*, *Vibrio cholerae* (*V. cholerae*), and *Shigella* species being the predominant pathogens observed in affected patients (2-4). These co- and multi-infections are commonly associated with more severe clinical symptoms, likely owing to the higher overall infectious load or the enhancement of the virulence of at least one of the infecting species (4-6).

We have recently studied one such virulence enhancement mechanism by exploring the interplay between *V. cholerae* and enteropathogenic *E. coli* (EPEC), which are two of the primary infectious drivers of gastroenteritis. Specifically, we found that EPEC enhances its virulence in the presence of *V. cholerae* through its ability to detect elevated concentrations of cholera autoinducer 1 (CAI-1), which is the primary quorum-sensing (QS) molecule produced by *V. cholerae* (7-9). In *V. cholerae*, CAI-1 is synthesized by the CqsA enzyme, secreted to the extracellular environment, and its concentration rises as the size of the *V. cholerae* population increases. Once a threshold concentration has been reached, CAI-1 binds to the CqsS receptor on *V. cholerae* cells to alter the transcription of virulence factors and biofilm development-related genes (8, 9).

EPEC relies on the type III secretion system (T3SS) to infect host cells (10). The T3SS is a large protein transport complex that many other pathogenic gram-negative bacteria use to form a nano-syringe structure. The T3SS translocates effectors directly into host cells, where they interfere with crucial cellular processes that ultimately promote bacterial replication and transmission (11-13). In EPEC, the T3SS is encoded on a large 35-kbp chromosomal pathogenicity island, known as the locus of enterocyte effacement (LEE) (14). The LEE consists of 41 genes, organized in seven operons (LEE1–LEE7), that encode structural proteins, regulators, and effector proteins (13, 15, 16). We discovered that EPEC T3SS activity, and hence its overall virulence, are enhanced in response to CAI-1 (7).

EPEC and *V. cholera* co-infections occur in the small intestine (17), which is colonized by a diverse population of microorganisms collectively referred to as the microbiome (18, 19). The gut microbiome regulates diverse physiological processes, such as food digestion and metabolite production, the maintenance of the gut mucosal barrier, and the prevention of pathogenic invasion (18-22). Microbiota-derived metabolites are essential for the regulation of the intestinal immune system and the maintenance of the gut microbiome homeostasis (23-26), thereby shaping human health and disease (27-29). In this study, we focused on indole, which is an amino-acid-derived metabolite that is produced from the degradation of tryptophan by a tryptophanase enzyme encoded by the *tnaA* gene that is found mainly in commensal bacteria such as *Bacteroides thetaiotaomicron* (23, 25, 26). Indole concertations are estimated to range as high as 1 mM in the human gastrointestinal tract (25, 30). These high indole concentrations have been shown to decrease enterohemorrhagic *E. coli* (EHEC) motility, biofilm formation, adherence to epithelial cells, and virulence gene expression, in addition to enhancing drug resistance of *Salmonella* *enterica* (25, 31-33).

In this study, we characterized EPEC responses to the presence of *V. cholerae* under conditions that better simulate the small intestine and examined whether microbiome-derived indole can alter the communication between these two pathogens. Interestingly, we discovered that although indole did not affect the growth or CAI-1 production of *V. cholerae*, it did interfere with the cross-talk between EPEC and *V. cholerae* by impairing the ability of EPEC to sense the presence of *V. cholerae* and to upregulate T3SS expression accordingly. Our results suggest that the microbiome can indirectly affect bacterial virulence by producing metabolites that influence pathogen communication, thus suggesting a tight connection between commensal bacteria, pathogens, and the diet.

**Materials and Methods**

**Bacterial strains**

The wild-type (WT) enteropathogenic *E. coli* (EPEC) O127:H6 strain E2348/69 (streptomycin-resistant) and the Δ*escN* null strain (Table 1) were grown at 37°C in Luria-Bertani (LB) broth (Sigma) supplemented with the appropriate antibiotics unless otherwise indicated. *V. cholerae* O1 In ET-122 (+) strains (WT, Δ*cqsA*, and the MM920 reporter strain – Table 1) were grown at 30°C in LB broth supplemented with appropriate antibiotics. *B. thetaiotaomicron* (Table 1) was grown at 37°C in Brain Heart Infusion (BHI, Sigma) broth under static, anaerobic conditions. The following antibiotics were used for this study: streptomycin (50 µg/mL) and tetracycline (12.5 µg/mL).

**Construction of the null Δ*cqsA V. cholerae* mutant strain**

Non-polar deletion of *cqsA* in *V. cholerae* O1 In ET-122 (+) was achieved by using the *sacB*-based allelic exchange method (34). Briefly, two PCR fragments corresponding to the flanking regions of *cqsA* (0.9 and 1.18 kb, from the 5’ and 3’ of *cqsA*, respectively) were generated with the corresponding primer pairs cqsA\_UF/cqsA\_UR and cqsA\_DF/ cqsA\_DR (Table 2). The fragments were then annealed using the cqsA\_UF/cqsA\_DR primer pair and cloned into the pRE112 suicide vector. The resultant pRE112 plasmid contained the *cqsA* flanking regions, with 94% of *cqsA* having been deleted. The plasmid was then transformed into the *E. coli* SM10λpir conjugative strain to be introduced into WT *V. cholerae* (35). After a sucrose selection process, *V. cholerae* colonies that were resistant to sucrose and susceptible to chloramphenicol were screened for the deletion of *cqsA* by PCR. The deletion of the *cqsA* gene was then confirmed by sequencing.

