**Microbiome-derived metabolite intercepts the communication between Enteropathogenic *E. coli* and *Vibrio cholerae***

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**Abstract**

The numbers of co- and multiple infections, with two or more infectious agents, in diarrheal samples are increasing, probably due to advances in bacterial diagnosis. These include bacterial species such as *Vibrio cholerae* (*V. cholerae*) and enteropathogenic *Escherichia coli* (EPEC), which infect the small intestine and are associated with a high mortality rate. It was previously reported that EPEC upregulates its virulence in the presence of *V. cholerae* by sensing and responding to elevated concentrations of *V. cholerae*'s primary quorum-sensing (QS) molecule, cholera autoinducer 1 (CAI-1). In this study, we examined this bacterial communication in the presence of indole, a major microbiome-derived metabolite found in 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 upregulate its virulence in response to *V. cholerae* presence. Furthermore, the co-culture of EPEC and *V. cholerae* in the presence of *B. thetaiotaomicron*, an indole-producing commensal bacteria, results in the depletion of EPEC virulence upregulation. According to these results, microbiome compositions or diets that influence indole gut concentrations might differently affect pathogen virulence 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 bacteria can benefit them and promote successful colonization. In this study, we showed that a pathogenic *E. coli* strain could detect and respond to the presence of *Vibrio cholerae* by enhancing its virulence. Interestingly, we found that a metabolite produced by commensal bacteria, called indole, can impair this bacterial communication and interfere with the ability of the bacterial pathogen to colonize the host. Our results suggest that intercepting pathogens' communication by microbiome-derived metabolites may be a broader phenomenon that could open an exciting new research venue for novel therapeutic discoveries.

**Introduction**

According to the world health organization, diarrheal diseases are still one of the leading causes of death of children under the age of five in developing countries, which account for more than 500,000 deaths annually. These infections were traditionally associated with a single infectious agent. However, along with the improvement of microbes' diagnosis, high numbers of co- and multi-infections, with two or more infectious agents, are detected in samples of diarrheal disease patients (1-3). These can reach up to 60% of all tested samples, with *Escherichia coli*, *Vibrio cholerae* (*V. cholerae*), and *Shigella* being the predominant bacterial species (2-4). These co- and multi-infections are commonly associated with more severe clinical symptoms, likely due to the higher infection load or the enhancement of the bacterial virulence of at least one of the infecting species (4-6).

We have recently studied one such virulence enhancement mechanism between *V. cholerae* and enteropathogenic *Escherichia coli* (EPEC), two primary infection agents of gastroenteritis, and discovered that EPEC upregulates its virulence in the presence of *V. cholerae*. We found that this upregulation is achieved by the ability of EPEC to sense and respond to elevated concentrations of *V. cholerae*'s primary quorum-sensing (QS) molecule, cholera autoinducer 1 (CAI-1) (7-9). In *V. cholerae*, CAI-1 is synthesized by the CqsA enzyme, secreted to the extracellular environment, and its concentration rises when the size *V. cholerae* population increases. Once a threshold concentration has been reached, CAI-1 binds to the *V. cholerae*'s CqsS receptor to modify the transcription of virulence factors and biofilm development genes (8, 9).

EPEC relay on a complex called type III secretion system (T3SS) for its ability 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 infection ability are enhanced in response to CAI-1 (7).

EPEC and *V. cholera* co-infections occur in the small intestine (17), which is colonized with a diverse population of microorganisms called collectively the microbiome (18, 19). The gut microbiome is involved in various physiological processes, such as food digestion and metabolites production, maintenance of the gut mucosal barrier, and prevention of pathogens invasions (18-22). The microbiome-derived metabolites are essential for the regulation of the intestinal immune system and the maintenance of the gut microbiome homeostasis (23-26); both have input on human health and disease (27-29). In this study, we focused on an amino-acid-derived metabolite called indole. This metabolite is produced from the breakage of the amino acid tryptophan by a tryptophanase enzyme (encoded by the *tnaA* gene), found mainly in commensal bacteria such as *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) (23, 25, 26). Indole concertation is estimated to reach up to 1 mM in the human GI tract (25, 30). Such a high concentration of indole was shown to decrease enterohemorrhagic *E. coli* (EHEC) motility, biofilm formation, adherence to epithelial cells, and virulence gene expression and enhance drug resistance of *Salmonella* *enterica* (25, 31-33).

