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Navigating contradictions: *Salmonella* Typhimurium chemotaxis amidst conflicting stimuli of the intestinal environment

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eLife Assessment

In this manuscript, Franco and colleagues present **compelling** evidence that fecal extracts containing high concentrations of indole, a known repellent, enhance rather than protect against invasion of colonic tissue by Salmonella. The authors describe **important** findings that lead to the conclusion that the competing effects of attractants present in fecal matter, including L-serine, also sensed by the Tsr chemoreceptor that senses indole, override the repulsive effect of indole.

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Abstract

Motile bacteria sense and avoid deleterious stimuli in their environment through chemorepulsion, a behavior that helps them locate permissive ecological niches. In the gut, indole is a bacteriostatic compound produced by the microbiota and is thought to act as a chemorepellent for invading pathogens, thereby protecting the host against infection. The principal reservoir of intestinal indole is fecal matter, a complex biological material that contains both attractant and repellent stimuli. Whether indole in its natural context is sufficient for pathogen chemorepulsion or host protection has remained unknown. Using an intestinal explant system, we show that while pure indole indeed suppresses an infection advantage mediated through chemotaxis for the enteric pathogen *Salmonella enterica* serovar Typhimurium, this effect is abolished in the presence of other chemoeffectors present in feces, including the chemoattractant L-Serine (L-Ser), in a manner dependent on the chemoreceptor Tsr. Live imaging reveals that although *S.* Typhimurium is repelled by pure indole, the pathogen is actually strongly attracted to human fecal matter despite its high indole content, and that this response is mediated by Tsr, which simultaneously senses both indole and L-Ser. Fecal attraction is conserved across diverse *Enterobacteriaceae* species that harbor Tsr orthologues, including *Escherichia coli*, *Citrobacter koseri*, *Enterobacter cloacae*, and clinical isolates of non-typhoidal *Salmonella*. In a defined system of fecal chemoeffectors,

we find that L-Ser and other fecal chemoattractants override indole chemorepulsion, but the magnitude of bacterial chemoattraction is controlled by indole levels. Together, these findings suggest that indole in its native context is not protective against enteric infection and that indole taxis actually benefits pathogens during infection by locating niches with low competitor density. Our study highlights the limitations of applying single-effector studies in predicting bacterial behavior in natural environments, where chemotaxis is shaped by the integration of multiple, often opposing, chemical signals.

Introduction

Motile bacteria that colonize the gastrointestinal tracts of humans and other animals employ chemotaxis to sense chemical effectors in the gut lumen and navigate to environments conducive to growth and colonization [1–5]. This process is controlled by chemoreceptor proteins, which recognize chemical effectors and transduce signals through a phosphorylation cascade to regulate flagellar rotation and swimming direction, ultimately determining the spatial and temporal patterns of bacterial colonization (**Fig. 1A**) [1, 2, 6, 7]. While many effectors have been studied and characterized in isolation as chemoattractants or chemorepellents [4, 7], natural environments like the gut contain complex mixtures of opposing signals. Only a handful of studies have investigated how bacteria navigate conflicting chemical gradients, and much remains to be learned about how bacteria prioritize these signals to direct their movement and colonization (**Fig. 1A**) [1, 4, 8–15].

A chemical effector of major importance for enteric bacterial communities is indole, an interbacterial signaling molecule that regulates diverse aspects of physiology and lifestyle [16–18]. Indole is excreted by gut microbiota as a byproduct of tryptophan metabolism and accumulates to millimolar levels in human feces (**Fig. 1A**) [16, 19, 20]. Indole is amphipathic and can transit bacterial membranes to regulate biofilm formation and motility, suppress virulence programs, and exert bacteriostatic and bactericidal effects at high concentrations [16–18, 20–24]. Indole was one of the earliest identified chemorepellents, and subsequent work has extensively explored its role in *Escherichia coli* chemotaxis, mostly examining responses to indole as a singular effector (Table S1) [8, 9, 11, 14, 18, 25]. Recent studies have advanced understanding of the molecular mechanisms underlying *E. coli* indole taxis and the involvement of the chemoreceptor taxis to serine and repellents (Tsr) (**Fig. 1A**) [8, 9, 14, 18].

From this body of research, the hypothesis emerged that indole from the gut microbiota functions to repel pathogens and restrict their growth as a mechanism of colonization resistance [8, 16–18, 23, 24]. If true, this could represent an intriguing avenue for cultivating microbiomes that are more robust against pathogen infiltration—a major area of interest for improving gut health [26, 27]. However, this hypothesis is based largely on observations of bacterial chemorepulsion in response to indole as a single effector, and it remains unclear whether chemotactic behavior is similar or altered in the presence of other intestinal effectors. For instance, fecal material, while rich in indole, also contains high concentrations of sugars and amino acids that serve as nutrients and chemoattractants—factors that could diminish, nullify, or have no influence on indole chemorepulsion [1, 21, 22, 28]. Although indole may suppress bacterial growth, nutrients derived from the host diet could offset this effect, allowing bacteria to tolerate indole and still benefit from colonizing indole-rich niches. Indeed, pathogens frequently succeed in establishing enteric infections, suggesting that they can tolerate indole or circumvent its effects under certain conditions. Thus, bacteria in the intestinal environment must navigate contradictory chemotaxis signals, and how they resolve these conflicts influences infection, pathogenesis, and host health in ways that remain to be elucidated. Furthermore, because indole taxis has only been studied in *E. coli*, it remains unconfirmed whether other enteric species even chemotactically sense or respond to indole (Table S1).

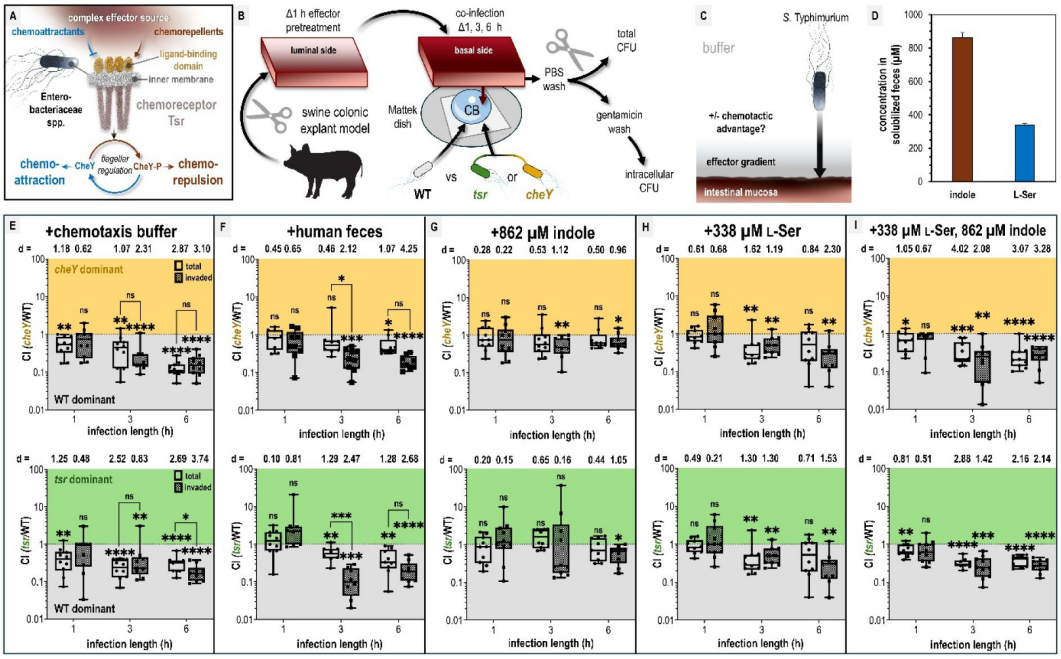


Fig. 1.

Chemotaxis-mediated infection advantages in the presence of fecal effectors.

A. Overview of the role of Tsr in coordinating responses to conflicting stimuli. B. Experimental design of colonic explant infections. See Materials & Methods for experimental details such as tissue dimensions. C. Conceptual model of the explant infection system. The effectors from the treated tissue (gray) diffuse into the surrounding buffer solution providing a gradient. Note that the bacteria are not immersed in the effector solution, and experience a local concentration during infection much lower than the effector pretreatment. Quantifications of tissue-associated bacteria reflect the ability of chemotaxis to provide an advantage (black arrow) in accessing the intestinal mucosa (reddish brown). D. Serine (presumed to be nearly 100% L-Ser, see Materials & Methods) and indole content of liquid human fecal treatments, as measured by mass spectrometry. E-I. Competitive indices (CI) of colony-forming units (CFUs) recovered from co-infected swine explant tissue, either from the total homogenate (open box and whiskers plots), or from tissue washed with gentamicin to kill extracellular and attached cells, which we refer to as the “invaded” intracellular population (checkered box and whisker plots), as indicated. Each data point represents a single experiment of a section of tissue infected with bacteria, normalized by tissue weight, and the CI of CFUs recovered from that tissue (n=7-10). Boxes show median values (line) and upper and lower quartiles, and whiskers show max and min values. Effect size (Cohen’s *d*) and statistical significance are noted for each experiment in relation to competitive advantage, i.e. deviation from a CI of 1 (not significant, ns; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). See also **Figure S1** for competition between WT and an invasion-inhibited mutant *invA*, and **Figure S2** for disaggregated CFU enumerations for each experimental group prior to CI calculation. Data S1 contains all numerical CFU measurements.

In this study, we aimed to (1) test the hypothesis that indole protects against intestinal infection and (2) determine how enteric pathogens use chemotaxis to navigate the complex mixture of opposing chemical cues present in fecal material, a major source of both indole and nutrients within the gut. We used *Salmonella enterica* serovar Typhimurium as a model pathogen, as it requires chemotaxis and the chemoreceptor Tsr for efficient infection and cellular invasion of intestinal tissue [29–35]. Tsr is of particular interest because it mediates responses to both chemorepellents and the chemoattractant L-Serine (L-Ser), suggesting an important role in integrating contradictory chemotactic signals for *S.* Typhimurium and other *Enterobacteriaceae* that possess Tsr orthologues (**Fig. 1A-B**) [1, 8, 9, 14, 18, 25, 36–39]. *S. Typhimurium* also differs from *E. coli* in that it lacks tryptophanase and cannot itself produce indole, thereby offering a novel perspective on indole taxis [40, 41]. Our findings reveal that bacterial chemotaxis within biologically relevant mixtures of effectors cannot be reliably inferred from studies of individual compounds alone, with important implications for understanding how chemotaxis influences pathogen behavior within the gut.

Results

Fecal indole is insufficient to protect against pathogen invasion in a colonic explant modelWe sought to test whether indole in human fecal matter protects against *Salmonella enterica* serovar Typhimurium infection and whether this involves indole chemorepulsion mediated by the chemoreceptor Tsr. *Salmonella* Typhimurium preferentially infects tissue of the distal ileum but also infects the cecum and colon in humans and animal models [42–46]. We presume that the amount of indole is greatest in the lower gastrointestinal tract, where tryptophan has been maximally digested by microbial tryptophanases. To mimic this environment, we developed a swine colonic explant model that simulates the architecture and size of adult human colonic tissue (**Fig. 1B**) [41, 47–51]. This model was based on a prior study using explant tissue to characterize cellular invasion via gentamicin washes, which kill extracellular and surface-attached bacteria (**Fig. 1B**) [52]. Gentamicin washing is also a commonly used method to quantify intracellular *Salmonella* Typhimurium populations in cell culture experiments [53, 54].

A section of colonic tissue was prepared for each experiment by gentle cleaning and then soaked with different effector solutions for 1 h: solubilized human feces, purified indole and/or L-Ser at fecal-relevant concentrations, or buffer as a control (see Materials & Methods for tissue dimensions and additional experimental details). Subsequently, the tissue was removed from the effector treatment and oriented with the luminal side downward onto a Mattek dish containing 300 µl of buffer with motile *S.* Typhimurium (**Fig. 1B**). To be clear, in this system the bacteria are not immersed in the effector treatment and experience an effector concentration far lower than used for the soak of the tissue prior to infection where the residual effector diffuses outward from the tissue into the much larger volume of buffer in which the cells are swimming (**Fig. 1C**). This establishes a chemical gradient which we can use to quantify the degree to which different effector treatments are permissive of pathogen association with, and cellular invasion of, the intestinal mucosa (**Fig. 1C**). Using this approach we sought to test infection based on fecal treatments and fecal-relevant concentrations of L-Ser and indole (**Fig. 1D**).

We employed a strategy of co-infections in order to compete and compare the advantages conferred by chemotaxis using *S.* Typhimurium strain IR715 wildtype (WT) and either a *cheY* mutant (motile but non-responsive to chemoeffector stimuli) or *tsr* deletion mutant (**Fig. 1B**, **Table 1**, see Materials & Methods) [30]. The functionality of these mutants has been previously confirmed through *in vivo* infection studies using genetically complemented strains [30, 31]. To assess the role of chemotaxis in infection, we quantified bacteria harvested from tissue homogenates at 1, 3, and 6 h post-infection (**Fig. 1C**, Materials & Methods) [55, 56]. Using this experimental setup, we found for tissue pretreated with fecal material that WT had a competitive advantage over an invasion-inhibited mutant (*invA*) in homogenates from gentamicin-washed

tissue, but no advantage in unwashed homogenates, supporting that gentamicin washing selects for intracellular bacteria [57]. For simplicity in discussing the explant infection data we refer to these two types of quantifications as "invaded" (i.e. *Salmonella* that have entered non-phagocytic host cells) and "total" bacteria in the figures, respectively (Fig. S1) [58].

In buffer-treated explant experiments, WT *S.* Typhimurium exhibits a 5- to 10-fold time-dependent advantage in colonization and cellular invasion compared to chemotactic mutants, indicating that chemotaxis, and specifically Tsr, promotes tissue colonization in this system (**Fig. 1E**, **Fig. S2A–B**, Data S1). The mechanism mediating this advantage isn't clear, but could arise from a combination of factors, including sensing of effectors emitted from the tissue, redox or energy taxis, and/or swimming behaviors that enhance infection [5], [30], [31], [35]. This experiment indicates that under baseline conditions, the intestinal mucosa is accessible to the pathogen. The hypothesis that indole protects against pathogen colonization predicts that feces, the major biological reservoir of gut indole, should confer protection against infection. Contrary to this prediction, we found that fecal treatment provided a similar infection advantage as buffer treatment, and this effect was mediated by chemotaxis and Tsr (**Fig. 1F**, **Fig. S2C–D**). One notable difference, however, was that fecal treatments yielded a higher competitive advantage for the WT invaded population over the total population at 3 hours compared to buffer treatment (comparing buffer and fecal treatments: WT vs *cheY*, $p = 0.18$ and $p = 0.02$; WT vs *tsr*, $p = 0.35$ and $p = 0.0004$, respectively; **Fig. 1E–F**, Data S1). That the phenotype fades at longer time points could relate to the effector gradient being eliminated by diffusion.

Analysis of the liquefied human fecal matter used in this study revealed an indole concentration of 862 μM, consistent with previously reported ranges (0.5–5 mM) (**Fig. 1D**; Materials & Methods) [16], [19]–[22]. When colonic tissue was treated with purified indole at this concentration, WT loses its competitive advantage over the chemotactic mutants (**Fig. 1G**, **Fig. S2E–F**, Data S1). Given that Tsr mediates attraction to L-Ser in both *Escherichia coli* and *Salmonella* Typhimurium, we hypothesized that L-Ser present in feces might negate the protective effect of indole [1], [36], [59], [60]. Treatment with 338 μM L-Ser alone, the concentration present in our fecal sample (**Fig. 1D**; Materials & Methods), conferred a WT advantage similar to buffer and fecal treatments (**Fig. 1H**) and WT also exhibits a colonization advantage when L-Ser is co-administered with indole (**Fig. 1I**, Data S1).