**Type III secretion (T3S) assay**

T3S assays were performed as previously described (36, 37). Briefly, WT EPEC and Δ*escN* strains were grown overnight at 37°C in LB broth with appropriate antibiotics. The overnight cultures were diluted 1:40 into either pre-warmed complete Dulbecco's modified Eagle's medium (DMEM, Biological Industries), referred to as optimal T3SS-inducing medium, or a 1:1 (v/v) DMEM:plain LB medium, referred to as semi-optimal T3SS-inducing medium, with this media being supplemented with DMSO, CAI-1 (50 μM), or indole (100-1000 μM). These cultures were grown for 6 h at 37°C under aerobic conditions (in a tissue culture incubator with 5% CO2) or anaerobic conditions (in a DonWhitley A35 anaerobic workstation, with a gas mixture of 5% H2, 10% CO2, and 85% N2). The optical density at 600 nm (OD600) of these cultures was measured before the cultures were centrifuged at 20,000 × g for 5 min to separate the bacterial pellets, which were dissolved in SDS-PAGE sample buffer, from the culture supernatants. The supernatants were filtered through a 0.22 μm low-protein-binding filter, normalized according to the bacterial OD600, and the secreted proteins present therein were precipitated with 10% (v/v) trichloroacetic acid (TCA) overnight at 4°C. The samples were then centrifuged at 18,000 × g for 30 min at 4°C, and secreted protein precipitates were dissolved in SDS-PAGE sample buffer, with the residual TCA being neutralized using saturated Tris. Samples were then analyzed on SDS-PAGE gels with Coomassie Blue staining (InstantBlue, Abcam) or via western blotting.

**Bacterial co- and multi-cultures**

EPEC and *V. cholerae* cultures were grown separately overnight at 37°C (EPEC) or 30°C (*V. cholerae*) in LB broth. *B. thetaiotaomicron* cultures were grown anaerobically overnight at 37°C in BHI broth. For co-culture assays performed under aerobic conditions, EPEC and *V. cholerae* overnight cultures were diluted 1:40 into semi-optimal T3SS-inducing medium (1:1 [v/v] DMEM: LB) and grown together in a tissue culture incubator (with 5% CO2) statically for 6 h, either alone or in the presence of indole (500 µM - Arcos Organics). For co- and multi-culture assays performed under anaerobic conditions, *B. thetaiotaomicron* overnight cultures were diluted 1:16 into 1:1 (v/v) DMEM:BHI medium and grown for 8 h. Then, WT EPEC alone or together with WT *V. cholerae* (each diluted 1:80) were added into *B. thetaiotaomicron* growth medium and cultured for an additional 6 h. In addition, samples of EPEC only, *B. thetaiotaomicron* only, and co-cultures of EPEC and *V. cholerae* with or without the presence of 500 µM indole, as well as the multi-culture combination of *B. thetaiotaomicron*, EPEC, and *V. cholerae* were added to 1:1 (v/v) DMEM:BHI medium and cultured for 6 h. These cultures were then separated into bacterial supernatants and pellets and processed as described above for the T3S assay.

**Bioluminescence (LuxR) assay**

The presence of CAI-1 in the culture media was determined by assessing light production mediated by the *V. cholerae* Δ*cqsA*Δ*luxP* reporter strain harboring the luxCDABE operon (MM920). *V. cholerae* MM920, WT *V. cholerae*, and *V. cholerae* Δ*cqsA* were grown overnight at 30°C in LB broth. The reporter strain was diluted 1:20 into fresh LB medium in white 96-well clear-bottom plates and was mixed with the supernatants of WT *V. cholerae*, Δ*cqsA* null strain, or WT *V. cholerae* grown in the presence of 500 µM indole. Plates were then incubated at 30°C with aeration, and light production and OD600 values were measured every 30 min (TECAN Infinity 200Pro). Plain LB and 10 µM CAI-1 were used as negative and positive controls, respectively. Luminescence signal values divided by OD600 values are presented as relative units (RU). The results represent the average values from three independent experiments.

**Western blotting**

Samples were separated via SDS-PAGE and transferred to nitrocellulose (pore size: 0.45 μm; Amersham Protran) or PVDF (pore size: 0.45 μm; Amersham Hybond) membranes. These blots were blocked for 1 h in 5% (w/v) skim milk-PBST (0.1% Tween in phosphate-buffered saline [PBS]), incubated for 1 h with appropriate primary antibodies (diluted in 5% skim milk-PBST) at room temperature, washed, and then incubated for 1 h with appropriate secondary antibodies (diluted in 5% skim milk-PBST) at room temperature. Chemiluminescence was detected with EZ-ECL reagents (Cyanagen). The optimal dilution for each antibody was determined as follows: mouse anti-DnaK (Abcam), diluted 1:1000; mouse anti-JNK (BD Pharmingen), diluted 1:1000; and mouse anti-actin (MPBio), diluted 1:10,000. Antibodies directed against T3SS components, including mouse anti-EspB, and mouse anti-Tir, were a generous gift from Prof. B. Brett Finlay (University of British Columbia, Canada) and Prof. Rebekah Devinney (University of Calgary, Canada). Horseradish peroxidase-conjugated (HRP)-goat anti-mouse (Abcam), diluted 1:10,000, was used as the secondary antibody for these analyses. Blots representative of at least three independent experiments are presented in the results section.