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

**Materials and Methods**

**Bacterial strains**

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), unless otherwise indicated, supplemented with the appropriate antibiotics. *Vibrio cholerae* O1 In ET-122 (+) strains (WT, Δ*cqsA* and MM920 reporter strain – Table 1) were grown at 30°C in LB broth supplemented with the appropriate antibiotics. *Bacteroides thetaiotaomicron* (Table 1) was grown at 37°C in Brain Heart Infusion (BHI, Sigma) in anaerobic conditions, statically. Antibiotics were used at the following concentrations: 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 generated by using the *sacB*-based allelic exchange method (34). Briefly, two PCR fragments of 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 primer pair cqsA\_UF/cqsA\_DR and cloned into pRE112 suicide vector. The resulting pRE112 plasmid contains the flanking regions of *cqsA*, with 94% of the *cqsA* deleted. The plasmid was then transformed into *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 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-heated full 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, and supplemented with DMSO, CAI-1 (50 μM) or indole (at 100-1000 μM concentrations). The cultures were grown for 6 h at 37°C under aerobic conditions (in a tissue culture incubator with 5% CO2) or anaerobic conditions (in DonWhitley A35 anaerobic workstation, with a gas mixture of 5% H2, 10% CO2, and 85% N2). The optical density at 600 nm of the cultures was measured (OD600) before the cultures were centrifuged at 20000 × g for 5 min to separate between bacterial pellets, which were dissolved in SDS-PAGE sample buffer, and the culture supernatants. The supernatants were filtered through a 0.22 μm low protein binding filter, normalized according to the bacterial OD600, and their protein content (proteins secreted into the culture medium) was 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; the precipitates of the secreted proteins were dissolved in an SDS-PAGE sample buffer, and the residual TCA was neutralized with saturated Tris. Samples were analyzed on SDS-PAGE gels with Coomassie Blue staining (InstantBlue, Abcam) or 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* culture was grown anaerobically overnight at 37°C in BHI broth. For co-culture assay performed under aerobic conditions, EPEC and *V. cholerae* overnight cultures were diluted 1:40 into a 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, alone or in the presence of indole (500 µM - Arcos organics). For co- and multi-culture assays performed under anaerobic conditions, *B. thetaiotaomicron* overnight culture was diluted 1:16 into a 1:1 (v/v) DMEM:BHI medium and grown for 8 h. Then, WT EPEC alone or with WT *V. cholerae* were added into the growth medium of *B. thetaiotaomicron* (each diluted 1:80) and grown for an additional 6 h. In addition, samples of EPEC only, *B. thetaiotaomicron* only, co-culture of EPEC and *V. cholerae* alone or in the presence of 500 µM indole, and the multi-culture combination of *B. thetaiotaomicron*, EPEC, and *V. cholerae* were added to 1:1 (v/v) DMEM:BHI medium and were grown for 6 h. The cultures were then separated into bacterial supernatants and pellets and processed similarly to that described above for the T3S assay.

**Bioluminescence (LuxR) assay**

The presence of CAI in the culture media was determined by following the light production of the reporter strain *V. cholerae* Δ*cqsA*Δ*luxP* 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. The plate was incubated at 30°C with aeration, and light production and OD600 were measured every 30 min (TECAN infinity 200pro). Plain LB and 10 µM CAI-1 were used as negative and positive controls, respectively. The values of the luminescence signal divided by the OD600 value are presented as relative units (RU). The results represent the average values of three independent experiments.