Our key takeaway from these experiments is that pretreatment of intestinal tissue with indole alone is unique in that the WT strain gains no infection advantage in this context (**Fig. 1G**). In contrast, under all other treatment conditions, the WT infects the tissue to a greater extent than the chemotactic mutants (**Fig. 1E-I**). Put another way, chemotaxis and Tsr enhance pathogen transit of the chemical gradient and increase access to the intestinal tissue in all conditions except when indole is the sole effector. This was surprising, given that other treatments contain the same concentration of indole and still permit a chemotaxis- and Tsr-mediated advantage (**Fig. 1**). To us, this suggests that bacterial perception of indole via chemotaxis differs fundamentally depending on whether indole is the only effector present or amidst other fecal effectors. Notably, only fecal treatment resulted in a greater competitive advantage for the invaded population than for the total population (**Fig. 1F**).

Enterobacteriaceae species are attracted to human feces despite high indole content

Having found that chemotaxis and Tsr mediate efficient infection, but do not confer an advantage when indole is the only effector, we next sought to understand the chemotactic behaviors orchestrated by Tsr in response to indole-rich feces. Given that feces represents the highest concentration of indole that *S.* Typhimurium is likely to encounter natively, we expected to observe chemorepulsion (**Fig. 1D**, Table S1) [8], [9], [14], [25], [39]. We employed the chemosensory injection rig assay (CIRA) for live-imaging of bacterial chemotaxis responses to a

Strain	*Reference/Source*
S. enterica Typhimurium IR715 nalidixic acid-resistant derivative of ATCC 14028	Rivera-Chávez, F. et al.[30]
S. enterica Typhimurium IR715 Δ*cheY::Tn10* (Tet^R)	Rivera-Chávez, F. et al.[30]
S. enterica Typhimurium IR715 Δ*tsr::pFR3* (Cm^R)	Rivera-Chávez, F. et al.[30]
S. enterica Typhimurium IR715 invA::pSW127 ($Carb^R$)	Thiennimitr, P. et al.[57]
S. enterica SARA1	Beltran, P. et al.[67]
S. enterica Newport M11018046001A	Shariat, N. et al.[68]
S. enterica Enteriditis 05E01375	Shariat, N. et al.[68]
E. coli BL21-DE3 Cat# 70954-3	Millipore-Sigma
E. coli MG1655 pXS-GFP (Amp^R)	This study
Enterobacter cloacae subsp. *cloacae* strain CDC 442-68 Cat# 13047	ATCC
Citrobacter koseri Frederiksen 4225-83 Cat# BAA-895	ATCC
Escherichia coli strain NCTC 9001 Cat# 11775	ATCC

Table 1

Bacterial Strains

source of effectors injected through a glass microcapillary [36]. The flow and dynamic nature of the gut lumen make this a suitable *in vitro* approach for modeling and studying enteric chemotaxis responses [36, 61].

In this assay, chemoattraction is observed as an influx of cells toward the effector source, whereas chemorepulsion is indicated by a decrease in local cell density (**Fig. S3**). As described previously, effector injection creates a steep chemical microgradient [36]. Using mathematical modeling of the diffusion of fecal-relevant concentrations of indole and L-Ser, we approximated the local concentrations experienced by bacteria at varying distances from the injection site. For most of the field of view, these concentrations fall within the picomolar to low nanomolar range (**Fig. 2A**; Materials & Methods) [36].

Over five minutes, we found both WT and *tsr* exhibit strong chemoattraction to fecal material, whereas *cheY* remains randomly distributed (**Fig. 2B**, Movie 1). By examining the radial distribution of the bacterial populations, we found WT more tightly centers around the treatment source than *tsr* (**Fig. 2C-E**, Movie 1). In terms of the rate of bacterial accumulation, the chemoattraction of *tsr* lags behind the WT for the first 120 s (**Fig. 2F-G**, Movie 1). We wondered how these deficiencies in fecal attraction might translate to direct competition, where different strains are experiencing the same treatment source simultaneously. To address this, we performed CIRA with solubilized human feces and two strains present in the same pond, which we tracked independently through fluorescent markers (**Fig. 3**) [36]. As expected, WT shows a strong chemoattraction response versus *cheY* (**Fig. 3A**, Movie 2). Interestingly, we found that when competed directly, WT vastly outperforms *tsr*, with the maximal bacterial distribution in proximity to the treatment source higher by about 4-fold (**Fig. 3B**, Movie 2). These data confirm that despite its high indole content, *S.* Typhimurium is attracted to human fecal material through chemotaxis, and this response involves Tsr, although not as the sole mediator. We expect the attraction of the *tsr* mutant is explained by the fact that *S.* Typhimurium possesses other chemoreceptors that detect glucose, galactose, ribose, and L-Asp as chemoattractants, which are also present in human feces [1, 7, 62–66].

Recent work highlights how genetic diversity among *Salmonella* strains and differences in Tsr expression, even within isogenic populations, modulate chemotaxis function [9, 32]. To gain a broader perspective on fecal taxis, we examined the responses among diverse non-typhoidal *Salmonella* serovars and strains responsible for human infections. Using dual-channel imaging, we compared *S.* Typhimurium IR715 with a clinical isolate of *S.* Typhimurium, SARA1, and found both strains exhibit attraction to feces, although SARA1 shows a slightly weaker response (**Fig. 3C**, Movie 3) [67]. We then tested a clinical isolate of *S.* Newport, an emerging cause of salmonellosis in the United States and Europe [68, 69]. This strain is strongly attracted to fecal material, with a tighter accumulation of cells at the treatment source than *S.* Typhimurium IR715 (**Fig. 3D**, Movie 3). We also examined a clinical isolate of *S.* Enteritidis, a zoonotic pathogen commonly transmitted from poultry, which displays weak attraction to fecal material (**Fig. 3E**, Movie 3) [69].

Next, we extended this analysis to other disease-causing *Enterobacteriaceae* that possess Tsr orthologues [36]. *E. coli* strain MG1655, commonly used for *in vitro* experiments, and *E. coli* NCTC 9001, a strain isolated from human urine and associated with urinary tract infections, both exhibited fecal attraction, although the response was more diffuse than that observed for *Salmonella* (**Fig. 4A–D**; Movies 4–5) [70]. The clinical isolate *Citrobacter koseri* strain 4225-83 also showed fecal attraction, with a tight association near the effector source (**Fig. 4E–F**; Movie 6). Lastly, *Enterobacter cloacae* CDC 442-68, a clinical isolate with uncharacterized chemotaxis behavior, appeared to exhibit fecal attraction as well, although this strain was not extensively tested due to limited motility under laboratory conditions (**Fig. S4**).

Fig. 2.

Salmonella Typhimurium exhibits attraction toward indole-rich liquid human fecal material.

A. Diffusion modeling showing calculated local concentrations in CIRA experiments with liquid human fecal material based on distance from the central injection source. B. Max projections of representative *S.* Typhimurium IR715 responses to a central source of injected liquid human fecal material. C-E. Bacterial population density over time in response to fecal treatment. The initial uniform population density in these plots is indicated with the blue line (time 0), and the final mean distributions with the red line (time 280 s), with the mean distributions between these displayed as a blue-to-red spectrum at 10 s intervals. F-G. Temporal analyses of area under the curve (AUC) or relative number of bacteria within 150 µm of the source. Effect size (Cohen's *d*) comparing responses of WT and *tsr* attraction at 120 s post-treatment is indicated. Data were collected at 30 °C. Data are means and error bars are standard error of the mean (SEM, n=3-5). See also Movie 1, Table S1, and **Figure S3**.

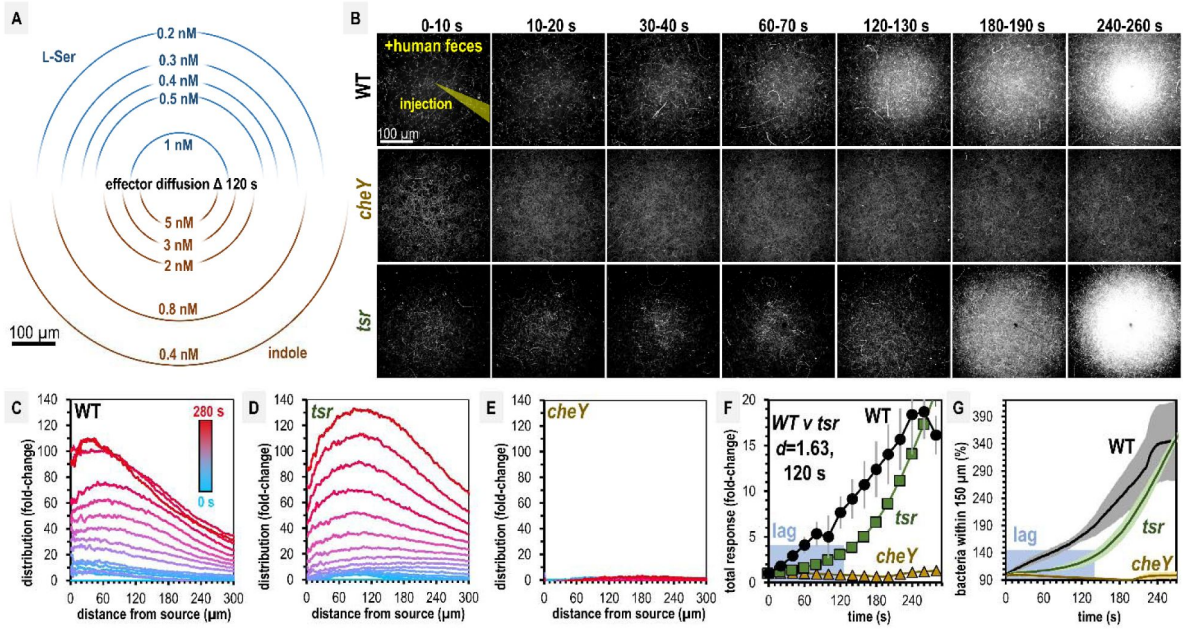
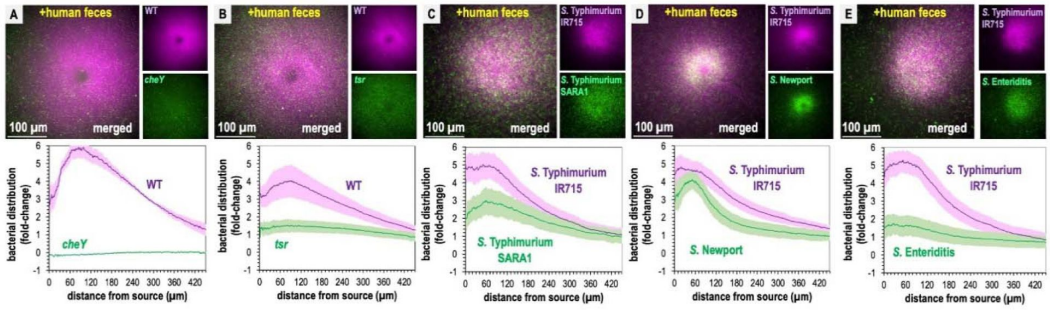


Fig. 3.

Non-typhoidal *Salmonella* exhibit fecal attraction.

A-E. Dual-channel imaging of chemotactic responses to solubilized human feces by WT *S.* Typhimurium IR715 (pink) and isogenic mutants or clinical isolate strains (green), as indicated. Shown are max projections at time 295-300 s post-treatment. Data were collected at 37 °C. Data are means and error bars are standard error of the mean (SEM, n=3-5). See also Movie 2, Movie 3.



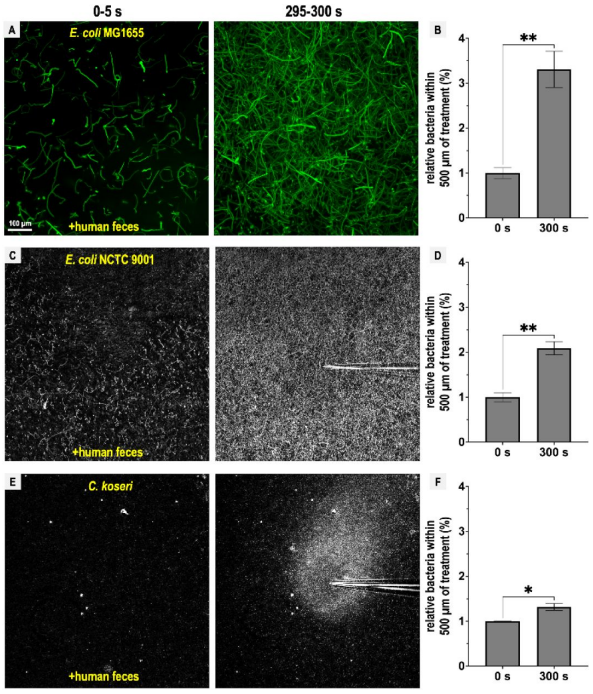


Fig. 4.

Representative *Enterobacteriaceae* exhibit fecal attraction.

Shown are max projections from CIRA experiments over 5 s before fecal treatment and after 5 minutes of treatment, as well as quantifications of bacteria within 500 µm of the treatment source at these same time points for *E. coli* MG1655 (A-B, GFP-reporter), *E. coli* NCTC 9001 (C-D, phase), and *C. koseri* CDC 4225-83 (E-F, phase). Data were collected at 37 °C. Data are means and error bars are standard error of the mean (SEM, n=3-5). See also Movie 4, Movie 5, Movie 6.

Overall, we find that Tsr mediates fecal attraction in *Salmonella*, and that this behavior is conserved among diverse *Enterobacteriaceae* that possess Tsr and are associated with human infections. Although the degree of attraction varies, none of the enteric pathogens or pathobionts tested exhibited chemorepulsion from feces, despite its high indole content.

Fecal chemoattractants override indole chemorepulsion

To better understand the relationship between indole and other fecal effectors in directing *S.* Typhimurium chemotaxis, we next employed a reductionist approach and developed a mixture of fecal effectors at physiological concentrations based on our measurements and the Human Metabolome Database (**Fig. 5**) [28]. Along with the chemorepellent indole (862 μM), we tested combinations of fecal chemoattractants including L-Ser (338 μM), sensed through Tsr; D-glucose (970 μM), D-galactose (78 μM), and ribose (28.6 μM), sensed through the chemoreceptor Trg; and L-aspartate (L-Asp, 13 μM), sensed through the chemoreceptor Tar [1, 7, 28, 36, 71, 72]. A low density of motile cells (A_{600} ~ 0.05) was used in the CIRA experiments to increase sensitivity for detecting attraction in response to different combinations of these fecal effectors (**Fig. 5**).

We observed that L-Ser was sufficient to negate indole chemorepulsion, and may even elicit attraction, although this was not statistically significant (**Fig. 5A–B**; Movie 7). When all effectors were present, bacteria were clearly attracted to the treatment (**Fig. 5C–D**; Movie 8), with a slightly reduced attraction in the absence of L-Ser (**Fig. 5E–F**; Movie 9). Interestingly, when all effectors were present but the concentration of indole was halved (431 μM), cells exhibited the greatest degree of attraction (**Fig. 5G–H**; Movie 10).

From these data, we conclude that the Tsr ligand L-Ser can override chemorepulsion from indole. However, this effect can also be mediated by other fecal effectors sensed through different chemoreceptors, providing an explanation for the reduced, but still appreciable, fecal attraction observed for the *tsr* mutant (**Fig. 3B**). While the overall responses to this mixture of fecal effectors can be characterized as attraction, the bacteria remain sensitive to indole levels, as reflected in the enhanced attraction observed in treatments with lower indole concentrations (**Fig. 5G–H**).

Mediation of opposing chemotactic responses by Tsr

We considered whether our inability to observe repulsion from fecal material and mixtures of fecal effectors might be due to *S.* Typhimurium not sensing indole as a chemorepellent, since this chemotactic response has only been previously described for *E. coli* (Table S1). We compared chemotaxis responses to either 5 mM L-Ser or 5 mM indole and found that *S.* Typhimurium responds rapidly to these two effectors as chemoattractants and chemorepellents, respectively (**Fig. S3H-I**). Treatment with 5 mM indole, a concentration at the upper end of what occurs in the human gut [22], induces rapid chemorepulsion with the bacteria vacating the region proximal to the source (**Fig. S3I**). Interestingly, the chemorepulsion response occurs faster than chemoattraction, with a zone of avoidance clearly visible within the first 10 s of indole exposure (**Fig. S3H-I**, Movie 11).