**Real-time quantitative polymerase chain reaction (qPCR)**

WT EPEC was grown overnight at 37°C in LB broth. The culture was diluted 1:50 into 1:1 (v/v) DMEM: plain LB medium supplemented with either DMSO, CAI-1 (50 μM), or indole (50 or 500 μM) and grown statically in a tissue culture incubator (with 5% CO2) for 2 h to the early exponential phase of growth. Bacteria (5 × 108 cells) were collected and RNA was extracted using the NucleoSpin Bacterial RNA isolation kit according to provided directions (Macherey-Nagel). RNA was examined for genomic DNA contamination and subjected to additional DNase I treatment when needed, followed by extraction using the TRIzol reagent. A total of 200 ng of RNA from each sample was taken for cDNA synthesis performed using the ProtoScript II First Strand cDNA Synthesis Kit (NEB) using a random primer mix. cDNA was examined for genomic DNA contaminations. Primer sequences used for qPCR are presented in Table 2. Melting curve analyses were used to ensure the specificity of each primer pair. All qPCR analyses were performed using SYBR Green I mix (Roche), sample cDNA, and a LightCycler 480 instrument (Roche) with the following thermocycler settings: 1 cycle at 95°C for 10 min, 40 cycles of 95°C for 15 s, cooling to 60°C for 10 s, followed by 72°C for 10 s. The resultant data were analyzed using the LightCycler 480 software to extract the critical threshold (CT) values. The relative expression levels of these target genes following these different treatments were normalized to the *rpoA* housekeeping gene and compared using the relative quantification method. Real-time data are presented as the fold change in expression levels.

**Translocation assay**

Translocation assays were performed as previously described by Baruch *et al*. (38) with slight modifications. Briefly, EPEC WT and Δ*escN* strains were pre-induced for 3 h under optimal T3SS-inducing conditions or 2 h under semi-optimal T3SS-inducing conditions, statically, in a CO2 tissue culture incubator in the presence or the absence of indole (50-1000 μM), CAI-1 (50 μM), or combination of both. HeLa cells (8 × 105 cells/well) were then infected with bacterial cultures at a multiplicity of infection (MOI) of 1:300 for 3 h (when pre-induced under optimal T3SS-inducing conditions) or for 2 h (when pre-induced under semi-optimal T3SS-inducing conditions). The cells were then washed with cold PBS and lysed with RIPA buffer. The lysed samples were collected, centrifuged at 18,000 × g for 5 min to remove unlysed cells, and subjected to western blotting analyses using anti-JNK and anti-actin (loading control) primary antibodies. Uninfected samples and samples from cells infected with the Δ*escN* mutant strain were used as negative controls.

**Results**

**Indole inhibits EPEC T3SS activity.** Previous studies have reported that indole can alter various bacterial processes, as in the case of its ability to inhibit the virulence of enteric pathogens such as EHEC and *Citrobacter rodentium* (25, 31). To examine the effect of indole on the T3SS activity, which is the primary virulence mechanism exhibited by EPEC, we grew WT EPEC under optimal T3SS-inducing conditions (DMEM, statically) in aerobic and anaerobic environments in the presence of different indole concentrations (Fig. 1). As the physiological concentration of indole in the gastrointestinal tract of humans and mice has been suggested to be as high as 1 mM (23, 32, 39-42), we examined the effects of indole concentrations ranging from 100-1000 µM. T3SS activity was assessed by measuring the ability of EPEC to secrete T3SS translocators (EspA, EspB, and EspD) into culture supernatants. We observed efficient secretion of these translocators by WT EPEC, whereas no translocators were detected in the supernatants collected from the Δ*escN* null strain, which harbor a deletion of the T3SS ATPase gene (Fig. 1). Analyses of the supernatants prepared from WT EPEC grown in the presence of indole concentrations at or above 300 μM exhibited reduced levels of secreted extracellular T3SS-associated proteins relative to DMSO vehicle control-treated samples (Fig. 1A). To better monitor the effects of indole on T3SS activity, we analyzed the supernatants and bacterial pellets via Western blotting using anti-EspB and anti-Tir antibodies, revealing that indole inhibited EspB secretion in a dose-dependent manner (Fig. 1B and 1C). We also assessed the expression of the T3SS effector protein Tir, which should be retained within the bacterial cells at this stage, by analyzing whole-cell bacterial pellets. This analysis revealed that indole similarly inhibited Tir expression in a dose-dependent manner within the bacterial pellets (Fig. 1B and 1C). DnaK levels were used to confirm equal levels of lysate loading in these different samples. To exclude the possibility that indole reduced bacterial virulence by inhibiting bacterial growth, we grew WT EPEC under optimal T3SS-inducing conditions in the presence (500 μM) or absence of indole and monitored optical density values over time, observing similar growth rates irrespective of the presence of indole (Fig. S1). Overall, these results indicated that physiological concentrations of indole inhibit EPEC T3SS secretion activity.