**Western blotting**

Samples were subjected to SDS-PAGE and transferred to nitrocellulose (pore size: 0.45 μm; Amersham Protran) or PVDF (pore size: 0.45 μm; Amersham Hybond) membranes. The blots were blocked for 1 h in 5% (w/v) skim milk-PBST (0.1% Tween in phosphate-buffered saline), incubated with the primary antibody (diluted in 5% skim milk-PBST) for 1 h at room temperature, washed, and then incubated with the secondary antibody (diluted in 5% skim milk-PBST) for 1 h at room temperature. Chemiluminescence was detected with EZ-ECL reagents (Cyanagen). The optimal dilution for each antibody was calibrated individually: 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, 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. Representative western blots of at least three independent experiments are presented in the results section.

**RNA extraction and qPCR analysis**

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 (early exponential growth phase). Bacteria (5 × 108 cells) were collected and subjected to RNA extraction with the NucleoSpin Bacterial RNA isolation kit according to the manufacturer's guidelines (Macherey-Nagel). RNA was examined for genomic DNA contaminations, subjected to additional DNase I treatment when needed, and extracted using TRIzol reagent. 200 ng of RNA from each sample were taken for cDNA synthesis by ProtoScript II First Strand cDNA Synthesis Kit (NEB) using a random primer mix. cDNA was examined for genomic DNA contaminations. The sequences of the primers used for the qPCR experiments are presented in Table 2. Melting curve analysis was used to ensure the specificity of each primer pair. RT-qPCR fluorescence reactions with the cDNA of the examined samples and the primers were diluted into SYBR Green I mix (Roche) and analyzed in the LightCycler 480 instrument (Roche). The reaction conditions for amplification were: 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 obtained data were analyzed by LightCycler 480 software to extract the critical threshold (CT) values. The transcription levels of the target genes following the 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 had been pre-induced for 3 h in optimal T3SS-inducing conditions or 2 h in 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 per well) were then infected with bacterial cultures at MOI of 1:300 for 3 h (when pre-induced in optimal T3SS-inducing conditions) or for 2 h (when pre-induced in 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 blot analysis with anti-JNK and anti-actin (loading control) 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 reported that indole alters various bacterial processes, including inhibiting bacterial virulence of enteric pathogens such as EHEC and *Citrobacter rodentium* (25, 31). To examine the effect of indole on EPEC's primary virulence mechanism, the T3SS activity, we grew WT EPEC under optimal T3SS-inducing conditions (Dulbecco's modified Eagle's medium (DMEM), statically) in aerobic and anaerobic environments in the presence of different indole concentrations (Fig. 1). Since the physiological concentration of indole in the gastrointestinal tract of humans and mice was suggested to reach up to one millimolar concentration (23, 32, 39-42), we examined the effect of indole at concentrations range of 100-1000 µM. T3SS activity was assessed in terms of the ability of EPEC to secrete T3SS translocators (EspA, EspB, and EspD) into the culture supernatant. Our results showed efficient secretion of the translocators by WT EPEC, while no translocators were detected in the supernatant collected from Δ*escN* null strain, which is deleted for the T3SS ATPase gene (Fig. 1). Analysis of the supernatants of WT EPEC grown in the presence of indole at 300 μM concentration or higher showed reduced secretion of T3SS proteins into the extracellular medium compared to a sample supplemented with DMSO alone (the solvent for the stock solution of indole; Fig. 1A). To better monitor the effect of indole on the T3SS activity, we analyzed the supernatants and bacterial pellets by western blot analysis using anti-EspB and anti-Tir antibodies. We detected that indole inhibits EspB secretion in a dose-dependent manner (Fig. 1B and 1C). We also evaluated the expression levels of the T3SS effector protein Tir, which should retain within the bacterial cells at this stage, by analyzing the whole-cell bacterial pellets. Here, we detected a similar dose-dependent inhibition of indole on Tir expression within the bacterial pellets (Fig. 1B and 1C). DnaK levels demonstrated equal loading of the lysates. 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 the optical density over time. We observed similar growth rates regardless of indole presence (Fig. S1). Overall, these results indicated that physiological concentrations of indole inhibit EPEC T3SS secretion activity.