We next wondered if perhaps our fecal treatments contained insufficient indole to elicit chemorepulsion from *S.* Typhimurium. To identify the effective source concentrations that drive indole chemorepulsion and understand the temporal dynamics of this response, we performed a titration of indole across 0.05-10 mM (**Fig. 6A-B**). At all concentrations tested, indole induces chemorepulsion, and the bacteria avoid the treatment source for the duration of the 5-minute experiment (**Fig. 6A-B**). At source concentrations exceeding 3 mM most motile cells vacate the field of view within 60 s (**Fig. 6A**). Integrating these chemorepulsion responses and fitting them to a Monod curve suggests that an indole source concentration of approximately 67 μM is

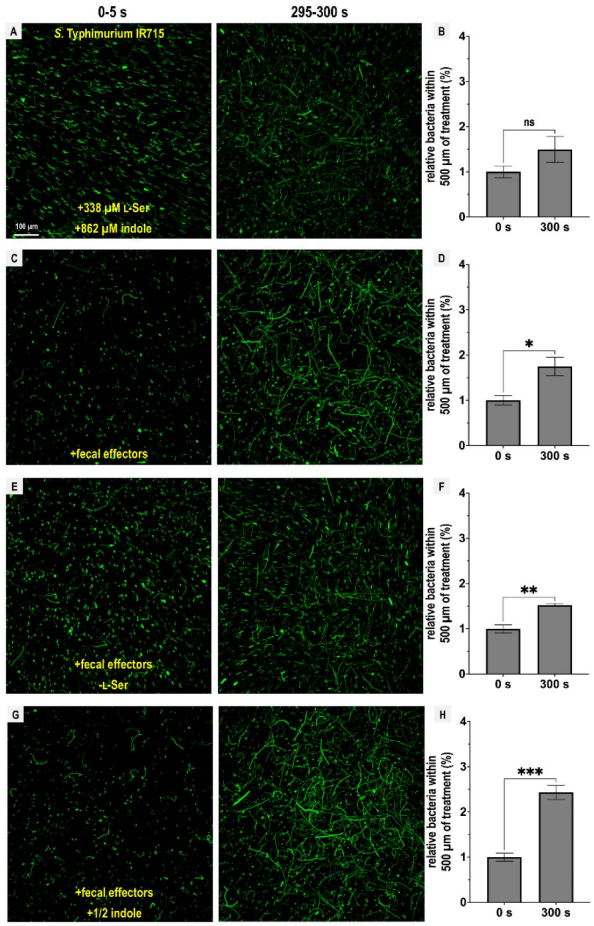


Fig. 5.

Chemotactic responses to defined fecal effector mixtures.

CIRA experiments with *S.* Typhimurium IR715 were performed with different combinations of fecal effectors (n=3-5). Shown are max projections from experiments over 5 s before fecal treatment and after 5 minutes of treatment as well as quantifications of bacteria within 500 µm of the treatment source at these same time points. Data are means and error bars are standard error of the mean (SEM, n=3-5). To achieve the greatest degree of sensitivity to differences in responses, experiments were performed using the same culture on the same day. The complete fecal effector mixture consists of indole (862 µM), L-Ser (338 µM), D-Glucose (970 µM), D-Galactose (78 µM), ribose (28.6 µM), and L-Asp (13 µM), modified to include or exclude certain effectors as indicated. See also Movie 7, Movie 8, Movie 9, and Movie 10 Data were collected at 30 °C.

sufficient for half-maximal ($K_{1/2}$) chemorepulsion (**Fig. 6C**). These data show that even though we observe strong attraction to fecal material, pure indole at the concentration present in fecal material, and far lower, is indeed a strong chemorepellent for *S.* Typhimurium.

Based on its function in *E. coli*, we hypothesized that both indole chemorepulsion and L-Ser chemoattraction for *S.* Typhimurium could be partly or fully mediated through Tsr [7, 8, 38]. We compared the chemotactic responses of the WT and *tsr* strains when exposed to sources of these effectors and found Tsr to be required for both chemorepulsion from indole and chemoattraction to L-Ser (**Fig. 6E-F**). The canonical mode of chemoreceptor effector recognition involves binding of the effector to the periplasmic ligand-binding domain (LBD) [7, 73], but the mechanism by which indole is sensed through Tsr in *Salmonella* has not been elucidated. We recently reported the first crystal structure of *S.* Typhimurium Tsr LBD, which clearly defines how the binding site recognizes the L-Ser ligand (PDB code: 8fyv), and we thought it unlikely indole can be accommodated at the same site [36]. To our knowledge, no prior study has tested whether the Tsr LBD binds indole directly, so we expressed and purified the LBD, corresponding to the soluble periplasmic portion, and performed isothermal titration calorimetry (ITC). These data show that no binding occurs between the Tsr LBD and indole (**Fig. 6G**).

We next wondered if indole acts as an allosteric regulator of the LBD, possibly through interacting with the L-Ser-bound form or interfering with L-Ser recognition. To address these possibilities, we performed ITC of 50 μM Tsr LBD with L-Ser in the presence of 500 μM indole and observed a robust exothermic binding curve and K_D of 5 μM, identical to the binding of L-Ser alone, which we reported previously (**Fig. 6H**) [36]. These data indicate that indole does not alter the Tsr LBD affinity for L-Ser. We conclude that Tsr senses indole through an atypical mechanism, which might either involve regulation through a solute-binding protein [8, 74], responsiveness to perturbation in the proton motor force [18], or binding to a different region other than the periplasmic LBD. Our findings reveal that while indole acts as a chemorepellent for *S.* Typhimurium in isolation, and is sensed through Tsr, its presence within fecal material mixed with other effectors is insufficient to elicit chemorepulsion.

Compromising between conflicting effector signals through chemohalation

Since Tsr mediates both chemoattraction to L-Ser and chemorepulsion from indole, we wondered at what threshold each response dominates, and how this behavior is regulated at the point of transition. To address these questions, we assessed responses to physiological mixtures of these effectors using 500 μM L-Ser and increasing concentrations of indole at L-Ser:indole molar ratios of 10:1, 1:1, or 1:10 (**Fig. 7A-D**, Movie 11). These experiments reveal a fascinating transition in the distribution of the pathogen population as a function of increasing chemorepellent, which occurs within minutes of exposure (**Fig. 7A-D**, Movie 11).

With only chemoattractant present, the bacterial population organizes tightly around the effector source (**Fig. 7A**, Movie 11). When indole is introduced at a concentration 10-fold lower than L-Ser, the bacterial distribution still exhibits chemoattraction but becomes more diffuse (**Fig. 7B**, Movie 11). At a 1:1 ratio of chemoattractant and chemorepellent, a different population structure emerges, in which the swimming bacteria are attracted toward the source but form a halo around the treatment with an interior region of avoidance (**Fig. 7C**, **Fig. 7E**, Movie 11). When the concentration of indole is 10-fold higher than L-Ser, the bacteria exhibit a wider zone of avoidance (**Fig. 7D-E**, Movie 11). Interestingly, whereas 5 mM indole on its own induces strong chemorepulsion (**Fig. S3I**, Movie 11), the addition of 10-fold lower L-Ser effectively converts the behavior to a null response (**Fig. 7D-E**, Movie 11). This demonstrates that even at the highest concentrations of indole *S.* Typhimurium might encounter in the gut, the presence of chemoattractant can override indole chemorepulsion.

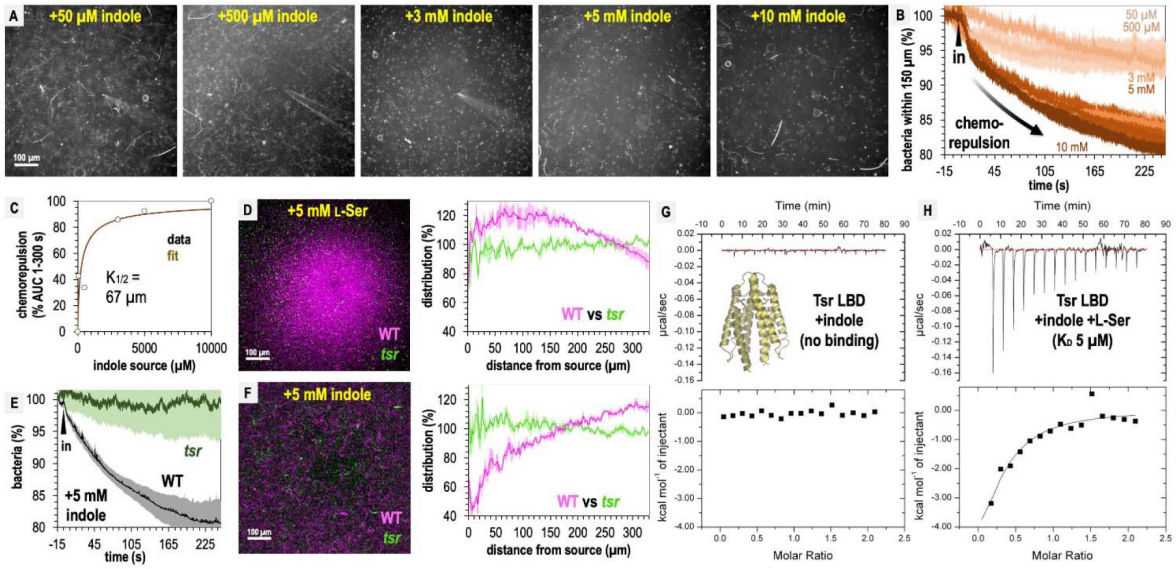


Fig. 6.

Tsr mediates indole chemorepulsion in *S.* Typhimurium.

A. Representative max projections of responses at 295-300 s of indole treatment. B-C. Quantification of chemorepulsion as a function of indole concentration (n=3-5). D-F. Comparison of WT and *tsr* mutant responses to L-Ser or indole. E. Shows a quantification of the relative number of cells in the field of view over time following treatment with 5 mM indole for a competition experiment with WT and *tsr* (representative image shown in F). Data were collected at 30 °C. G-H. Isothermal titration calorimetry (ITC) experiments with 50 µM *S.* Typhimurium Tsr ligand-binding domain (LBD) and indole, or with L-Ser in the presence of 500 µM indole. Data are means and error bars are standard error of the mean (SEM, n=3-5). AUC indicates area under the curve.

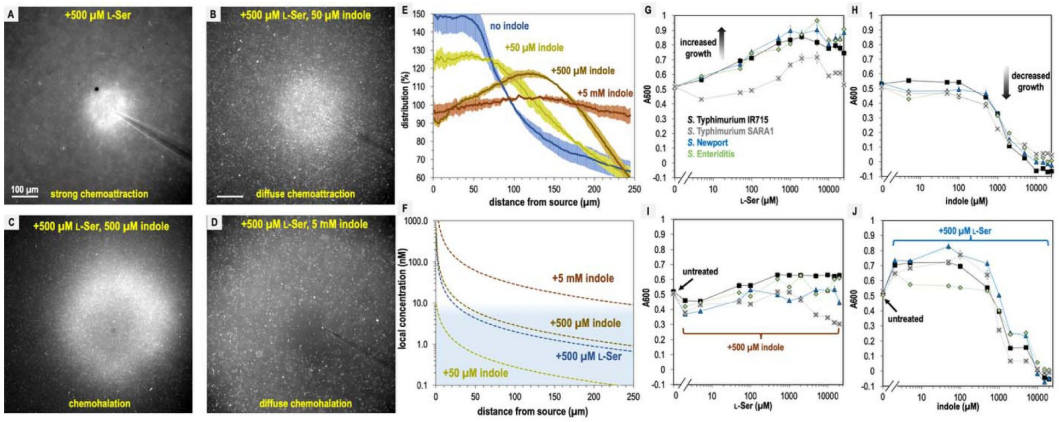


Fig. 7.

S. Typhimurium mediates distinct chemotactic responses based on the ratio of L-Ser to indole.

A-D. Representative max projections of responses to treatments of L-Ser and indole at 295-300 s, as indicated. E. Relative bacterial distribution in response to treatments of 500 µM L-Ser and varying amounts of indole, from panels A-D, with the mean value normalized to 100%. Data were collected at 30 °C. Data are means and error bars are standard error of the mean (SEM, n=3-5). F. Diffusion modeling of local effector concentrations based on sources of 5 mM indole (dark brown), 500 µM L-Ser (blue), 500 µM indole (light brown), and 50 µM indole (yellow) are shown as dashed lines. The approximate local concentration of indole that elicits a transition in chemotactic behavior is highlighted in light blue. G-H. Bacterial growth as a function of L-Ser or indole, at the time point where the untreated culture reaches A_{600} of 0.5. I-J. Bacterial growth +/- pretreatment with 500 µM indole or L-Ser, and increasing concentrations of indole or L-Ser, as indicated at the time point where the untreated culture reaches A_{600} of 0.5. Data are means and error bars are standard error of the mean (SEM, n=8-24). See also Movie 11.

The intermediate responses to opposing effector mixtures bear similarities to CIRA experiments with fecal material, some of which also exhibited a halo-like structure around the treatment source (**Fig. 3**, Movie 2, Movie 3). Previous studies have also observed responses that are an intermediate behavior between chemattraction and chemorepulsion, and have been referred to by a variety of names [9, 14, 15]. There exists no consensus descriptor for taxis of this nature, and so we suggest expanding the lexicon with the term "chemohalation," in reference to the halo formed by the cell population, and which is congruent with the commonly used terms chemoattraction and chemorepulsion. We expect chemohalation is a compromise between the chemoattraction driven by L-Ser and the chemorepulsion driven by indole. Across these experiments, the interior zone of avoidance roughly corresponds to where the local concentration of indole is calculated to exceed 10 nM (**Fig. 4E-F**).

L-Ser enables resilience to indole-mediated growth inhibition

We questioned why non-typhoidal *Salmonella* are attracted to a biological solution with high amounts of indole, a chemical reported to inhibit bacterial growth [17, 75, 76]. We examined how growth is affected by 0-25 mM indole or L-Ser in a background of minimal media (MM, Materials & Methods). As expected, increasing amounts of the nutrient L-Ser provide a growth advantage for all *Salmonella* strains analyzed, with maximal benefit achieved by approximately 500 μM (**Fig. 7G**). Equivalent treatments with indole show tolerance up to approximately 1 mM, with growth inhibition occurring in the 1-5 mM range and lethality occurring at indole concentrations greater than 5 mM (**Fig. 7H**). However, adding L-Ser in a background of 500 μM indole provides only a small growth enhancement (**Fig. 7I**), and addition of 500 μM L-Ser increases tolerance for indole up to about 1 mM, above which indole toxicity is unavoidable (**Fig. 7J**). It appears that the relative attraction to combinations of these effectors relates to their propensity to enhance or inhibit growth, with more permissive conditions eliciting a greater degree of chemoattraction. Overall, the bacteria still obtain growth benefits from L-Ser so long as the concentration of indole is under 1 mM.

Discussion

Bacteria in the human gastrointestinal tract encounter complex chemical landscapes that contain both chemoattractants and chemorepellents [4, 28, 36]. However, chemotaxis responses are often studied in isolation, outside of their biological and ecological contexts, which can lead to an over- or underestimation of the roles specific interactions play in natural settings. In the present work, we contribute to an emerging understanding of how bacteria navigate conflicting chemotaxis stimuli and relate these chemotactic compromises to enteric infection and pathogen growth [9, 15, 77].

In this study, we show that despite the microbial metabolite indole being a strong chemorepellent in isolation (**Fig. 6**), fecal indole is insufficient to elicit pathogen chemorepulsion, i.e. pathogens do not swim away from fecal material, or protect against cellular invasion in an explant model (**Fig. 1**, **Fig. 2**, **Fig. 3**, **Fig. 4**). Instead, it appears pathogens employ indole taxis as a means to regulate the magnitude of their attraction toward sources of intestinal nutrients (**Fig. 5**). *In vivo*, we expect that the bacteria are attracted to indole-rich fecal material, and it is simply a matter of the degree of attraction and which sites are prioritized among those accessible to the invading pathogen. This finding revises our understanding of indole taxis during enteric infection, suggesting that, rather than impairing pathogen infection as others have proposed [8, 23], indole chemorepulsion serves a useful function for pathogens and enables them to integrate information about local microbial competitors into their chemotaxis responses. This, in turn, allows pathogens to prioritize niches with abundant nutrients and reduced microbial competition.