**Indole impairs the enhancement of EPEC virulence in response to *V. cholerae* growth under co-culture conditions.** We previously reported that EPEC modulates its virulence in response to the size of *V. cholerae* populations by sensing and responding to elevated concentrations of CAI-1 (7). To evaluate EPEC virulence when grown in co-culture with *V. cholerae*, we inoculated these bacterial strains into a 1:1 (v/v) mixture of DMEM and Luria-Bertani (LB) broth. This mixture corresponds to semi-optimal T3SS-inducing conditions, as it induces only partial T3SS activation and leaves room for an additional T3SS enhancement. As expected, we observed elevated EspB secretion and Tir expression levels in EPEC and *V. cholerae* co-culture samples relative to these levels in the pure EPEC culture sample (Fig. 2A). DnaK levels in the bacterial pellets again confirmed equal sample loading. To reaffirm that EPEC T3SS upregulation is CAI-1 dependent, we generated a *V. cholerae* Δ*cqsA* mutant strain in which the CAI-1 synthase gene *cqsA* had been deleted, and examined the ability of these bacteria to alter EPEC T3SS responses. EPEC co-cultured with the *V. cholerae* Δ*cqsA* mutant strain exhibited weak EspB secretion and no Tir expression, with these levels more closely resembling those for pure EPEC cultures (Fig. 2A). These results confirmed that the upregulation of EPEC T3SS activity is CAI-1 dependent.

To characterize EPEC T3SS responses in the presence of *V. cholerae* under conditions that better simulate the human gastrointestinal tract, we performed co-culture experiments in the presence of indole. The addition of indole (500 µM) to the co-culture of EPEC andWT *V. cholerae* completely abolished EspB secretion and Tir expression (Fig. 2A), indicating that indole impairs the ability of EPEC to sense the presence of *V. cholerae* and upregulate its T3SS accordingly. To validate that the inability of EPEC to respond to *V. cholerae* presence was not due to the effects of indole on CAI-1 production, we assessed the CAI-1 concentrations produced by *V. cholerae* grown in the presence or absence of indole using the MM920 *V. cholerae* reporter strain, which contains the *V. harveyi* *lux*CDABE luciferase operon that is activated by CAI-1 (43). We incubated this reporter strain with supernatants prepared from WT *V. cholerae* grown in the presence (500 µM) or absence of indole and measured light production over time. Supernatants prepared from the Δ*cqsA* *V. cholerae* strain and synthetic CAI-1 were used as negative and positive controls, respectively. While no light production was observed from the reporter strain grown in presence of Δ*cqsA V. cholerae* supernatants, a strong signal was detected when the reporter strain was grown in the presence of WT *V. cholerae* supernatant irrespective of the presence or absence of indole (Figure 2B). These results indicate that indole did not alter CAI-1 production. To further exclude the possibility that indole reduced EPEC T3SS responses due to its effect on *V. cholerae* growth, we compared bacterial growth rates in the presence (500 μM) or absence of indole by monitoring the optical density over time. We observed similar growth rates regardless of indole presence (Fig. S2). Our results thus suggested that indole impairs the ability of EPEC to upregulate its T3SS activity in response to the presence of *V. cholerae*.

**Indole interferes with EPEC responses to CAI-1 at high micromolar concentrations.** To study the interplay between indole, CAI-1, and EPEC T3SS responses, we examined T3SS activity levels at various synthetic CAI-1/indole molar ratios. To more sensitively detect the enhancement T3SS activity, we cultured EPEC under semi-optimal T3SS-inducing conditions that do not induce full T3SS activation, providing the opportunity for further T3SS upregulation. These bacterial cultures were then separated into supernatants and bacterial pellet samples and were analyzed to detect EspB secretion (supernatants) and Tir expression (bacterial pellets). As expected, we observed elevated levels EspB secretion and Tir expression when WT EPEC were cultured in the presence of CAI-1 (50 µM) relative to DMSO control (Fig. 3A). Moreover, a similar elevation was observed for EPEC grown in the presence of CAI-1 and indole at a 1:1 molar ratio (Fig. 3A). However, at higher indole concentrations, the T3SS-upregulating effects of CAI-1 were curtailed in a dose-dependent manner. WT EPEC samples grown in the presence of a 1:10 ratio of CAI-1 and indole exhibited the complete elimination of EspB secretion and Tir expression (Fig. 3A). DnaK levels within the bacterial pellets confirmed equal sample loading for these analyses.

To better determine the antagonistic effect of indole on CAI-1 responses, we cultured WT EPEC under optimal T3SS-inducing conditions that promote a maximal T3SS response. Under these conditions, we found that indole promoted a similar dose-dependent inhibition of EspB secretion and Tir expression levels, with a maximal effect at a 1:10 CAI-1/indole ratio (Fig. 3B). Furthermore, although residual EspB secretion and Tir expression were observed in the presence of indole (500 µM), no upregulation was observed in the presence of CAI-1 (Fig. 3B). These results suggested that indole not only inhibits T3SS per se but also, at high micromolar concentrations, neutralizes the ability of CAI-1 to upregulate EPEC T3SS activity.