**Indole impairs EPEC virulence upregulation in response to *V. cholerae* growth in co-culture.** We previously reported that EPEC modulates its virulence according to *V. cholerae*'s population size 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 the bacterial strains into a 1:1 (v/v) mixture of DMEM and Luria-Bertani (LB). This mixture is considered semi-optimal for T3SS-inducing conditions, as it generates only a partial T3SS activation and leaves room for an additional T3SS enhancement. As expected, we observed elevated EspB secretion and Tir expression levels in the co-culture sample of EPEC and *V. cholerae* relative to their levels in the pure EPEC culture sample (Fig. 2A). DnaK levels in the bacterial pellets demonstrated equal sample loading. To confirm that EPEC T3SS upregulation is CAI-1 dependent, we created a *V. cholerae* Δ*cqsA* mutant strain, deleted for the CAI-1 synthase gene *cqsA*, and examined its ability to alter T3SS response. Co-culture of EPEC with *V. cholerae* Δ*cqsA* mutant strain showed weak EspB secretion and no Tir expression that overall resembled the levels detected for EPEC pure culture(Fig. 2A). These results confirmed that the upregulation of EPEC T3SS activity is CAI-1 dependent.

To characterize EPEC T3SS response in the presence of *V. cholerae* under conditions that better simulate the human gastrointestinal tract, we performed co-cultured experiments in the presence of indole. Addition of indole (500 µM) to the co-culture of EPEC andWT *V. cholerae* completely abolished EspB secretion and Tir expression (Fig. 2A). These results indicate that indole impairs the ability of EPEC to sense *V. cholerae* presence and upregulate its T3SS accordingly. To validate that the inability of EPEC to respond to *V. cholerae* presence was not due to indole effect on CAI-1 production, we assessed the CAI-1 concentration produced by *V. cholerae* grown in the presence or absence of indole. For that purpose, we employed the *V. cholerae* reporter strain, MM920, which contains the *V. harveyi* *lux*CDABE luciferase light emission operon that is activated by CAI-1 (43). We incubated the reporter strain with supernatants of WT *V. cholerae* strain grown in the presence (500 µM) or absence of indole and measured light production over time. The supernatant of 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* supernatant, a strong signal was detected when the reporter strain was grown in the presence of WT *V. cholerae* supernatant regardless of the presence or the 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 response due to its effect on *V. cholerae* growth, we compared bacterial growth rate 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). Thus, our results suggested that indole impairs EPEC's ability to upregulate its T3SS activity in response to *V. cholerae* presence.

**Indole interferes with EPEC response to CAI-1 at high micromolar concentrations.** To study the interplay between indole, CAI-1, and EPEC T3SS response, we examined the T3SS activity at various synthetic CAI-1/indole molar ratios. To detect T3SS upregulation activity, in a more sensitive experimental setup, we grew EPEC under semi-optimal T3SS-inducing conditions that do not induce full T3SS activation, and therefore make ample room for T3SS upregulation. The bacterial cultures were then separated into supernatants and bacterial pellet samples and analyzed for EspB secretion (supernatants) and Tir expression (bacterial pellets). As expected, we observed elevated EspB secretion and Tir expression when WT EPEC was grown in the presence of CAI-1 (50 µM) compared to DMSO only (Fig. 3A). Moreover, a similar elevation was observed for EPEC grown with CAI-1 and indole at a 1:1 molar ratio (Fig. 3A). However, at higher indole concentrations, the T3SS upregulation effect of CAI-1 has been curtailed in a dose-dependent manner. WT EPEC samples grown in a 1:10 ratio of CAI-1 and indole showed a complete elimination of EspB secretion and Tir expression (Fig. 3A). DnaK levels within the bacterial pellets demonstrated equal sample loading.