Interpretations of explant infections and the functions of chemotaxis and Tsr

Our explant experiments can be thought of as testing whether a layer of effector solution is permissive to pathogen entry to the intestinal mucosa, and whether chemotaxis provides an advantage in transiting this chemical gradient to associate with, and invade, the tissue (**Fig. 1C**, **Fig. S5**). This behavior is probabilistic, and given sufficient time even chemotactic-deficient cells will contact the tissue. This is reflected in that all treatments showed substantial infection by all strains in terms of absolute CFUs isolated from homogenates (Fig. S2). If we compare the probability of chemotaxis-mediated transit of the effector gradients we tested, greatest is for fecal treatment, which among all treatments showed the highest degree of intracellular invasion (at 3 h post-infection, **Fig. 1**). Then, buffer, L-Ser, and L-Ser + indole treatments are similarly permissive and chemotaxis enhances infection in these backgrounds as well (**Fig. 1**, **Fig. S5**). That chemotaxis provides an advantage in the buffer treated background, without any added chemoeffector, is interesting and could simply be from effectors emitted from the host tissue (**Fig. S5**). For instance, there is evidence that intestinal tissue, and host cells more broadly, can release L-Ser and other amino acids, particularly in the context of tissue injury which could be caused by *Salmonella* epithelial invasion in these experiments [1, 36, 59, 78]. Altogether, chemotaxis enhances the transit of the effector gradients mentioned above to access the host tissue (**Fig. S5**).

The explant system offers new insights into whether indole is protective against pathogen infection. First, indole treatment does negate the infection advantage conferred by chemotaxis, which was a unique effect among the treatments we tested (**Fig. 1**, **Fig. S5**). This is an interesting result, somewhat mirroring what others have seen in cell culture [23], and indicates that the indole gradient does not increase the likelihood of transit for chemotactic cells. In assessing the total bacteria isolated from homogenates we see no evidence that indole protects against infection, since the bacteria counts are prevalent and similar to other treatments, though this could be different at lower multiplicities of infection (Fig. S2). Second, this effect is only observed with indole as the sole effector, but not when the same concentration of indole is present within fecal material, as it exists in the intestinal environment, or co-treatment with the fecal effector L-Ser (**Fig. 1**, **Fig. S5**). Thus, the loss of the chemotactic advantage observed with indole treatment is reversed in the presence of chemoattractant stimuli, suggesting that this effect is unlikely to occur *in vivo*. It is also worth noting that the residual effector concentrations experienced by the bacteria in the explant experiments were very low (**Fig. 1C**), and so we do not think the effects we see are due to impacts on bacterial growth. It is unclear whether there would ever be a situation *in vivo* where indole is the dominant effector, and so the behavior of bacteria swimming away from a source of pure indole may be somewhat artificial (**Fig. 6**). These data, overall, do not support indole chemorepulsion as a mechanism of colonization resistance against pathogens, although indole is known to reduce virulence through other mechanisms [8, 22, 23, 75].

New insights into indole taxis from non-E. coli systems

Indole is a key regulator of enteric microbial communities, known to modulate motility and virulence, and is highly abundant in fecal matter due to the metabolic activity of the microbiota [8, 16–18, 21–24]. While *E. coli* has served as an important model system for elucidating the mechanisms of indole chemorepulsion (Table S1) [8, 18, 23, 25], no prior work has examined how indole sensing is integrated alongside multiple other intestinal effectors, nor whether these behaviors are conserved across clinical isolates of disease-causing species. Here, we address these gaps by providing confirmatory evidence for some earlier predictions and evidence that challenges others, refining our understanding of how indole influences pathogen behavior through chemotaxis within the intestinal environment.

Perhaps our most striking finding is that fecal material, the native reservoir of the strong chemorepellent indole, does not elicit chemorepulsion in the form of bacteria swimming away (**Fig. 2**, **Fig. 3**, **Fig. 4**). Instead, a representative panel of diverse *Enterobacteriaceae* pathogens and pathobionts exhibited fecal attraction (**Fig. 3**, **Fig. 4**), demonstrating that enteric species associated with disease are undeterred by indole in its natural context, i.e., when mixed with other fecal chemoattractants. Through analyses showing that indole chemorepulsion is easily overridden by the presence of intestinal nutrients we surmise that the benefits associated with fecal material typically outweigh the deleterious effects of indole (**Fig. 5**, **Fig. 7**). This conclusion is further supported by growth analyses indicating that *Salmonella* tolerates indole when sufficient nutrients are available (**Fig. 7**).

We used *Salmonella* Typhimurium as a model to dissect the mechanisms underlying fecal attraction and indole sensing. Regarding the latter, our findings largely confirm previous studies in *E. coli*, showing that the chemoreceptor Tsr is required for indole taxis (**Fig. 6**, Table S1) [8, 18, 25]. However, we do add some new dimensions to understanding indole taxis. First, for *E. coli* the involvement of Tar in indole sensing has been reported [8], but we see no equivalent function for *S.* Typhimurium. Yet, we previously showed *S.* Typhimurium WT and *tsr* are both readily attracted to L-Asp, supporting the presence of a functional Tar under the same experimental conditions as we test here (**Fig. 6**) [36]. We do not know the reason for this outcome, but note different assays were used, and this could also reflect variation between the chemotaxis systems of these two bacteria. Second, while Tsr serves as the sensor for indole in *Salmonella*, it is also a key mediator of fecal attraction through its role in sensing L-Ser, which is also abundant in fecal material (**Fig. 1**, **Fig. 2**, **Fig. 3**, **Fig. 6**). Third, we are the first to visualize and quantify the rapid temporal dynamics of indole chemorepulsion (**Fig. 5**, **Fig. 6**, **Fig. S3I**; Movie 11). For responses to pure indole a clear zone of avoidance around the treatment appears within 10 seconds of exposure, much faster than chemoattraction to L-Ser, suggesting the cells have the ability to rapidly flee deleterious conditions (**Fig. 6**, **Fig. S3I**; Movie 11). Lastly, we also investigated whether indole sensing occurs through the canonical chemoreceptor mechanism of direct binding to the Tsr ligand-binding domain (LBD). Our data shows it does not, nor does indole antagonize or inhibit L-Ser binding to the LBD (**Fig. 6**). While these findings do not resolve the molecular mechanism of indole sensing, they eliminate two plausible models that, to our knowledge, have not been previously tested. Overall, our data support the hypothesis that Tsr employs a non-canonical mechanism to sense indole [8, 18, 74].

Having confirmed the role of Tsr in mediating indole chemorepulsion in *S.* Typhimurium, and shown it to function similarly as in *E. coli* [8, 9, 14], and having demonstrated that diverse *Enterobacteriaceae* are attracted to indole-rich fecal material, we expect that the behaviors described here are representative of the many enteric species that possess Tsr orthologues, which we mapped in a previous study [36]. As we report here, there does seem to be a large variety in the magnitude of fecal attraction by different 'wild' enteric pathogens, which could reflect adaptations to different host intestinal environments and microbiota communities and may influence pathogenesis (**Fig. 3**, **Fig. 4**). While foundational insights into indole taxis have come from model bacterial systems, continued progress in understanding the role of chemotaxis in human disease will benefit from extending such analyses to a broader range of clinically relevant bacterial species and strains.

Function of indole taxis in enteric invasion

In the context of non-typhoidal *Salmonella* infections, it is clear there exists complex relationships between chemotactic sensing of effectors, bacterial growth, and invasion (**Fig. S5**). In addition to the factors we have investigated, it is already well-established in the literature that the vast metabolome in the gut contains many chemicals that modulate *Salmonella* cellular invasion, virulence, growth, and pathogenicity [79–81]. As it pertains specifically to sensing the opposing effectors L-Ser and indole, we propose that Tsr directs bacteria toward the highest ratio of attractant to repellent accessible in the local environment, with fine-tuning of navigation

occurring through regulation of the magnitude of attraction and chemohalation. In addition to sensing these two effectors, our data indicate that fecal attraction involves other stimuli, including L-Asp sensing through Tar and sugar sensing through Trg (**Fig. 5**). Ultimately, the dual sensing of opposing effectors by Tsr serves to improve pathogen fitness through colonizing niches rich in nutrients, signaled by local L-Ser concentrations, and seeking niches with low microbial competition, indicated by local indole concentrations.

Navigating contradictory stimuli in nature

The scenario we investigated here of *S.* Typhimurium encountering high concentrations of opposing chemotactic stimuli in the intestinal environment is just one example of the complex chemical landscapes that bacteria navigate in nature. To better understand the "decision-making" process underlying chemotaxis in the presence of conflicting effectors, we examined physiological mixtures of the fecal metabolites indole and L-Ser and recorded a series of real-time videos capturing behavioral transitions as a function of effector concentration (**Fig. 7**; Movie 11). These videos reveal that, upon sensing conflicting stimuli, the bacterial population structure rapidly evolves based on the attractant-to-repellent ratio, displaying a spectrum of behaviors: chemoattraction, diffuse chemoattraction, chemohalation, diffuse chemohalation, and chemorepulsion (**Fig. 7**; Movie 11).

These dynamic, micron-scale chemohalation patterns reflect a behavioral compromise between attraction and repulsion and would be difficult or impossible to detect without live imaging, which may explain why they were previously unappreciated in binary models of chemotaxis [11]. In fact, many chemotaxis assays that use indirect methods of quantification, such as growth, would not be able to distinguish between chemoattraction and chemohalation since they both involve in increase in bacteria overt time. To be clear, we suggest chemohalation as a new term to generally describe intermediate chemotaxis responses to conflicting stimuli that are neither chemoattraction nor chemorepulsion, but others have contributed to studying how chemotaxis functions in confounding chemical landscapes [9, 14, 15]. The chemohalation responses reported here most closely resemble the "trade-off" response previously described in *E. coli* exposed to attractant–repellent mixtures [9]. Interestingly, that study also described a "bet-hedging" response, in which a subpopulation remained attracted despite the presence of a chemorepellent, but we did not observe this behavior in our system [9].

In addition to our work here, there are other examples of chemohalation responses to complex biological stimuli of the gastrointestinal environment. Recently, we reported on *Enterobacteriaceae* chemotactic sensing of blood serum, which bacteria encounter during intestinal bleeding events, and those responses appear to involve chemohalation [36]. Chemohalation is also seen in the case of the gastric pathogen *Helicobacter pylori* responding to mixtures of urea, a chemoattractant, and acid, a chemorepellent, conflicting effectors it encounters near the stomach mucosa [15, 82]. The functional significance of chemohalation remains to be understood, but could be a method of fine-tuning colonization bias such that nutrients can be acquired while not approaching too closely to a deleterious stimulus. Continuing to investigate chemohalation behaviors and understanding how they coordinate bacterial colonization may provide important insights into how chemotaxis confers fitness advantages in natural environments.

Limitations of this study

This study provides insights into the roles of chemotaxis in directing the behaviors of *Enterobacteriaceae* species in response to fecal material and indole, however, several limitations should be considered. First, our analyses of pathogen enteric infection were performed using swine colonic explants, which do not fully recapitulate the complexity of *in vivo* infection dynamics in the human gut. While explant assays offered insights into the relationship between chemotaxis and tissue colonization, these experiments exhibited variability. To mitigate this, we used multiple tissue sections from a single animal to improve experimental consistency. However,

this approach limits our ability to assess how inter-host variability might influence bacterial responses. Future studies using distal ileum tissue, a major site of *S.* Typhimurium cellular invasion known to contain distinct chemical features, may provide further insight into the functions of indole taxis during infection [42]. Another experimental limitation is the difference in timescales between our assays. Chemotaxis experiments were conducted over approximately 5-minutes, whereas tissue explant experiments required several hours for significant differences in colonization and cellular invasion to be observed. Thus, there are effects from chemotactic adaptation and replication that we do not elucidate here. Lastly, while we confirmed that non-typhoidal *Salmonella* are attracted to human fecal material, we only determined the dependency on Tsr, and sensing of L-Ser, in our model strain (IR715). Although we predict that *Enterobacteriaceae* also use Tsr for fecal attraction, this remains uncertain without targeted genetic analyses in each strain background.

Materials & Methods

All methods were carried out in accordance with relevant guidelines, regulations, and state and federal law. Experimental protocols were approved by the Institutional Biosafety Committee (IBC) of Washington State University (#1372).

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. As previously described [36], bacteria intended for chemotaxis assays were grown overnight in tryptone broth (TB) with antibiotic selection, as appropriate. Motile bacteria were prepared with a 1:1000 back-dilution and grown shaking for approximately 4 hours at 37° C to reach A_{600} of 0.5. Cells were centrifuged, washed, and resuspended in a chemotaxis buffer (CB) containing 10 mM potassium phosphate (pH 7), 10 mM sodium lactate, and 100 μM EDTA to A_{600} of 0.2 and rocked gently at the temperatures indicated in figure legends until fully motile, typically 1-2 h. For *in vitro* growth analyses, cultures were grown overnight in Lysogeny Broth (LB) at 37° C. Subsequently, 5 μl of A_{600} 2.0 cells were used to inoculate 200 μl of minimal media (MM), containing 47 mM Na_2HPO_4, 22 mM KH_2PO_4, 8 mM NaCl, 2 mM $MgSO_4$, 0.4% glucose (w/v) 11.35 mM $(NH_4)_2SO_4$, 100 μM $CaCl_2$ and L-Ser and/or indole at the described concentrations, and cultured in a 96-well microtiter plate. Cultures were grown at 37° C and monitored by A_{600} readings at 5-minute intervals.

Chemosensory injection rig assay (CIRA)

CIRA was performed as described previously [36]. Briefly, an Eppendorf Femtotip 2 microcapillary containing the treatment of interest was lowered into a pond of 50 μl of motile cells using a Sutter micromanipulator. An injection flow of effector into the pond at approximately 300 fl per minute was achieved using a Femtojet 4i set to P_c 35. Solubilized fecal treatments were prepared by dissolving 1 g of commercially obtained human feces (Lee Biosolutions) in 10 ml of CB. The solution was clarified by centrifugation at 10,000 g for 20 minutes, followed by sterile filtration through a 0.2 μm filter. Treatment solutions of indole and L-Ser were also diluted into CB and sterile-filtered before application. Videos were recorded using an inverted Nikon Ti2 microscope with heated sample chamber at 37 °C.

CIRA microgradient modeling

Modeling the microgradient generated through CIRA was performed as described earlier [36], based on the continual injection and diffusion of an effector from a fixed-point source. Briefly, diffusion is modeled as a 3D process where the diffusing substance is gradually and continuously

introduced at a fixed point within a large surrounding fluid volume. The substance is prepared at a concentration of M_S (typically between 0.5 µM and 5 mM) and injected at a volume rate of Q = 305.5 fl/min. The species then diffuses into the ambient fluid with a diffusion constant D:

$$C(r,t) = \frac{q}{4\pi Dr} erfc \frac{r}{2\sqrt{Dt}}$$

Here, r is the distance from the point source, t is the time from initial injections, q is the injection rate of the species (equal to M_sQ), and C is the species concentration. In earlier work [36], we reported using fluorescent dye that the concentrations predicted by this model appear to be accurate within 5% in the range of 70-270 µm from the source, whereas at distances less than 70 µm the measured concentrations are about 10% lower than predicted. At the point where the effector treatment is injected into the larger volume the local concentration drops precipitously, hence why the concentration reported at distance 0 is not that of the concentration within the microcapillary.

Isothermal titration calorimetry ligand binding studies (ITC)

Purification of *S.* Typhimurium Tsr LBD was performed as described previously [36]. ITC experiments were performed using a Microcal ITC200 instrument (GE Healthcare). Either 500 µM indole or L-Ser was titrated in 2.5 µL injections into a 200 µL sample cell containing 50 µM Tsr LBD. For the indole/L-Ser competition experiment, 500 µM indole was added to both the titrant and sample cell, thus providing a constant excess background concentration of indole. For all experimental conditions, blank titrations were also collected in which indole or L-Ser was titrated into a cell containing buffer alone. All experiments were performed using thoroughly degassed samples at 25 °C in 50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.5. The reference power was set to 5 µcal/sec. The resulting power curves were integrated using the Origin analysis software included with the instrument. The heat of dilution was subtracted from each point using the blank. A single-site binding model was then fit to the data, floating parameters describing the binding enthalpy (ΔH), equilibrium constant (K_D), and apparent binding stoichiometry (n). The instrument software was used for this purpose.