**Indole inhibits the CAI-1-induced upregulation of EPEC T3SS genes.** To examine whether indole affects the transcription of T3SS genes, we cultured WT EPEC under semi-optimal T3SS-inducing conditions. We then added CAI-1 alone or together with indole at a 1:10 molar ratio and evaluated the transcription of three representative LEE genes; *tir* - the first translocated effector encoded on the LEE5 operon, and two T3SS translocators, *espA* and *espB*, encoded on the LEE4 operon. As observed previously, we detected significantly elevated levels of these T3SS transcripts when cells were cultured in the presence of CAI-1 as compared to DMSO alone (Fig. 4). However, bacteria grown in the presence of CAI-1 and indole at a 1:10 molar ratio exhibited the almost complete abrogation of the transcription of all three genes (Fig. 4). These results indicated that indole reduces T3SS activity by downregulating the transcription of T3SS genes, and this reduction is not relieved even under CAI-1-inducing conditions.

**Microbiome-derived indole impairs the ability of EPEC to sense and respond to the presence of *V. cholerae*.** To further examine the effect of indole on communication between EPEC and *V. cholerae*, we performed a multi-bacteria culture assay that more closely resembles the intestinal environment. We employed the indole-producing commensal bacteria *Bacteroides thetaiotaomicron* to simulate natural indole production (25, 26, 44). *B. thetaiotaomicron* was sub-cultured together with EPEC and *V. cholerae* under anaerobic conditions in a 1:1 (v/v) mixture of DMEM and BHI. These semi-optimal T3SS-inducing conditions were conducive to the growth of all three bacterial strains. We then compared the T3SS activity of EPEC grown under multi-culture conditions to that of EPEC grown as a pure culture or co-cultured with either *B. thetaiotaomicron* or *V. cholerae*, as shown in Figure 5A. A *B. thetaiotaomicron* pure culture served as a negative control.

As expected, the co-culture of EPEC and *V. cholerae* induced higher levels of T3SS secretion activity compared to that of EPEC pure culture (Fig. 5B). However, the co-culture of EPEC and *V. cholerae* in medium pre-incubated with *B. thetaiotaomicron* (grown for 8 h as a pure culture) completely abolished EPEC T3SS activity and resembled the EspB/Tir levels detected for samples of EPEC and *V. cholerae* co-culture grown in the presence of 500 µM indole (Fig. 5B). In addition, the co-culture of EPEC and *B. thetaiotaomicron* eliminated EspB secretion and Tir expression (Fig. 5B). DnaK levels in the prepared bacterial pellets confirmed equal sample loading for these analyses. These results further supported our previous observations by demonstrating that microbiome-derived indole inhibits EPEC T3SS secretion activity and disrupts crosstalk between EPEC and *V. cholerae*.

When *B. thetaiotaomicron*, EPEC, and *V. cholerae* were simultaneously co-cultured without providing *B. thetaiotaomicron* an opportunity to produce indole before EPEC and *V. cholerae* are added to the growth medium was associated with higher levels of EspB secretion and Tir expression as compared to the multi-culture sample established following the 8-h pre-culture of *B. thetaiotaomicron* (Fig. 5B). This suggested that the belated production of indole limits its effect on the T3SS response. Unsurprisingly, the *B. thetaiotaomicron* pure culture sample was negative for DnaK expression as the utilized anti-DnaK antibody reacts primarily with DnaK proteins derived from *E. coli* or closely related bacteria, such as *V. cholerae*. To exclude the possibility that *B. thetaiotaomicron* cultures inhibited EPEC and *V. cholerae* growth, we compared the bacterial counts following co-culture and multi-culture growth by plating these cells on selective medium. We found that EPEC and *V. cholerae* counts were unaffected by the presence of *B. thetaiotaomicron* (Fig. S3). Therefore, we conclude that microbiome-derived indole can interfere with the crosstalk between EPEC and *V. cholerae* pathogens. While both EPEC and *V. cholerae* can produce indole, this has been reported to occur primarily during their stationary growth phase, which is less relevant to this experimental setup (26).

**Indole inhibits the ability of EPEC to translocate effector proteins into host cells.** To further evaluate the effects of indole on EPEC virulence, we utilized a bacterial infection model that examines the ability of WT EPEC to infect HeLa cells and promote the translocation of effectors into host cells. We infected HeLa cells with EPEC strains grown under optimal T3SS-inducing conditions, in the presence of varying concentrations of indole, and monitored the cleavage of host cell-derived c-Jun N-terminal kinase (JNK), which is degraded by a translocated effector protein NleD (38). As expected, HeLa cells infected with WT EPEC cultures exhibited JNK degradation, in contrast to the uninfected HeLa samples and samples infected with Δ*escN* EPEC strain (Fig. 6A). However, HeLa cells infected with WT EPEC cultures pre-incubated with indole at a concentration at or above 500 μM exhibited reduced JNK degradation and higher levels of full-length JNK (Fig. 6A). These results suggested that indole inhibits the virulence of EPEC by interfering with the ability of its T3SS to translocate effector proteins into host cells.