To better determine the antagonist effect of indole on CAI-1 response, we grew WT EPEC under optimal T3SS-inducing conditions that induce maximal T3SS response. Under these conditions, we observed indole generated 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 upregulated transcription of T3SS genes induced by CAI-1.** To examine whether indole affects the transcription level of T3SS genes, we cultured WT EPEC strain under semi-optimal T3SS-inducing conditions. We added CAI-1 alone or together with indole at a 1:10 molar ratio and evaluated the transcription levels 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 before, we detected significantly elevated transcription levels of T3SS genes in the presence of CAI-1 compared to DMSO only (Fig. 4). However, bacteria grown in the presence of CAI-1 and indole at a 1:10 molar ratio exhibited almost complete abrogation of transcription of all three genes (Fig. 4). These results indicated that indole reduces T3SS activity by downregulating the transcription of T3SS genes. This reduction is not relieved even under CAI-1 inducing conditions.

**Microbiome-derived indole impairs the ability of EPEC to sense and respond to *V. cholerae* presence.** To further examine the effect of indole on EPEC and *V. cholerae* communication, we performed a multi-bacteria culture assay that better resembles the intestinal environment. We employed the indole-producing commensal bacteria *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*) to simulate natural indole production (25, 26, 44). *B. thetaiotaomicron* was sub-cultured together with EPEC and *V. cholerae* and grown anaerobically in a 1:1 (v/v) mixture of DMEM and BHI. These conditions allow the growth of all three bacterial strains and are semi-optimal T3SS-inducing conditions. We compared the T3SS activity of EPEC grown under multi-culture conditions to that of EPEC grown as a pure culture or co-culture with either *B. thetaiotaomicron* or *V. cholerae* (experimental setup is presented in Fig. 5A). *B. thetaiotaomicron* pure culture was used 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 (Figure 5B). However, 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 the sample 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 bacterial pellets demonstrated equal sample loading. These results further supported our previous observations by showing that microbiome-derived indole inhibits EPEC T3SS secretion activity and the crosstalk between EPEC and *V. cholerae*.

Multi-culturing of *B. thetaiotaomicron*, EPEC, and *V. cholerae* simultaneously, without providing *B. thetaiotaomicron* an opportunity to produce indole before EPEC and *V. cholerae* are added to the growth medium, showed higher EspB secretion and Tir expression levels compared to the multi-culture sample after eight-hour pre-growth of *B. thetaiotaomicron* (Fig. 5B). These results suggested that the belated production of indole limits its effect on the T3SS response. Not surprisingly, *B. thetaiotaomicron* pure culture sample was negative for DnaK expression since anti-DnaK antibody reacts mainly with *E. coli* DnaK or DnaK from 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 them on selective medium. We observed similar bacterial counts of EPEC and *V. cholerae* regardless of *B. thetaiotaomicron* presence (Fig. S3). Therefore, we conclude that the microbiome-derived indole can interfere with the crosstalk between EPEC and *V. cholerae* pathogens. While both EPEC and *V. cholerae* can produce indole, it was reported to occur mainly during their stationary growth phase, which is less relevant for this experimental setup (26).

**Indole inhibits EPEC's ability to translocate effector proteins into host cells.** To further evaluate the effect 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 indole at the various concentration, and monitored the cleavage level of a host cell protein c-Jun N-terminal kinase (JNK), which is degraded by a translocated effector called NleD (38). As expected, HeLa cells infected with WT EPEC cultures showed an evident degradation of JNK, in contrast to the uninfected HeLa sample and the sample infected with Δ*escN* EPEC strain (Fig. 6A). However, HeLa cells infected with WT EPEC cultures pre-incubated with indole at 500 μM concentration or higher, showed lower levels of JNK degradation and a higher level of full-length JNK (Fig. 6A). These results suggested that indole inhibits the virulence of EPEC by interfering with its T3SS ability to translocate effector proteins into host cells.

As we previously reported that CAI-1 enhances EPEC's ability to infect HeLa cells and translocate effectors into host cells (7), we examined whether this enhancement is altered in the presence of indole. We, therefore, monitored the cleavage pattern of JNK of HeLa cells infected with EPEC strains grown under semi-optimal T3SS conditions (to ensure we can detect virulence enhancement) incubated with both CAI-1 and indole at 1:1 and 1:10 molar ratio. As expected, HeLa cells infected with WT EPEC in the presence of CAI-1 showed higher degradation of JNK compared to infection of WT EPEC only. While high JNK degradation levels were still observed for the sample 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 in a 1:10 ratio (Fig. 6A). These results suggested that indole downregulates EPEC virulence enhancement induced by CAI-1.