Quantification of indole and serine in human fecal samples

Solubilized human feces was prepared as described above for CIRA and analyzed by mass spectrometry to determine the molar serine content as a service through the University of Washington Mass Spectrometry Center. This measurement reflects total serine, of which close to 100% is expected to be L-Ser [36]. As described in earlier work, the indole content of solubilized human fecal samples was determined using a hydroxylamine-based calorimetric assay with purified indole as a reference and standard [83].

Explant infection assays

Swine intestinal tissue was acquired from the descending colon of an 8-week-old animal, pursuant to animal protocol ASAF #7128, approved through the Washington State University IACUC. Before infection, an approximately 20 by 20 mm piece of swine intestinal explant tissue was gently washed with PBS to remove fecal matter. Next, the tissue section was bathed in chemoeffector solution (solubilized human fecal matter (Lee Biosolutions), a mixture of 338 µM L-Ser and 862 µM indole, 338 µM L-Ser alone, 862 µM indole alone, or chemotaxis buffer) in a 6-well tissue culture plate (Celltreat) and incubated at 4° C for 1 h. Then, tissue was transferred to a 35 mm Mattek dish where the luminal side of the tissue was exposed to a bacterial solution containing a 1:1 mixture (~10^9 CFU each) of WT *S.* Typhimurium IR715 and either the isogenic *tsr* or *cheY* mutant, suspended in CB at a volume of 300 µl. The tissue was then incubated in the dish with the competing bacteria at 37 °C and 5% CO_2 for 1, 3, or 6 h. After, half of the tissue was transferred into screwcap tubes containing 500 µl LB media and 5-10 2.3 mm zirconia beads (BioSpec Products) on ice and homogenized using a Bead Mill 24 (Fisher Scientific) at 6.5 m/s for 60 s, repeated four

times. To enumerate the "invaded" bacteria, the other half of the tissue was washed in PBS and incubated in PBS containing 100 μg/ml gentamicin for 1 h at 37 °C and 5% CO_2, then washed twice in PBS, as done previously [56], [84], [85]. The homogenization process was then repeated for the gentamicin-treated tissue. CFUs were enumerated by plating 10-fold dilutions on LB agar plates containing the appropriate antibiotic [56], [86]. Competitive index values were calculated by dividing the number of mutant CFUs by the number of WT CFUs for each treatment and time point [87], [88].

Quantification of CIRA data

Videos of chemotactic responses were quantified as described previously [36]. The number of cells in each frame were calculated by determining a fluorescence intensity ratio per cell for frames pre-treatment and extrapolated using the 'plot profile' function of ImageJ. The distribution of the bacteria was calculated using the Radial Profile ImageJ plugin. Local background subtraction was performed based on experiments with the non-chemotactic *cheY* strain to control for autofluorescence in solubilized fecal samples.

Statistical Analyses

Competitive indices (CIs) for explant experiments were calculated for each treatment group at each time point. Log-transformed CI values were obtained by taking the logarithm ($\log_{10}$) of the original CI measurements. These log-transformed values were then subjected to statistical analysis. First, a one-sample t-test was performed to determine whether the mean of the log-transformed CIs significantly differed from zero. In cases where the assumption of normality was violated, the non-parametric Wilcoxon rank sum test was applied as an alternative. Effect size was assessed using Cohen's *d* and calculated using the same log-transformed CIs. To determine p-values between total and invaded populations at 3 and 6 h and for comparing relative bacteria % within 500 μm of the treatment source in **Figs. 5**-**6**, unpaired t-tests were employed.

Supplemental Information

Species, Strain(s)	Indole Treatment [a]	Reported Response [b]	Experimental Method(s)	Chemotaxis & Motility Proteins Involved in Response	Ref.
E. coli, strain RP437	≤1 mM >1 mM	chemorepulsion chemoattraction	tethered cell assay [c]	Tsr, Tar	[8]
E. coli, strain MG1655	1 mM ± 0.5-0.1 mM L-Ser	"bet-hedging" [d]	diffusion-based assay into long channel with fluorescent imaging	Tar, Tsr	[9]
E. coli, strain RP437	500 µM	chemorepulsion, overridden in presence of 500 µM Autoinducer-2	flow-based microfluidic chemotaxis device coupled to a gradient generator	Tar, Tsr	[14]
E. coli, strain RP437	≤1 mM >1 mM	decreased motility slower flagellar rotation	tethered cell assay [c]	Non-CheY dependent	[18]
E. coli, strain O157:H7 CDC EDL933	500 µM	chemorepulsion	agarose plug chemotaxis assay imaged every 5 minutes for 30 minutes	MotB and FliD	[25]
S. Typhimurium, strain ATCC14028s	1 mM	decreased motility	swimming motility agar plates [c]	MotA	[23]
S. Typhimurium, strain ATCC14028s	1 mM	decreased motility	swimming motility agar plates [c]	FlhC	[24]

[a] Source concentration, or ranges, used in experiment.

[b] The motility or chemotactic response as reported by the study authors; note that some methods employed may not be able to distinguish between responses as a consequence bacterial growth versus chemotaxis.

[c] The assay may have limitations in its ability to report on either rapid temporal responses, or localization to or from an effector source.

[d] In this work, we refer to behaviors of this type as "chemohalation" to be similar to the widely-used terms chemoattraction and chemorepulsion.

Table S1.

Summary of prior studies related to indole chemotaxis.

Data S1. Enumeration of colony-forming units (CFUs) from explant studies.

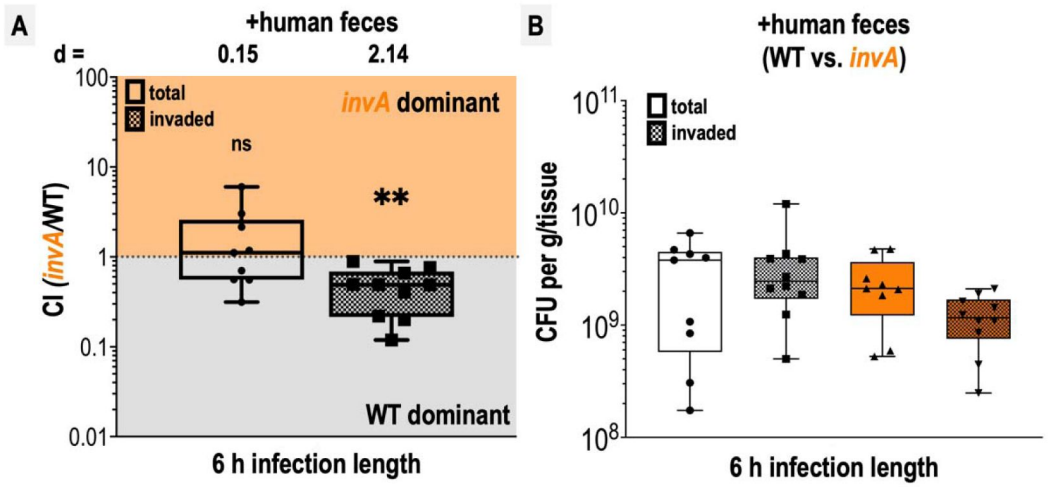


Fig. S1.

Competition of WT and *invA* in explant infections.

A. Competitive indices (CI) of colony-forming units (CFUs) recovered from co-infected swine explant tissue, either from the total homogenate (total, open box and whiskers plots), or from tissue washed with gentamicin to kill extracellular and attached cells (invaded, checkered box and whisker plots), as indicated. Each data point represents a single experiment and the CI of CFUs recovered from that tissue (n=9-10). Boxes show median values (line) and upper and lower quartiles, and whiskers show max and min values. Effect size (Cohen's *d*) and statistical significance are noted for each experiment in relation to competitive advantage, i.e. deviation from a CI of 1 (not significant, ns; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). B. Disaggregated CFU enumerations for each experimental group prior to CI calculation.

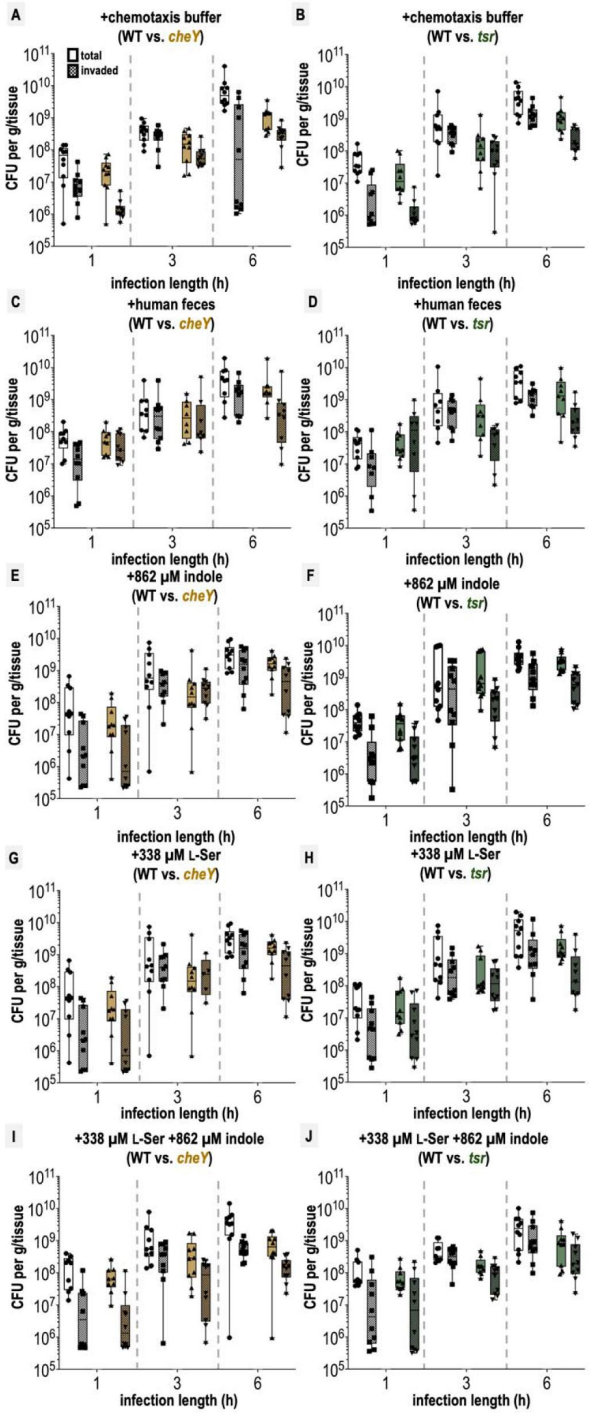


Fig. S2.

Colony-forming units (CFU) recovered from swine colonic explant infections.

Shown are disaggregated CFU enumerations for each co-infection experiment with *S.* Typhimurium IR715 WT and mutant strains following tissue treatments, as indicated. CFUs extracted either using the entire tissue homogenate (total) or extracted from tissue treated with a gentamicin wash to kill exterior non-invaded cells (invaded) are noted separately (see Materials & Methods). Each data point represents CFUs recovered from a single explant experiment (n=8-10). Box and whisker plots represent the sample median (line), the edges are the upper and lower quartiles, and whiskers extend to the max and min values. The limit of detection was 1 x 10^5 CFUs, and experiments at or below this limit are not included here. See Data S1 for numeric values.

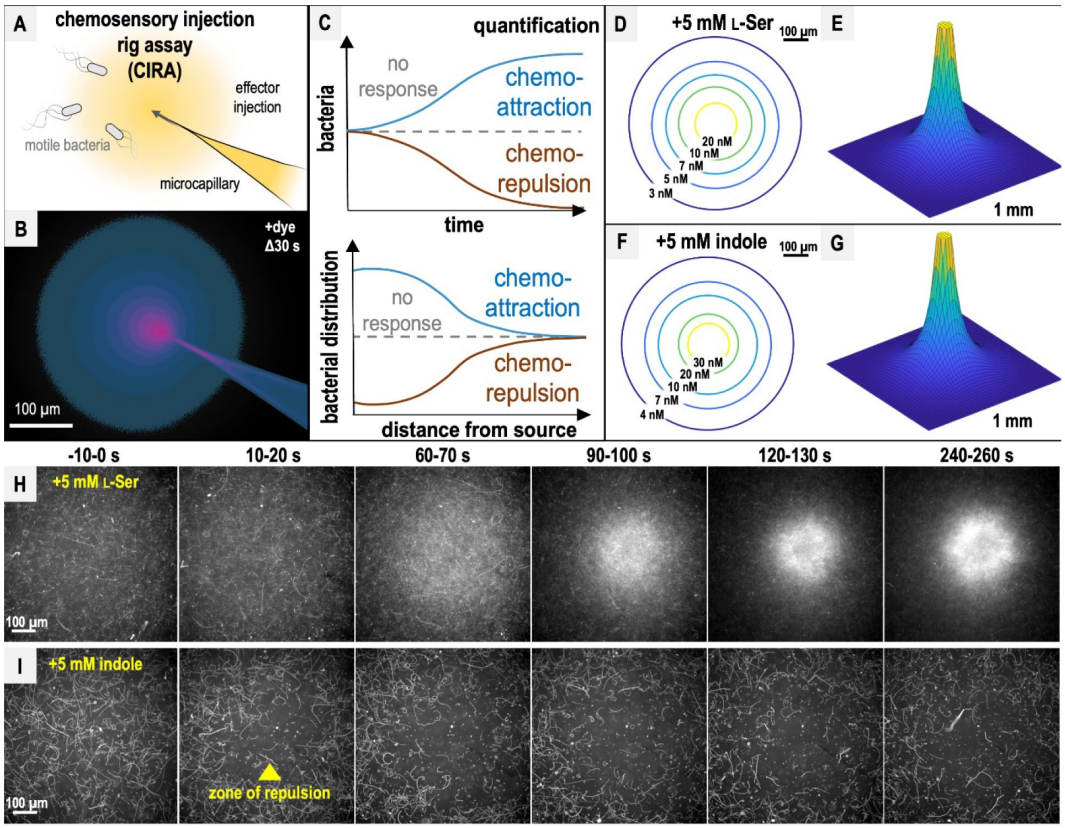


Fig. S3.

CIRA design, diffusion modeling, and responses to L-Ser or indole.

A. CIRA experimental design. B. CIRA microgradient diffusion model, simulated with a source of 1.13 mM A488 dye after 30 s of injection. Image rendered on a pink (100% source concentration)-blue-black (0%) color intensity scale, based on previous work [36]. C. CIRA quantification overview, with blue lines corresponding to chemoattraction, null chemotactic response corresponding to gray dashed lines, and chemorepulsion corresponding to brown lines. D-G. Microgradient models of the local effector concentration for treatments with 5 mM L-Ser or indole, respectively, at Δ300 s. Color gradient corresponds to gradually decreasing effector concentrations from yellow to blue. H-I. Representative 10 s max projections of *S.* Typhimurium IR715 response to sources of L-Ser (strong chemoattraction) or indole (strong chemorepulsion) using CIRA. The glass microcapillary that injects the treatment solution is centered within the field of view. Note that cells contain a plasmid expressing mPlum (see Materials & Methods), and fluorescence data are collected in the far-red channel, so the glass microcapillary is poorly visible.

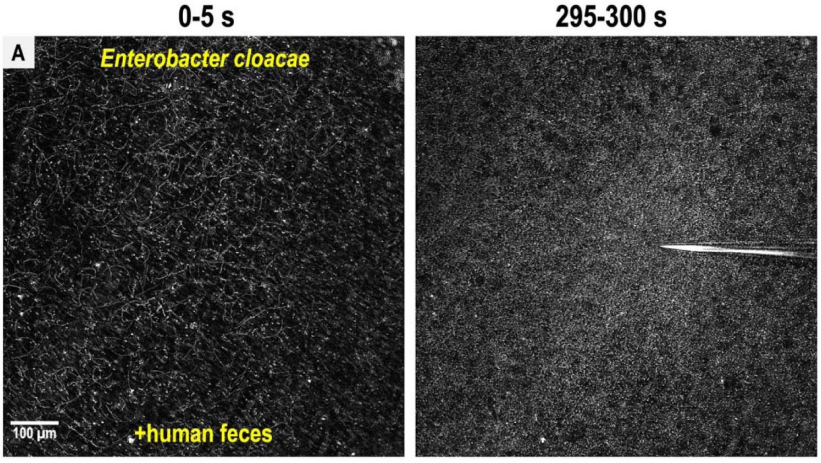


Fig. S4.