As we previously reported that CAI-1 enhances the ability of EPEC to infect HeLa cells and to translocate effectors into host cells (7), we further examined whether this enhancement was altered in the presence of indole. To that end, we monitored the cleavage patterns of JNK in HeLa cells infected with EPEC strains grown under semi-optimal T3SS conditions (to ensure that there was an opportunity for enhanced virulence) incubated with both CAI-1 and indole at a 1:1 or 1:10 molar ratio. As expected, HeLa cells infected with WT EPEC in the presence of CAI-1 exhibited higher levels of JNK degradation as compared to those infected with WT EPEC alone. While high JNK degradation levels were still observed for the samples infected with WT EPEC incubated with CAI-1 and indole at a 1:1 ratio, complete inhibition of JNK degradation was detected for EPEC incubated with CAI-1 and indole at a 1:10 ratio (Fig. 6A). These results suggested that indole suppresses the enhancement of EPEC virulence induced by CAI-1.

**Discussion**

The gastrointestinal microbiome plays a critical role in human health, in part because it provides colonization resistance against pathogenic bacteria (45, 46). This is achieved owing to the ability of the microbiome to produce various metabolites (e.g., essential vitamins, carbohydrates, peptides, proinflammatory cytokines, and lipopolysaccharides) that enhance host immunity (24, 45-48). In addition, these microbiome-derived metabolites can directly inhibit pathogen virulence mechanisms and therefore protect against bacterial infections. For example, *Bacteroides thuringiensis* and commensal *E. coli* synthesize peptides known as bacteriocins that inhibit the virulence of *Enterococcus* *faecalis, Klebsiella pneumonia*, *Salmonella*, and EHEC (49-52).

The ability of indole, a microbiome-derived metabolite, to directly inhibit bacterial virulence and reduce the infection capabilities of several enteric pathogens has previously been demonstrated (25, 33, 53). In this study, we extended this finding to EPEC and found that indole directly inhibits EPEC T3SS secretion activity, at physiological concentrations under aerobic and anaerobic conditions (Fig. 1 and 5). These results are in keeping with a previous study that reported that indole and its derivatives alter the motility, biofilm formation, and Shiga toxin production activities of various pathogenic *E. coli* strains (54).

In this study, we examined not only whether indole was able to inhibit bacterial virulence, but also whether it could interfere with the bacterial communication-mediated enhancement of such virulence. For that purpose, we used an EPEC and *V. cholerae* co-infection model, having previously demonstrated that these two pathogens time their virulence to reduce their competition and coordinate their infectious processes (7). We have previously suggested that this communication is mediated by CAI-1, the primary QS molecule produced by *V. cholerae*. Here, we confirmed this model by generating a *V. cholerae* mutant strain in which the CAI-1 synthase gene, *cqsA*, had been deleted such that these bacteria were deficient for CAI-1 production. Unlike co-culture with WT *V. cholerae,* co-culture of EPEC and *V. cholerae* Δ*cqsA* did not induce the upregulation of EPEC T3SS activity (Fig. 2), thus confirming that this bacterial communication is mediated through CAI-1.

Using this inter-bacterial communication system, we discovered that the addition of indole or *B. thetaiotaomicron*, which produce indole, to the EPEC and *V. cholerae* completely co-culture model system completely disrupted the communication between these pathogens and resulted in the ablation of EPEC T3SS activity upregulation (Fig. 2 and Fig. 5). We observed that the ability of CAI-1 to upregulate EPEC virulence was neutralized in an indole-dependent manner, primarily at higher indole concentrations (Fig. 3 and Fig. 5). This novel finding provides a possible explanation for the variability found among individuals with respect to their susceptibility to bacterial infections. This suggests that differences in microbiome composition can account for variations in gastrointestinal indole concentrations, which in turn can inhibit virulence and alter the communication between gut pathogens, ultimately supporting or interfering with the process of bacterial infection.

This finding is in line with previous studies demonstrating a link between microbiome composition and certain gut-associated diseases such as inflammatory bowel disease, obesity, type 2 diabetes, and even cancer (55, 56). The deliberate alteration of the microbiota may thus offer potential as a therapeutic tool (56). Given that our results demonstrate that microbiome-derived indole was sufficient to interfere with pathogen communication and disrupt the ability of pathogens to coordinate infections, it is logical to assume that the enrichment of indole-producing bacterial species within the microbiome will provide more robust colonization resistance, particularly against simultaneous infection with multiple enteric pathogens (1-3). In addition, to promote indole production by these strains, the consumption of a protein-rich diet should be encouraged, as indole is produced via the metabolism of tryptophan. A protein-rich diet may thus aid in preventing bacterial infections. Furthermore, indole has the potential to be developed into a postbiotic supplement, which is defined as a bioactive compound naturally produced by the gut microbiome that has been shown to improve human health.