**Discussion**

The GI microbiome has a critical role in human health as it provides colonization resistance against pathogenic bacteria (45, 46). This is imposed by 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 reduce bacterial infections. For example, *Bacteroides thuringiensis* and commensal *E. coli* synthesize peptides called 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 was previously demonstrated (25, 33, 53). In this study, we extended this finding to EPEC and showed 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 production of Shiga toxins of various pathogenic *E. coli* strains (54).

Nevertheless, in this study, we examined not only whether indole can inhibit bacterial virulence mechanisms but also whether it can interrupt virulence upregulation, mediated by bacterial communication. For that purpose, we used a co-infection model of EPEC and *V. cholerae*, which previously demonstrated that the two pathogens time their virulence to reduce their competition and coordinate their infection (7). We have previously suggested that this communication is mediated by CAI-1, the primary QS molecule of *V. cholerae*. Here, we confirmed this proposition, by creating a *V. cholerae* mutant strain deleted for the CAI-1 synthase gene, *cqsA*, that is deficient in CAI-1 production. Co-culture of EPEC and *V. cholerae* Δ*cqsA* did not induced upregulation of EPEC T3SS activity similarly to co-culture with WT *V. cholerae* (Fig. 2), thus confirming that the bacterial communication is mediated through CAI-1.

Using this inter-bacterial communication system, we discovered that the addition of indole or *B. thetaiotaomicron* culture, which produce indole over time, to the co-culture model of EPEC and *V. cholerae* completely disrupts the communication between the pathogens and results in the depletion of EPEC T3SS activity upregulation (Fig. 2 and Fig. 5). We observed that the ability of CAI-1 to upregulate EPEC virulence has been neutralized in an indole-dependent manner, mainly at higher indole concentrations (Fig. 3 and Fig. 5). This novel finding provides a possible explanation to the variability found among individuals regarding their susceptibility to bacterial infections. According to our discovery, different microbiome compositions can account for various indole gut concentrations, which in return can inhibit virulence and alter the communication between gut pathogens that can either support or interfere with the bacterial infection.

This finding is in keeping with previous studies that showed that the microbiome composition is linked to certain gut-associated diseases such as inflammatory bowel disease, obesity, type 2 diabetes and even cancer (55, 56). Therefore, microbiome alteration can potentially be used as a therapeutic tool (56). Since our findings demonstrate that microbiome-derived indole reduced pathogen communication and pathogens’ ability to coordinate infection, it is logical to assume that enrichment of the microbiome with indole-producing bacterial species will provide stronger colonization resistance especially against multi-infections of enteric pathogens (1-3). In addition, to promote indole production by these strains, rich protein diet should be encouraged, as indole is produced from metabolism of tryptophan. Therefore, consumption of rich-protein diet should assist in preventing bacterial infections. Furthermore, indole have the potential to become postbiotics supplement, which is defined as a bioactive compound naturally produced by the gut microbiome that has shown to improve human health.

The ability of bacteria to respond to chemical signals that indicate the bacterial population size of the same and/or different species, is vital for group behavior coordination that is required for the infection process and survival within the host. Interference with this process can therefore be a powerful strategy to fight bacterial infection. An example of such interference was previously described by Xavier and Bassler, where *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* miscalculation of its population size and interferes with the ability of the bacteria to properly respond to changes in their cell population density, which are often crucial for successful bacterial-host relationships. In this study, we described an additional mechanism to intercept with bacterial communication, using a microbiome-derived metabolite. This observation that gut-microbiome species produce a specific component that has an important role in protecting the gut health can represent a broad phenomenon and might provide a novel method to fight infectious diseases. Therefore, research into CAI-1 and indole signaling, including finding CAI-1 receptor in EPEC and determining whether indole is antagonist to this receptor remains to be investigated. Understanding indole direct and indirect effect on bacterial virulence will assist in developing 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*) 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 by 12% SDS-PAGE with Coomassie staining **(A)** or western blot analysis with 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. Also indicated is the location of EspC, which is not secreted via the T3SS.