***Enterobacter cloacae* exhibits fecal attraction.**

A. Shown are max projections over 5 s from a CIRA experiment from time -5 s before fecal treatment and after 5 minutes of treatment with liquid human feces. Data were collected using phase-contrast.

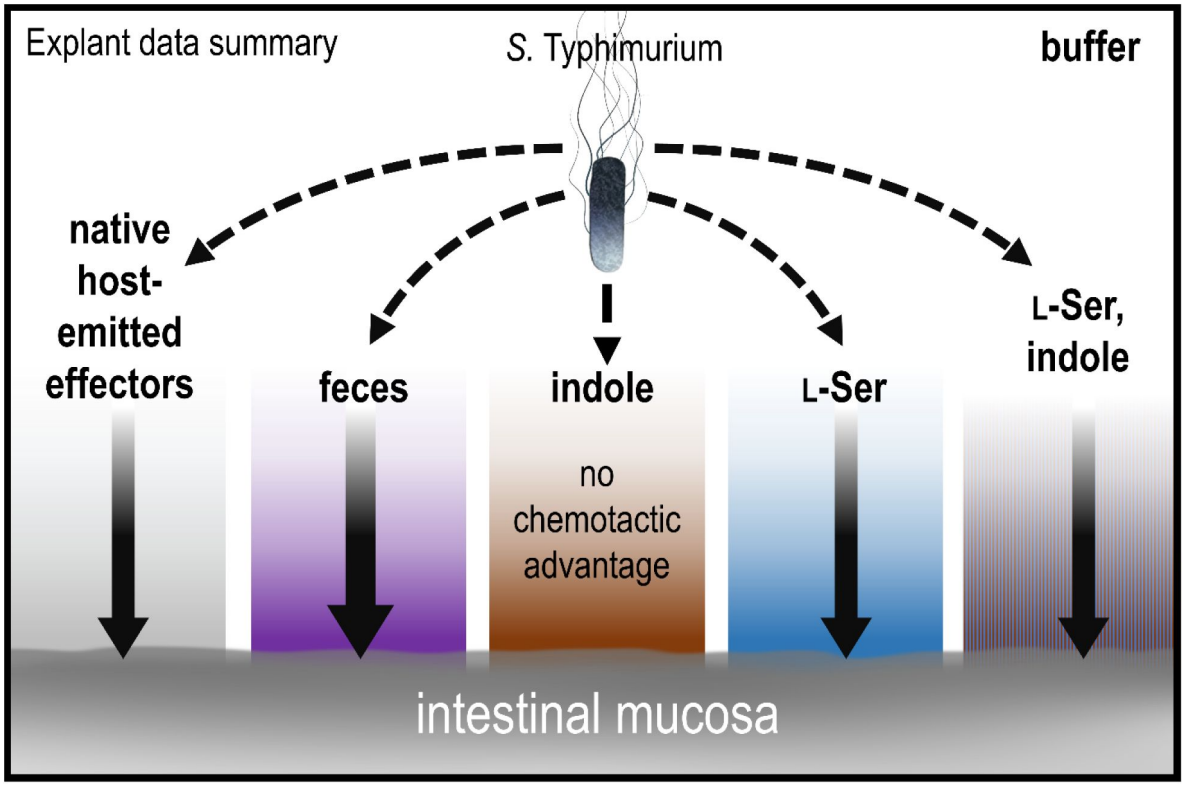


Fig. S5.

Model and summary for explant infection data.

Based on analyses in this study, we provide this summary of the role of chemotaxis in mediating infection advantages for different tissue pretreatments. The strength of chemotactic advantage for transiting each chemical gradient and accessing the host tissue is indicated by the width of the solid black arrows. The baseline level of chemotactic advantage seen in buffer treatments may be from effectors emitted from the host tissue (gray gradient). Other gradients containing fecal chemoattractants show a similar level of chemotactic advantage, with the highest being for fecal treatment (purple). Only soaking the tissue in pure indole results in a chemical gradient for which chemotaxis and Tsr do not provide an infection advantage. Note that bacteria are exposed only to low concentrations of residual effectors that remain after the tissue is soaked and then transferred to 300 µl of buffer for infection; they are not immersed in the more concentrated effector solution. See also **Fig. 1**, Data S1, and Fig. S2.

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Additional information

Author Contributions

K.F. performed the microgradient modeling and explant experiments. A.B. conducted the CIRA experiments and purification of Tsr-LBD. Z.G. performed bacterial growth experiments. M.S. and M.J.H. performed the ITC experiments. All authors contributed to data analyses and writing of the manuscript.

Movie figure legends

Movie 1. Chemotactic response of *S.* Typhimurium IR715 to solubilized human feces. Representative CIRA experiments showing *S.* Typhimurium IR715 WT and mutant strains responding to a source over 300 s (shown at 10x speed) Viewable at: *https://www.youtube.com/watch?v=BqUcRN3YwjU*

Movie 2. Chemotactic response of *S.* Typhimurium IR715 WT and chemotactic mutant strains to solubilized human feces. Representative CIRA experiments showing competition between *S.* Typhimurium IR715 (mPlum) and *cheY*, or *tsr*, as indicated (GFP), over 300 s. Viewable at: *https://www.youtube.com/watch?v=D5JL46b4lsI*

Movie 3. Chemotactic response of *S. enterica* clinical isolates to solubilized human feces. Representative CIRA experiments showing competition between *S.* Typhimurium IR715 (mPlum) and clinical isolates, as indicated (GFP), responding to a source of solubilized human feces over 300 s. Viewable at: *https://www.youtube.com/watch?v=dLsFDV0XgpY*

Movie 4. Chemotactic response of *E. coli* MG1655 to solubilized human feces. Representative CIRA experiment over 300 s. Viewable at: *https://youtube.com/shorts/WH6tabDbrw4?feature=share*

Movie 5. Chemotactic response of *E. coli* NCTC 9001 to solubilized human feces. Representative CIRA experiment over 300 s. Viewable at: *https://youtube.com/shorts/yzU2M4Z_Yf4?feature=share*

Movie 6. Chemotactic response of *C. koseri* 4225-83 to solubilized human feces. Representative CIRA experiments with treatment sources as indicated, over 300 s. Viewable at: *https://youtube.com/shorts/s_ybO0xcIDw?feature=share*

Movie 7. Chemotactic response of *S.* Typhimurium IR715 to L-Ser and indole treatment at fecal-relevant concentrations. Representative CIRA experiment over 300 s. Viewable at: *https://youtube.com/shorts/4UEYoBS6jIQ?feature=share*

Movie 8. Chemotactic response of *S.* Typhimurium IR715 to complete mixture of fecal effectors. Representative CIRA experiment over 300 s. Viewable at: *https://youtube.com/shorts/Yd14m3sI6Pw?feature=share*

Movie 9. Chemotactic response of *S.* Typhimurium IR715 to mixture of fecal effectors lacking L-Ser. Representative CIRA experiment over 300 s. Viewable at: *https://youtu.be/5QM116BrHhQ*

Movie 10. Chemotactic response of *S.* Typhimurium IR715 to mixture of fecal effectors with 0.5x indole. Representative CIRA experiment over 300 s. Viewable at: *https://youtube.com/shorts/OqH0HE2rYIE?feature=share*

Movie 11. Chemotactic response of *S.* Typhimurium IR715 to L-Ser and indole treatments. Representative CIRA experiments with treatment sources as indicated, over 300 s. Viewable at: *https://www.youtube.com/watch?v=bNQMqF2QMek*

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Additional files

Data S1

References

1. Zhou B., Szymanski C. M., Baylink A (2023) **Bacterial chemotaxis in human diseases** *Trends in Microbiology* **31**:453–467 Google Scholar

2. Keegstra J. M., Carrara F., Stocker R (2022) **The ecological roles of bacterial chemotaxis** *Nature Reviews Microbiology* https://doi.org/10.1038/s41579-022-00709-w | Google Scholar

3. Bi S., Sourjik V (2018) **Stimulus sensing and signal processing in bacterial chemotaxis** *Current Opinion in Microbiology* **45**:22–29 Google Scholar

4. Matilla M. A., Krell T (2018) **The effect of bacterial chemotaxis on host infection and pathogenicity** *FEMS Microbiology Reviews* **42** Google Scholar

5. Matilla M. A., Gavira J. A., Krell T (2023) **Accessing nutrients as the primary benefit arising from chemotaxis** *Current Opinion in Microbiology* **75**:102358 Google Scholar

6. Matilla M. A., Krell T. (2017) **Chemoreceptor-based signal sensing** *Current Opinion in Biotechnology* **45**:8–14 Google Scholar

7. Ortega Á., Zhulin I. B., Krell T (2017) **Sensory Repertoire of Bacterial Chemoreceptors** *Microbiol Mol Biol Rev* **81**:e00033–17 Google Scholar

8. Yang J., et al. (2020) **Biphasic chemotaxis of Escherichia coli to the microbiota metabolite indole** *Proc. Natl. Acad. Sci. U.S.A* **117**:6114–6120 Google Scholar

9. Livne N., Vaknin A (2022) **Collective responses of bacteria to a local source of conflicting effectors** *Sci Rep* **12**:4928 Google Scholar

10. Keller E. F., Segel L. A (1971) **Traveling bands of chemotactic bacteria: A theoretical analysis** *Journal of Theoretical Biology* **30**:235–248 Google Scholar

11. Adler J., Tso W.-W (1974) **'Decision'-Making in Bacteria: Chemotactic Response of Escherichia coli to Conflicting Stimuli** *Science* **184**:1292–1294 Google Scholar

12. Zhang X., et al. (2019) **Escape band in Escherichia coli chemotaxis in opposing attractant and nutrient gradients** *Proc Natl Acad Sci U S A* **116**:2253–2258 Google Scholar

13. Kalinin Y., Neumann S., Sourjik V., Wu M (2010) **Responses of Escherichia coli Bacteria to Two Opposing Chemoattractant Gradients Depend on the Chemoreceptor Ratio** *Journal of Bacteriology* **192**:1796 Google Scholar

14. Englert D. L., Manson M. D., Jayaraman A (2009) **Flow-Based Microfluidic Device for Quantifying Bacterial Chemotaxis in Stable, Competing Gradients** *Appl Environ Microbiol* **75**:4557–4564 Google Scholar

15. Huang J. Y., Sweeney E. G., Guillemin K., Amieva M. R (2017) **Multiple Acid Sensors Control Helicobacter pylori Colonization of the Stomach** *PLOS Pathogens* **13**:e1006118 Google Scholar

16. Li X., Zhang B., Hu Y., Zhao Y (2021) **New Insights Into Gut-Bacteria-Derived Indole and Its Derivatives in Intestinal and Liver Diseases** *Front. Pharmacol* **12**:769501 Google Scholar

17. Lee J.-H., Lee J (2010) **Indole as an intercellular signal in microbial communities** *FEMS Microbiol Rev* **34**:426–444 Google Scholar

18. Gupta R., et al. (2022) **Indole modulates cooperative protein–protein interactions in the flagellar motor** *PNAS Nexus* **1**:pgac035 Google Scholar

19. Darkoh C., Chappell C., Gonzales C., Okhuysen P (2015) **A Rapid and Specific Method for the Detection of Indole in Complex Biological Samples** *Appl Environ Microbiol* **81**:8093–8097 Google Scholar

20. Chappell C. L., et al. (2016) **Fecal Indole as a Biomarker of Susceptibility to Cryptosporidium Infection** *Infect Immun* **84**:2299–2306 Google Scholar

21. Ye X., et al. (2022) **Dual Role of Indoles Derived From Intestinal Microbiota on Human Health** *Front. Immunol* **13** Google Scholar

22. Kumar A., Sperandio V (2019) **Indole Signaling at the Host-Microbiota-Pathogen Interface** *mBio* **10**:e01031–19 Google Scholar

23. Kohli N., et al. (2018) **The microbiota metabolite indole inhibits Salmonella virulence: Involvement of the PhoPQ two-component system** *PLOS One* **13**:e0190613 Google Scholar

24. Nikaido E., et al. (2012) **Effects of indole on drug resistance and virulence of Salmonella enterica serovar Typhimurium revealed by genome-wide analyses** *Gut Pathog* **4** Google Scholar

25. Bansal T., et al. (2007) **Differential Effects of Epinephrine, Norepinephrine, and Indole on Escherichia coli O157:H7 Chemotaxis, Colonization, and Gene Expression** *Infect Immun* **75**:4597–4607 Google Scholar

26. Bai X., et al. (2023) **Engineering the gut microbiome** *Nat Rev Bioeng* **1**:665–679 Google Scholar

27. Wolter M., et al. (2021) **Leveraging diet to engineer the gut microbiome** *Nat Rev Gastroenterol Hepatol* **18**:885–902 Google Scholar

28. (no date) **Human Metabolome Database** https://hmdb.ca/

29. Rogers A. W. L., Tsolis R. M., Bäumler A. J (2020) **Salmonella versus the Microbiome** *Microbiology and Molecular Biology Reviews* **85**:e00027–19 Google Scholar

30. Rivera-Chávez F., et al. (2013) **Salmonella Uses Energy Taxis to Benefit from Intestinal Inflammation** *PLoS Pathog* **9**:e1003267 Google Scholar

31. Rivera-Chávez F., et al. (2016) **Energy Taxis toward Host-Derived Nitrate Supports a Salmonella Pathogenicity Island 1-Independent Mechanism of Invasion** *mBio* **7**:e00960–16 Google Scholar

32. Gül E., et al. (2024) **Salmonella T3SS-2 virulence enhances gut-luminal colonization by enabling chemotaxis-dependent exploitation of intestinal inflammation** *Cell Rep* **43**:113925 Google Scholar

33. Winter S. E., Lopez C. A., Bäumler A. J (2013) **The dynamics of gut-associated microbial communities during inflammation** *EMBO Reports* **14**:319–327 Google Scholar

34. Winter S. E., et al. (2010) **Gut inflammation provides a respiratory electron acceptor for Salmonella** *Nature* **467**:426–429 Google Scholar

35. Cooper K. G., et al. (2021) **Regulatory protein HilD stimulates Salmonella Typhimurium invasiveness by promoting smooth swimming via the methyl-accepting chemotaxis protein McpC** *Nat Commun* **12**:348 Google Scholar

36. Glenn S. J., et al. (2024) **Bacterial vampirism mediated through taxis to serum** *eLife* **12**:RP93178 https://doi.org/10.7554/eLife.93178 | Google Scholar

37. Piñas G. E., DeSantis M. D., Cassidy C. K., Parkinson J. S (2022) **Hexameric rings of the scaffolding protein CheW enhance response sensitivity and cooperativity in Escherichia coli chemoreceptor arrays** *Science Signaling* **15**:eabj1737 Google Scholar

38. Burt A., et al. (2020) **Complete structure of the chemosensory array core signalling unit in an E. coli minicell strain** *Nat Commun* **11**:743 Google Scholar

39. Chen X., Bi S., Ma X., Sourjik V., Lai L (2022) **Discovery of a New Chemoeffector for Escherichia coli Chemoreceptor Tsr and Identification of a Molecular Mechanism of Repellent Sensing** *ACS Bio Med Chem Au* **2**:386–394 Google Scholar

40. Vega N. M., Allison K. R., Samuels A. N., Klempner M. S., Collins J. J. (2013) **Salmonella typhimurium intercepts Escherichia coli signaling to enhance antibiotic tolerance** *Proc. Natl. Acad. Sci. U.S.A* **110**:14420–14425 Google Scholar

41. Roager H. M., Licht T. R (2018) **Microbial tryptophan catabolites in health and disease** *Nat Commun* **9**:3294 Google Scholar

42. Chowdhury R., Pavinski Bitar P. D., Chapman H. M., Altier C (2023) **Salmonella Invasion Is Controlled by Competition among Intestinal Chemical Signals** *mBio* **14**:e00012–23 Google Scholar