The ability of bacteria to respond to chemical signals that indicate the population size of that bacterial species or other potential competing species is vital for the coordination of group behavior that is required for the infection process and survival within a given host. Interference with this process can therefore be a potent means of combatting bacterial infection. An example of such interference was previously described by Xavier and Bassler, who found that *E. coli* interfere with *V. cholerae* and *V. haryeyi* QS signaling by actively internalizing their QS molecule, AI-2 (57). This mechanism results in *Vibrio* species miscalculating their population size, thereby interfering with the ability of these bacteria to properly respond to changes in their cell population density, with these responses often being crucial for successful bacterial-host relationships. In this study, we described an additional mechanism whereby a microbiome-derived metabolite can interfere with bacterial communication. The observation that gastrointestinal commensal species produce a specific component that plays an important role in promoting gut health may be representative of a broader phenomenon, potentially highlighting a novel approach to combatting infectious diseases. As such, further studies of CAI-1 and indole signaling are warranted, including efforts to define the EPEC CAI-1 receptor and to determine whether indole acts as an antagonist of this receptor. A more detailed understanding of the direct and indirect effects of indole on bacterial virulence will aid in the development of novel anti-virulence therapeutics.

**Figure Legends**

Figure 1. **Indole inhibits EPEC T3SS activity in a dose-dependent manner.** Wild type (WT) EPEC and *escN* null-mutant (Δ*escN*) EPEC were grown under optimal T3SS-inducing conditions aerobically **(A-B)** and anaerobically **(C)** for 6 h in the presence of various concentrations of indole. Bacterial supernatants and pellets were separated, normalized, and analyzed via 12% SDS-PAGE with Coomassie staining **(A)** or western blotting analyses performed using anti-EspB, anti-Tir, and anti-DnaK antibodies **(B-C)**. In panel A, the T3SS-secreted translocators, EspA, EspB, and EspD, are marked on the right of the gel. The location of EspC, which is not secreted via the T3SS, is also marked.

Figure 2. **Indole interferes with the crosstalk between EPEC and *V. cholerae*.** **(A)** Pure overnight cultures of EPEC and *V. cholerae* strains were sub-cultured in fresh 1:1 (v/v) DMEM: LB as single or mixed cultures. These cultures were grown in the presence or absence of indole under semi-optimal T3SS-inducing conditions for 6 h, and then the bacterial pellets and supernatants (bacterial sup) were separated, normalized, and analyzed. The secreted proteins were concentrated from collected supernatants and analyzed via 12% SDS-PAGE and western blotting using an anti-EspB antibody. The expression of the effector protein Tir, which should remain present primarily within the bacterial cytoplasm at this stage, was analyzed by subjecting the bacterial pellets to SDS-PAGE and western blotting using an anti-Tir antibody. Samples were also probed with anti-DnaK to confirm equal loading. **(B)** Relative light production was used as means of assessing the levels of CAI-1 produced by WT *V. cholerae* in the absence or presence of indole (500 µM) and by Δ*cqsA* *V. cholerae* strain. Synthetic CAI-1 (10 µM) was used as a positive control. Data are averaged from three replicates of a representative experiment.

Figure 3. **Indole competes with CAI-1 to influence EPEC T3SS activation.** Wild type (WT) EPEC and *escN* null-mutant (Δ*escN*) EPEC were grown for 6 h under semi-optimal **(A)** or optimal **(B)** T3SS-inducing conditions in the presence of CAI-1 (50 µM) and various concentrations of indole (50-500 µM). The secreted proteins were concentrated from bacterial culture supernatants (bacterial sup) of the bacterial cultures and analyzed via 12% SDS-PAGE and western blotting using an anti-EspB antibody. The expression of the effector protein Tir, was analyzed by subjecting the bacterial pellets to SDS-PAGE and western blotting using an anti-Tir antibody. Samples were also probed with anti-DnaK to confirm the equal loading of lysates.

Figure 4. **Indole suppresses the upregulation of EPEC T3SS genes induced by CAI-1.** WT EPEC was grown for 2 h under semi-optimal T3SS-inducing conditions in the presence of 0.5% (v/v) DMSO(dark gray bars),CAI-1 (white bars), indole(black bars), or both CAI-1 and indole (light gray bars). mRNA levels for the T3SS genes, *espB*, *espA*, and *tir*, were measured via qPCR. mRNA levels are presented relative to those of WT EPEC grown in the ​presence of DMSO (dark gray bars). Data are averaged from three replicates of a representative experiment; error bars correspond to the standard error of the mean; \*\*P < 0.005.

Figure 5. ***B. thetaiotaomicron*-derived indole inhibits the enhancement of EPEC T3SS activity upon co-culture with *V. cholerae*.** **(A)** A schematic overview of the bacterial combinations and indole supplementation approach used for this experiment. WT EPEC was sub-cultured in a mixture of 1:1 (v/v) DMEM:BHI medium as pure culture, a co-culture with *V. cholerae*, a co-cultured with *B. thetaiotaomicron*, or a multi-culture with both *V. cholerae* and *B. thetaiotaomicron* (EPEC and *V. cholerae* were added either at time 0 or after 8 h of *B. thetaiotaomicron* growth as a pure culture). One of the EPEC and *V. cholerae* co-cultures was supplemented with indole (500 µM). All cultures were grown anaerobically for 6 h and the bacterial pellets and supernatants (bacterial sup) were separated, normalized, and analyzed. **(B)** The secreted proteins were concentrated from culture supernatants and analyzed via 12% SDS-PAGE and western blotting using an anti-EspB antibody. The expression of the effector protein Tir, was analyzed by subjecting the bacterial pellets to SDS-PAGE and western blotting using an anti-Tir antibody. Samples were also probed with anti-DnaK to confirm the equal loading of lysates.