Figure 2. **Indole interferes with the crosstalk of EPEC and *V. cholerae*.** **(A)** Pure overnight cultures of EPEC and *V. cholerae* strains were sub-cultured into fresh 1:1 (v/v) DMEM: LB mixture as single or mixed cultures. The 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 supernatants of bacterial cultures and analyzed by 12% SDS-PAGE and western blot analysis using anti-EspB antibody. The expression of the effector protein, Tir, which should remain mostly within the bacterial cytoplasm at this stage, was analyzed by subjecting the bacterial pellets to SDS-PAGE and western blot analysis using an anti-Tir antibody. Samples were also probed with anti-DnaK to demonstrate equal loading. **(B)** Relative light production as means to determine the level 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 positive control. The figure presents average values of three replicates of a representing experiment.

Figure 3. **Indole competes with CAI-1 on EPEC T3SS activation.** Wild type (WT) EPEC and *escN* null-mutant (Δ*escN*) 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 (bacterial sup) were concentrated from the supernatants of the bacterial cultures and analyzed by 12% SDS-PAGE and western blot analysis using an anti-EspB antibody. The expression of the effector protein, Tir, was analyzed by subjecting the bacterial pellets to SDS-PAGE and western blot analysis using an anti-Tir antibody. Samples were also probed with anti-DnaK to confirm 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 in 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 of the T3SS genes, *espB*, *espA* and *tir*, were measured by qRT-PCR. mRNA levels are presented relative to those of WT EPEC grown in the ​presence of DMSO (dark gray bars). The figure presents average values of three replicates from a representing experiment; bars represent standard error; \*\*P < 0.005.

Figure 5. ***B. thetaiotaomicron*-derived indole inhibits EPEC elevated T3SS activity when co-cultured with *V. cholerae*.** **(A)** A color scheme of the bacterial combinations and supplemented indole used in this experiment. WT EPEC was sub-cultured in a mixture of 1:1 (v/v) DMEM:BHI medium as pure culture, co-culture with *V. cholerae*, co-cultured with *B. thetaiotaomicron*, or as multi-culture with both *V. cholerae* and *B. thetaiotaomicron* (EPEC and *V. cholerae* were added either at time 0 or after 8 h of growth of *B. thetaiotaomicron* as a pure culture). One of the co-cultures of EPEC and *V. cholerae* was supplemented with indole (500 µM). All cultures were grown anaerobically for 6 h and then the bacterial pellets and supernatants (bacterial sup) were separated, normalized, and analyzed. **(B)** The secreted proteins were concentrated from the supernatants of the bacterial cultures and analyzed by 12% SDS-PAGE and western blot analysis using an anti-EspB antibody. The expression of the effector protein, Tir, was analyzed by subjecting the bacterial pellets to SDS-PAGE and western blot analysis using an anti-Tir antibody. Samples were also probed with anti-DnaK to confirm 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 EPEC WT and Δ*escN* 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 blot analysis using anti-JNK and anti-actin (loading control) antibodies. JNK and its degradation fragments are indicated at the right of the gel. **(B)** Western blot analysis of JNK degradation pattern following HeLa infection with EPEC WT and Δ*escN* 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 medium (*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 biological independent experiments.

Figure S1A. **Indole does not affect EPEC growth.** Growth curves of 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 medium 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.** Pure overnight cultures of EPEC and *V. cholerae* strains were sub-cultured into fresh 1:1 (v/v) DMEM:BHI mixture as pure co- or multi-cultures with *B. thetaiotaomicron*. The cultures were grown under semi-optimal T3SS inducing conditions for 6 h and plated on LB plates containing chloramphenicol for EPEC growth (A) and carbenicillin for *V. cholerae* growth (B). The plates were incubated overnight in 37°C and the bacterial colony forming units (CFU) are presented.

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