43. Ehrhardt K., Grassl G. A (2022) **Mouse Model to Study Salmonella-Induced Colitis** *Methods Mol Biol* **2427**:201–213 Google Scholar

44. Grassl G. A., Valdez Y., Bergstrom K. S. B., Vallance B. A., Finlay B. B (2008) **Chronic enteric salmonella infection in mice leads to severe and persistent intestinal fibrosis** *Gastroenterology* **134**:768–780 Google Scholar

45. Radlinski L. C., et al. (2024) **Salmonella virulence factors induce amino acid malabsorption in the ileum to promote ecosystem invasion of the large intestine** *Proceedings of the National Academy of Sciences* **121**:e2417232121 Google Scholar

46. Mandal B. K., Mani V (1976) **Colonic involvement in salmonellosis** *The Lancet* **307**:887–888 Google Scholar

47. Kronsteiner B., et al. (2013) **Helicobacter pylori Infection in a Pig Model Is Dominated by Th1 and Cytotoxic CD8 + T Cell Responses** *Infect Immun* **81**:3803–3813 Google Scholar

48. Smith A. C., Swindle M. M (2006) **Preparation of Swine for the Laboratory** *ILAR Journal* **47**:358–363 Google Scholar

49. Poutahidis T., et al. (2001) **Helicobacter pylori -induced Gastritis in Experimentally Infected Conventional Piglets** *Vet Pathol* **38**:667–678 Google Scholar

50. Boyen F., et al. (2009) **Porcine in vitro and in vivo models to assess the virulence of Salmonella enterica serovar Typhimurium for pigs** *Lab Anim* **43**:46–52 Google Scholar

51. Boyen F., et al. (2008) **Coated fatty acids alter virulence properties of Salmonella Typhimurium and decrease intestinal colonization of pigs** *Veterinary Microbiology* **132**:319–327 Google Scholar

52. Köppen K., et al. (2023) **Ex vivo infection model for Francisella using human lung tissue** *Front. Cell. Infect. Microbiol* **13** Google Scholar

53. Bomjan R., Zhang M., Zhou D (2019) **YshB Promotes Intracellular Replication and Is Required for Salmonella Virulence** *Journal of Bacteriology* **201** https://doi.org/10.1128/jb.00314-19 | Google Scholar

54. Birhanu B. T., Park N.-H., Lee S.-J., Hossain M. A., Park S.-C (2018) **Inhibition of Salmonella Typhimurium adhesion, invasion, and intracellular survival via treatment with methyl gallate alone and in combination with marbofloxacin** *Veterinary Research* **49**:101 Google Scholar

55. MacBeth K. J., Lee C. A (1993) **Prolonged inhibition of bacterial protein synthesis abolishes Salmonella invasion** *Infect Immun* **61**:1544–1546 Google Scholar

56. Sharma A., Puhar A (2019) **Gentamicin Protection Assay to Determine the Number of Intracellular Bacteria during Infection of Human TC7 Intestinal Epithelial Cells by Shigella flexneri** *Bio-protocol* **9** Google Scholar

57. Thiennimitr P., et al. (2011) **Intestinal inflammation allows Salmonella to use ethanolamine to compete with the microbiota** *Proc. Natl. Acad. Sci. U.S.A* **108**:17480–17485 Google Scholar

58. Haneda T., et al. (2009) **The Capsule-Encoding viaB Locus Reduces Intestinal Inflammation by a Salmonella Pathogenicity Island 1-Independent Mechanism** *Infection and Immunity* **77**:2932–2942 Google Scholar

59. Kitamoto S., et al. (2020) **Dietary l-serine confers a competitive fitness advantage to Enterobacteriaceae in the inflamed gut** *Nature Microbiology* **5**:116–125 Google Scholar

60. Sugihara K., Kamada N (2020) **The role of dietary L-serine in the regulation of intestinal mucus barrier during inflammation** *Inflammatory Bowel Diseases* **26**:S42–S42 Google Scholar

61. Perkins A., Tudorica D. A., Amieva M. R., Remington S. J., Guillemin K (2019) **Helicobacter pylori senses bleach (HOCl) as a chemoattractant using a cytosolic chemoreceptor** *PLoS Biol* **17**:e3000395 Google Scholar

62. Ranque C. L., et al. (2020) **Examination of Carbohydrate Products in Feces Reveals Potential Biomarkers Distinguishing Exclusive and Nonexclusive Breastfeeding Practices in Infants** *J Nutr* **150**:1051–1057 Google Scholar

63. Ke Z., Lu Z., Li Q., Tong W (2024) **Intestinal glucose excretion: A potential mechanism for glycemic control** *Metabolism* **152**:155743 Google Scholar

64. Larke J. A., et al. (2023) **Dietary Intake of Monosaccharides from Foods is Associated with Characteristics of the Gut Microbiota and Gastrointestinal Inflammation in Healthy US Adults** *The Journal of Nutrition* **153**:106–119 Google Scholar

65. Shen X., et al. (2022) **Asparagine, colorectal cancer, and the role of sex, genes, microbes, and diet: A narrative review** *Front Mol Biosci* **9**:958666 Google Scholar

66. Yoo W., et al. (2024) **Salmonella Typhimurium expansion in the inflamed murine gut is dependent on aspartate derived from ROS-mediated microbiota lysis** *Cell Host & Microbe* **32**:887–899 Google Scholar

67. Beltran P., et al. (1991) **Reference collection of strains of the Salmonella typhimurium complex from natural populations** *Journal of General Microbiology* **137**:601–606 Google Scholar

68. Shariat N., et al. (2013) **Subtyping of Salmonella enterica Serovar Newport Outbreak Isolates by CRISPR-MVLST and Determination of the Relationship between CRISPR-MVLST and PFGE Results** *J Clin Microbiol* **51**:2328–2336 Google Scholar

69. Ferrari R. G., et al. (2019) **Worldwide Epidemiology of Salmonella Serovars in Animal-Based Foods: a Meta-analysis** *Appl Environ Microbiol* **85**:e00591–19 Google Scholar

70. Wiles T. J., et al. (2018) **Modernized Tools for Streamlined Genetic Manipulation and Comparative Study of Wild and Diverse Proteobacterial Lineages** *mBio* **9**:e01877–18 Google Scholar

71. Falke J. J., Hazelbauer G. L (2001) **Transmembrane signaling in bacterial chemoreceptors** *Trends Biochem Sci* **26**:257–265 Google Scholar

72. Lai R.-Z., et al. (2005) **Cooperative Signaling among Bacterial Chemoreceptors** *Biochemistry* **44**:14298–14307 Google Scholar

73. Gavira J. A., et al. (2020) **How Bacterial Chemoreceptors Evolve Novel Ligand Specificities** *mBio* **11**:e03066–19 Google Scholar

74. Matilla M. A., Ortega Á., Krell T (2021) **The role of solute binding proteins in signal transduction** *Computational and Structural Biotechnology Journal* **19**:1786–1805 Google Scholar

75. Chimerel C., Field C. M., Piñero-Fernandez S., Keyser U. F., Summers D. K (2012) **Indole prevents Escherichia coli cell division by modulating membrane potential** *Biochim Biophys Acta* **1818**:1590–1594 Google Scholar

76. Melander R. J., Minvielle M. J., Melander C (2014) **Controlling bacterial behavior with indole-containing natural products and derivatives** *Tetrahedron* **70**:6363–6372 Google Scholar

77. Moore J. P., Kamino K., Kottou R., Shimizu T. S., Emonet T (2024) **Signal integration and adaptive sensory diversity tuning in Escherichia coli chemotaxis** *Cell Systems* **15**:628–638 Google Scholar

78. Gül E., et al. (2023) **Intraluminal neutrophils limit epithelium damage by reducing pathogen assault on intestinal epithelial cells during Salmonella gut infection** *PLOS Pathogens* **19**:e1011235 Google Scholar

79. Antunes L. C. M., et al. (2014) **Antivirulence activity of the human gut metabolome** *mBio* **5**:e01183–1114 Google Scholar

80. Peixoto R. J. M., et al. (2017) **Repression of Salmonella Host Cell Invasion by Aromatic Small Molecules from the Human Fecal Metabolome** *Appl Environ Microbiol* **83**:e01148–17 Google Scholar

81. Lamas A., Regal P., Vázquez B., Cepeda A., Franco C. M (2019) **Short Chain Fatty Acids Commonly Produced by Gut Microbiota Influence Salmonella enterica Motility** *Biofilm Formation, and Gene Expression. Antibiotics* **8**:265 Google Scholar

82. Huang J. Y., et al. (2015) **Chemodetection and Destruction of Host Urea Allows Helicobacter pylori to Locate the Epithelium** *Cell Host & Microbe* **18**:147–156 Google Scholar

83. Sugihara K., et al. (2022) **Mucolytic bacteria license pathobionts to acquire host-derived nutrients during dietary nutrient restriction** *Cell Reports* **40**:111093 Google Scholar

84. Chandra K. (2022) **Gentamicin Protection assay (Intracellular Survival Assay) for Salmonella Typhimurium/Typhi v1** *protocols.io* https://doi.org/10.17504/protocols.io.b4zmqx46 | Google Scholar

85. Rivera Calo J., Rubinelli P. M., Ricke S. C. (2024) **Gentamicin Susceptibility and Comparison of Adhesion and Invasion of Caco-2 and HD11 Cell Lines by Salmonella enterica Serotypes** *Applied Sciences* **14**:3305 Google Scholar

86. Ben-David A., Davidson C. E (2014) **Estimation method for serial dilution experiments** *Journal of Microbiological Methods* **107**:214–221 Google Scholar

87. Macho A. P., Zumaquero A., Ortiz-Martín I., Beuzón C. R. (2007) **Competitive index in mixed infections: a sensitive and accurate assay for the genetic analysis of Pseudomonas syringae –plant interactions** *Molecular Plant Pathology* **8**:437–450 Google Scholar

88. Auerbuch V., Lenz L. L., Portnoy D. A (2001) **Development of a competitive index assay to evaluate the virulence of Listeria monocytogenes actA mutants during primary and secondary infection of mice** *Infect Immun* **69**:5953–5957 Google Scholar

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Reviewer #1 (Public review):

Summary:

The study shows, perhaps surprisingly, that human fecal homogenates enhance the invasiveness of Salmonella typhimurium into cells of a swine colonic explant. This effect is only seen with chemotactic cells that express the chemoreceptor Tsr. However, two molecules sensed by Tsr that are present at significant concentrations in the fecal homogenates, the repellent indole and the attractant serine, do not, either by themselves or together at the concentrations in which they are present in the fecal homogenates, show this same effect. The authors then go on to study the conflicting repellent response to indole and attractant response to serine in a number of different in vitro assays.

Strengths:

The demonstration that homogenates of human feces enhance the invasiveness of chemotactic Salmonella Typhimurium in a colonic explant is unexpected and interesting. The authors then go on to document the conflicting responses to the repellent indole and the attractant serine, both sensed by the Tsr chemoreceptor, as a function of their relative concentration and the spatial distribution of gradients.

Weaknesses:

The authors do not identify what is the critical compound or combination of compounds in the fecal homogenate that gives the reported response of increased invasiveness. They show it is not indole alone, serine alone, or both in combination that have this effect, although both are sensed by Tsr and both are present in the fecal homogenates. Some of the responses to conflicting stimuli by indole and serine in the in vitro experiments yield interesting results, but they do little to explain the initial interesting observation that fecal homogenates enhance invasiveness.

https://doi.org/10.7554/eLife.106261.2.sa2

Reviewer #3 (Public review):

Summary:

In this manuscript, Franco and colleagues describe careful analyses of Salmonella chemotactic behavior in the presence of conflicting environmental stimuli. By doing so, the authors describe that this human pathogen integrates signals from a chemoattractant and a chemorepellent into an intermediate "chemohalation" phenotype.

Strengths:

The study was clearly well-designed and well-executed. The methods used are appropriate and powerful. The manuscript is very well written, and the analyses are sound. This is an interesting area of research, and this work is a positive contribution to the field.

Weaknesses:

No significant weaknesses noted.

https://doi.org/10.7554/eLife.106261.2.sa1

Author response:

The following is the authors' response to the original reviews

We thank the reviewers and editors for their careful consideration of our work and pointing out areas where the current version lacked clarity or necessary experiments. Based on the reviews we have made the following significant changes to the revised version:

(1) Revised the text to focus on the distinct pathogen responses to indole in isolation versus fecal material.

We believe the key takeaway from this work is that the native context of a given effector, in this case indole, can elicit markedly different bacterial responses compared to the pure compound in isolation. This is because natural environments contain multiple, often conflicting, stimuli that complicate predictions of overall chemotactic behavior. For example, while indole has been proposed to mediate chemorepulsion and contribute to colonization

resistance against enteric pathogens, our findings challenge this model. We provide evidence that feces, the intestinal source of indole, actually induces attraction, and that indole taxis may in fact benefit the pathogen through prioritizing niches with low microbial competition. Put another way, the biological reservoir of indole, fecal material, generates an attraction response but indole regulated the degree of attraction.

Most current understanding of chemotaxis is based on responses to individual, purified effectors. Our study highlights the need to investigate chemotactic responses in the presence of native mixtures, which better reflect the complexity of natural environments and may reveal new functional insights relevant for disease.

Reviewer comments indicated that these core points above were not clearly conveyed in the previous version, and that the manuscript's logical flow needed improvement. In this revised version, we have substantially rewritten the text and removed extraneous content to sharpen the focus on these central findings. We have also aligned our discussion more closely with the experimental data. While we appreciated the reviewers' thoughtful suggestions, we chose not to expand on topics that fall outside the scope of our current experiments.

(2) Provide new chemotaxis data with mixtures of fecal effectors (Fig. 5).

Related to the above, the reviewers and editors brought up concerns that our discovery of pathogen fecal attraction was underexplored. Although we showed Tsr to be important for mediating fecal attraction, even the tsr mutant showed attraction to a lesser degree, and the reviewers noted that we did not identify what other fecal attractants could be involved.

Fecal material is a complex biological material (as noted by Reviewer 3) and contains effectors already characterized as chemoattractants and chemorepellents. It would be ideal to be able to perform some experiment where individual effectors are removed from fecal material and then quantify chemotaxis. We considered methods to do this but ultimately found this approach unfeasible. Instead, we employed a reductionist approach and developed a synthetic approximate of fecal material containing a mixture of known chemoeffectors at fecal-relevant concentrations (Fig. 5). We used this defined system as a way to test the specific roles of the Tsr effectors L-Ser (attractant) and indole (repellent) in relation to glucose, galactose, and ribose (sensed through the chemoreceptor Trg), and L-Asp (sensed through the chemoreceptor Tar). We chose these effectors as they have reasonable structure-function relationships established in prior work, and had information available about their concentrations in fecal material. We present these data as a new Figure 5, and also provide videos clearly showing the responses to each treatment (Movies 7-10).

This defined system provided several new insights that help understand and model indole taxis amidst other fecal effectors. First, the complete effector mixture, like fecal treatment, elicits attraction. Second, L-Ser is able to negate indole chemorepulsion in cotreatments of the two effectors, and also other chemoattractants in the absence of L-Ser also negate this repulsion, albeit to a lesser degree, helping to explain why the tsr mutant still shows attraction to fecal material. Lastly, we also show that the degree of attraction in this system is controlled by indole, with mixtures containing greater indole showing less attraction. We feel this is an important addition to the study because it provides a new view on how indole-taxis functions in pathogen colonization; rather than causing the pathogen to swim away (like pure indole does) indole helps the pathogen rank and prioritize its attraction to fecal effector mixtures, biasing navigation toward lower indolecontaining niches.

We also acknowledge that this defined system does not capture all possible interactions. Indeed, there are even a few chemoreceptors in Salmonella for which the sensing functions remain poorly understood. Nonetheless, we believe the data offer mechanistic context for understanding fecal attraction and suggest that factors beyond Tsr, L-Ser, and indole also contribute to the observed behaviors, aligning with other data we present.

(3) Provide new data that show that E. coli MG1655, and disease-causing clinical isolate strains of the Enterobacteriaceae Tsr-possessing species E. coli, Citrobacter koseri, and Enterobacter cloacae exhibit fecal attraction (Fig. 4).