Figure 6. **Indole reduces the ability of EPEC to translocate NleD into host cells, even in the presence of CAI-1. (A)** HeLa cells were infected with WT and Δ*escN* EPEC strains grown under optimal T3SS-inducing conditions in the presence of various indole concentrations (100 – 1000 µM) for 3 h. Cells were washed, and their proteins were extracted and subjected to western blotting analysis using anti-JNK and anti-actin (loading control) antibodies. JNK and its degradation fragments are indicated to the right of the gel. **(B)** Western blotting analysis of JNK degradation patterns following HeLa infection with WT and Δ*escN* EPEC strains grown under semi-optimal T3SS-inducing conditionsin the absence or presence of CAI-1 (50 µM) and indole (50 or 500 µM) for 2 h.

Table 1. Strains used in this study

|  |  |  |
| --- | --- | --- |
| **Strain** | **Description** | **Reference** |
| WT EPEC | EPEC strain E2348/69, streptomycin resistant | (58) |
| EPEC Δ*escN* | Non-polar deletion of *escN* | (59) |
| *Vibrio cholerae* | *V. cholerae* biotype El-Tor serotype Inaba O1 In ET-122 (+) | (60) |
| *Vibrio cholerae* Δ*cqsA* | Non-polar deletion of *cqsA in V. cholerae* | This study |
| *V. cholerae* MM920 reporter strain | *V. cholerae* biotype EI-Tor serotype Δ*cqsA*Δ*luxQ* with pBB1 cosmid containing the *V. harveyi* luxCDABE operon (tetracycline resistant) | (53) |
| *Bacteroides thetaiotaomicron* | *B. thetaiotaomicron* (Distaso) ATCC 29148 | (61) |
| *E. coli* SM10λpir | For bacterial conjugation | (35) |

Table 2. Sequences of primers used in this study

|  |  |  |  |
| --- | --- | --- | --- |
| **Null mutant** | **Primer name** | **Primer sequence** | **Reference** |
| *V.cholerae ΔcqsA* | cqsA\_UF | GAGCTCGATATCGCATGCTGCCCCCTTCACAAGC | This study |
| cqsA\_UR | CACCGTAGTTGACCGCATCATCAGGAAGTTGAGGCTTG |
| cqsA\_DF | CAAGCCTCAACTTCCTGATGATGCGGTCAACTACGGTG |
| cqsA\_DR | CAAGCTTCTTCTAGAGGTACCCGCAGGGAGAACTACTGC |
| **Gene** | **Primer name** | **Primer sequence** | **Reference** |
| *Rpoa* | rpoA\_qPCR\_F  rpoA\_qPCR\_R | GGCGCTCATCTTCTTCCGAAT  CGCGGTCGTGGTTATGTG | (62) |
| *espB* | EspB\_qPCR\_F  EspB\_qPCR\_R | GGCTCTTTTGCTGCCATTAATAGC  TCTGCTGCATCTGCAATACC | (7) |
| *espA* | EspA\_qPCR\_F  EspA\_qPCR\_R | GTGCGAATGCGAGTACTTCGAC  TTGCAGCCTGAAAAACACCGAGT | (7) |
| *Tir* | Tir\_qPCR\_F  Tir\_qPCR\_R | GGACCCTCTGCATTTCGTGTTG  GTCCCCCGGTAAAAACAAATCTG | (7) |

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**Supplementary**

**Bacterial growth:**

EPEC and *V. cholerae*were grown overnight in LB broth, supplemented with the appropriate antibiotics, in a shaking incubator at 37°C or 30°C, respectively. Bacteria were inoculated 1:100 into pre-warmed DMEM medium (WT EPEC) or LB broth (*V. cholerae*)in the presence of either DMSO or 500 µM indole. The absorbance at 600 nm was monitored over time (TECAN Infinity 200Pro plate reader). The results represent average values from three independent biological replicate experiments.

Figure S1A. **Indole does not affect EPEC growth.** Growth curves for WT EPEC grown under optimal T3SS-inducing conditions at 37°C in the absence (○) or presence of 500 μM indole (●). Optical density at 600 nm was measured over time.

Figure S2A. **Indole does not affect *V. cholerae* growth.** WT *V. cholerae* was grown in LB broth at 30°C in the absence (○) or presence of 500 μM indole (●). Optical density at 600 nm was measured over time.

Figure S3A. **EPEC and *V. cholerae* growth in single, co-, and multi-culture conditions.** Pure overnight cultures of EPEC and *V. cholerae* strains were sub-cultured in fresh 1:1 (v/v) DMEM:BHI as pure, co-, or multi-cultures together with *B. thetaiotaomicron*. These cultures were grown under semi-optimal T3SS-inducing conditions for 6 h and then plated on LB plates containing chloramphenicol for EPEC growth (A) or carbenicillin for *V. cholerae* growth (B). The plates were incubated overnight at 37°C, and bacterial colony-forming units (CFUs) were then counted.

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