An important new finding from this study is our direct test of whether indole-rich fecal material elicits repulsion. Contrary to expectations, given that for E. coli indole is a wellcharacterized strong chemorepellent, we show that fecal material instead elicits attraction in non-typhoidal Salmonella.

Reviewers raised the question of whether our observations regarding indole taxis and attraction to indole-rich feces in Salmonella are similar or relevant to E. coli. While a full dissection of indole taxis in E. coli is beyond the scope of this study and has been the focus of extensive prior research, we sought to address this point by examining whether other enteric pathogens respond similarly to the native indole reservoir, fecal material. To this end, we present new data demonstrating that, like S. Typhimurium, E. coli and other representative enteric pathogens and pathobionts possessing Tsr are also attracted to indole-rich feces (Fig. 4, Movies 4–6, Fig. S4).

Notably, these new results represent some of the first characterizations of chemotactic behavior in the clinical isolates we examined, including E. coli NTC 9001 (a urinary tract infection isolate), Citrobacter koseri, and Enterobacter cloacae, adding another element of novelty to this work.

(4) Repeated all of the explant Salmonella Typhimurium infection studies and added a new experimental control competition between WT and an invasion-deficient mutant (invA).

Although our new colonic explant system was noted as a novelty and strength of this work, it was also seen as a weakness in that some of the results were surprising and difficult to link to chemotactic behavior. Reviewer 3 also brought up the need to be clear about our usage of the term ‘invasion’ in reference to S. Typhimurium entering nonphagocytic host cells, and requested we test an invasion-inhibited mutant (which we do in new experiments, now Fig. S1). We also note that some of the interpretations of these data were made challenging by result variability.

To help address these issues we performed additional replicates for all of our explant experiments (contained within Figure 1, Fig. S1-S2, and Data S1), to provide greater power for our analyses. These new data provide a clearer view of this system that revise our interpretations from the prior version of this study. While treatment with indole alone does suppress the WT advantage over chemotactic mutants for both total colonization and cellular invasion, essentially all other treatments have a similar result with a timedependent increase in both colonization and invasion, dependent on chemotaxis and Tsr. A remaining unique feature of fecal treatment is an increase in the cellular invaded population of the cells at 3 h post-infection. As requested by Reviewer 3, we provide new experimental data showing that in competitions between WT and an invasion-deficient mutant (invA), with fecal material pretreatment, we see the WT has an advantage only for the gentamicin-treated qualifications, providing some support that our model selects for the invaded sub-population. Although we note that the invA still can invade through alternative mechanisms (as discussed in earlier work such as here: https://doi.org/10.1111/1574-6968.12614), so the relative amount of presumed cellular invasion is less than WT, and not zero, in our experiments (Fig. S1).

One point of confusion in the previous version of the text was the assay design for the explant experiments, which is important to understand in order to interpret the results. During the explant infection bacteria are not immersed in the effector treatment solution, rather the tissue is soaked in the effector solution beforehand and then exposed to a 300 µl buffer solution containing the bacteria. This means that the bacteria experience only the residue of that treatment at concentrations far lower. We have added clarity about this through revising

Fig. 1 to include a conceptual diagram of the assay (Fig. 1C), and added a new supplementary Fig. S5 that summarizes the explant data in this same conceptual model. We provide detail on the method in the text in lines 115-137. In describing the results, and synthesizing them in the discussion, we now state:

Line 112: “This establishes a chemical gradient which we can use to quantify the degree to which different effector treatments are permissive of pathogen association with, and cellular invasion of, the intestinal mucosa (Fig. 1C).”

And, a new section in the discussion devoted to describing the explant infections:

Line: 366: “Our explant experiments can be thought of as testing whether a layer of effector solution is permissive to pathogen entry to the intestinal mucosa, and whether chemotaxis provides an advantage in transiting this chemical gradient to associate with, and invade, the tissue (Fig. 1C, Fig. S5).”

As mentioned above, we have honed the text to focus on the disparity between the effects of indole alone versus treatments with indole-rich feces to help clarify how these data advance our understanding of the indole taxis in directing pathogenesis. While our explant studies still confirm the role of factors other than L-Ser, indole, and Tsr in directing Salmonella infection and cellular invasion, we now include further analyses of other fecal effectors (described above) that provide some insights into how fecal effectors have some redundancy in their impact.

> ***Public Reviews:***
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> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The study shows, perhaps surprisingly, that human fecal homogenates enhance the invasiveness of Salmonella typhimurium into cells of a swine colonic explant. This effect is only seen with chemotactic cells that express the chemoreceptor Tsr. However, two molecules sensed by Tsr that are present at significant concentrations in the fecal homogenates, the repellent indole and the attractant serine, do not, either by themselves or together at the concentrations in which they are present in the fecal homogenates, show this same effect. The authors then go on to study the conflicting repellent response to indole and attractant response to serine in a number of different in vitro assays.*
>
> *Strengths:*
>
> *The demonstration that homogenates of human feces enhance the invasiveness of chemotactic Salmonella Typhimurium in a colonic explant is unexpected and interesting. The authors then go on to document the conflicting responses to the repellent indole and the attractant serine, both sensed by the Tsr chemoreceptor, as a function of their relative concentration and the spatial distribution of gradients.*

Thank you for your summary and acknowledgement of the strengths of this work. We hope the revised text and additional data we provide further improve your view of the study.

> *Weaknesses:*
>
> *The authors do not identify what is the critical compound or combination of compounds in the fecal homogenate that gives the reported response of increased invasiveness. They show it is not indole alone, serine alone, or both in combination that have this effect, although both are sensed by Tsr and both are present in the fecal homogenates. Some of the responses to conflicting stimuli by indole and serine in the in vitro experiments yield*

> *interesting results, but they do little to explain the initial interesting observation that fecal homogenates enhance invasiveness.*

Thank you for noting these weaknesses. We have provided new data using a defined mixture of fecal effectors to further investigate the roles of L-Ser, indole, and other effectors present in feces that we did not initially study. We have refined our discussion of these results to hopefully improve the clarity of our conclusions. We show now both in explant studies (Fig. 1I) and chemotaxis responses to a defined fecal effector system (Fig. 5) that L-Ser is able to abolish both the suppression of indole-mediated WT advantage and also indole chemorepulsion, respectively. We also show the latter can be accomplished by other fecal chemoattractants (Fig. 5). This is in line with our earlier finding that Tsr, the sensor of indole and L-Ser, is an important mediator of fecal attraction but not the sole mediator.

As this reviewer points out, there are indeed other factors mediating invasion that we do not elucidate here, but we do note these possibilities in the text (lines: 125-127):

"This benefit may arise from a combination of factors, including sensing of host-emitted effectors, redox or energy taxis, and/or swimming behaviors that enhance infection [5,30,31,35]."

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The manuscript presents experiments using an ex vivo colonic tissue assay, clearly showing that fecal material promotes Salmonella cell invasion into the tissue. It also shows that serine and indole can modulate the invasion, although their effects are much smaller. In addition, the authors characterized the direct chemotactic responses of these cells to serine and indole using a capillary assay, demonstrating repellent and attractant responses elicited by indole and serine, respectively, and that serine can dominate when both are present. These behaviors are generally consistent with those observed in E. coli, as well as with the observed effects on cell invasion.*
>
> *Strengths:*
>
> *The most compelling finding reported here is the strong influence of fecal material on cell invasion. Also, the local and time-resolved capillary assay provides a new perspective on the cell's responses.*

Thank you for acknowledging these aspects of the study.

> *Weaknesses:*
>
> *The weakness is that indole and serine chemotaxis does not seem to control the fecal-mediated cell invasion and thus the underlying cause of this effect remains unclear.*
>
> *In addition, the fact that serine alone, which clearly acts as a strong attractant, did not affect cell invasion (compared to buffer) is somewhat puzzling. Additionally, wild-type cells showed nearly a tenfold advantage even without any ligand (in buffer), suggesting that factors other than chemotaxis might control cell invasion in this assay, particularly in the serine and indole conditions. These observations should probably be discussed.*

Addressed above.

> *Final comment. As shown in reference 12, Tar mediates attractant responses to indole, which appear to be absent here (Figure 3J). Is it clear why? Could it be related to receptor expression?*

Thank you for noting this. We now mention this in the discussion. In the course of this work, we encountered a number of apparent inconsistencies, or differences, between what we were observing with S. Typhimurium and what had been reported previously in studies of Tsr function in E. coli. We indeed noted that some studies had investigated a role of Tar for indole taxis (in E. coli), hence why we determined whether, and confirmed, that Tsr is required for indole taxis for S. Typhimurium (Fig. 6).

We do not know the reason for this apparent difference between the two bacteria, but we have previously shown with our same strain of S. Typhimurium IR715, under the same growth assay, and preparation protocol, that L-Asp is a strong chemoattractant for both WT and the tsr mutant (see Glenn et al. 2024, eLife, Fig. 5G: https://iiif.elifesciences.org/lax:93178%2Felife-93178-fig5-v1.tif/full/1500,/0/default.jpg).

This supports that this strain of Salmonella indeed has a functional Tar present and is expressed at a level sufficient for sensing L-Asp. So, if Tar generally mediates indole sensing we do not know why we would not see that in Salmonella. Hence, we do not see any role for Tar in indole chemorepulsion in our strain of study, which is different than reported for E. coli, but we cannot confirm the reason.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *In this manuscript, Franco and colleagues describe careful analyses of Salmonella chemotactic behavior in the presence of conflicting environmental stimuli. By doing so, the authors describe that this human pathogen integrates signals from a chemoattractant and a chemorepellent into an intermediate "chemohalation" phenotype.*
>
> *Strengths:*
>
> *The study was clearly well-designed and well-executed. The methods used are appropriate and powerful. The manuscript is very well written and the analyses are sound. This is an interesting area of research and this work is a positive contribution to the field.*

Thank you for your comments.

> *Weaknesses:*
>
> *Although the authors do a great job in discussing their data and the observed bacterial behavior through the lens of chemoattraction and chemorepulsion to serine and indole specifically, the manuscript lacks, to some extent, a deeper discussion on how other effectors may play a role in this phenomenon. Specifically, many other compounds in the mammalian gut are known to exhibit bioactivity against Salmonella. This includes compounds with antibacterial activity, chemoattractants, chemorepellers, and chemical cues that control the expression of invasion genes. Therefore, authors should be careful when making conclusions regarding the effect of these 2 compounds on invasive behavior.*

Thank you for this comment, and we agree with your point. We hope we have revised the text and provided new data to address your concern. We have also chosen for clarity to keep our text close to our experimental data and so have refrained from speculating about some topics, even though you are absolutely correct about the immense complexity of these systems.

> *It is important that the word invasion is used in the manuscript only in its strictest sense, the ability displayed by Salmonella to enter non-phagocytic host cells. With that in mind, authors should discuss how other signals that feed into the control of Salmonella invasion can be at play here.*

Thank you for your recommendation. We have revised the text to hopefully be clearer on our meaning of invasion in regard to Salmonella entering non-phagocytic host cells, essentially changing our usage to 'cellular invasion' throughout.

It is also a commonly-used phrase in reference to enteric infections and the colonization resistance conferred by the microbiome to refer to 'invading pathogens' (i.e. invasion in the sense of a new microbe colonizing the intestines), For instance, this recent review on Salmonella makes use of the term invading pathogen (https://www.nature.com/articles/s41579-021-00561-4). We acknowledge the confusion by this dual use of the term. We have mostly removed our statements using invasion in this context. We hope our language is clearer in this revised version.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *It was difficult to understand the true intent or importance of the study described in this manuscript. The first figure in the paper showed that a Salmonella Typhimurium strain lacking either CheY, and thus incapable of any chemotaxis, or the Tsr chemoreceptor, and thus incapable of sensing serine or indole, was modestly inferior to the wild-type version of that strain in invading the cells of a swine colonic explant. It then showed that, in the presence of a human fecal homogenate, the wild-type strain had a much greater advantage in invading the colonic cells. Thus, the presence of the fecal homogenate significantly increased invasiveness in a way that depends on chemotaxis and the Tsr chemoreceptor.*
>
> *As human feces were determined to contain 882 micromolar indole and 338 micromolar serine, the effects of those concentrations of either indole or serine alone or in combination were tested. The somewhat surprising finding was that neither indole nor serine alone nor in combination changed the result from the experiment done with just buffer in the colonic explant.*
>
> *The clear conclusion of this initial study is that both chemotaxis in general and chemotaxis mediated by Tsr improve the invasiveness of S. Typhimurium. They provide a much bigger advantage in the presence of human feces. However, two molecules present in the feces that are sensed by Tsr, serine, and indole, seem to have no effect on invasiveness either alone or in combination.*
>
> *At this point, the parsimonious interpretation is that there is something else in human feces that is responsible for the increased invasiveness, and the authors acknowledge this possibility. However, they do not take what appears to be the obvious approach: to look for additional factors in human feces that might be responsible, either by themselves or in combination with indole and/or serine, for the increased invasiveness. Instead, they carry out a detailed examination of the counteracting effects of indole as a repellent and of serine as an attractant as a function of their relative concentrations and their spatial distributions.*

Thank you for your comments. In our revised version, we have undertaken some additional studies of other fecal effectors that help better understand the relationship between L-Ser and indole, but also the roles of other chemoattractants (glucose, galactose, ribose, L-Asp) in

mediating fecal attraction (Fig. 5). We agree with the reviewer and conclude that fecal attraction and the cell invasion phenotype mediated by fecal treatment are influenced by factors other than only Tsr, indole, and L-Ser. Our new data do show that L-Ser is sufficient to block both the invasion suppression effects of indole (negating the WT advantage) and also indole chemorepulsion, therefore making our detailed examination of the counteracting effects more relevant for understanding this system.

> *What they find is what other studies have shown, primarily with S. Typhimurium's relative, the gamma-proteobacterium Escherichia coli.*
>
> *At high indole and low serine concentrations, the repulsion by indole wins out. At low indole and high serine concentrations, attraction by serine wins out. What is perhaps novel is what happens at an intermediate ratio of concentrations. Repulsion by indole dominates at short distances from the source, so there is a zone of clearing. At longer distances, attraction by serine dominates, so there is an accumulation of cells in a "halo" around the zone of clearing. Thus, assuming that serine and indole diffuse equally, the repulsive effect of indole dominates until its concentration falls below some critical level at which the concentration of serine is still high enough to exert an attractive effect.*
>
> *They go on to show, using ITC, that serine binds to the periplasmic ligand-binding domain (LBD) of Tsr, something that has been studied extensively with very similar E. coli Tsr.*
>
> *They also show that indole does not bind to the Tsr LBD, which also is known for E. coli Tsr.*
>
> *This would be newsworthy only if the results were different for S. Typhimurium than for E. coli. As it is, it is merely confirmatory of something that was already known about Tsr of enteric bacteria.*
>
> *An idea that the authors introduce, if I understand it correctly, is that a repellent response to something in feces, perhaps indole, drives S. Typhimurium chemotactically competent cells out of the colonic lumen and promotes invasion of the bacteria into the cells of the colonic lining. If the feces contain both an attractant and a repellent, bacteria might be attracted by the feces to the lining of the intestine and then enter the colonic cells to escape a repellent, perhaps indole. That is an interesting proposition.*
>
> *In summary, I think that the initial experimental approach is fine. I do not understand the failure to follow up on the effect of the fecal homogenates in promoting invasion by chemotactic bacteria possessing Tsr. It seems there must be something else in the homogenates that is sensed by Tsr. Other amino acids and related compounds are also sensed by Tsr. Perhaps it is energy or oxygen taxis, which is partially mediated by Tsr, as the authors acknowledge.*
>
> *Much of the work reported here is quasi-repetitive with work done with E. coli Tsr. Minimally, previous work on E. coli Tsr should be explained more thoroughly rather than dealt with only as a citation.*

Thank you for your comments.

We would like to confirm our agreement that E. coli and S. enterica indeed possess similarities. They are Gammaproteobacteria and inhabit/infect the gut. But also we note they diverged evolutionarily during the Jurassic period (ca. 140 million years ago, see: PMC94677). In the context of colonizing humans, the former is a pathobiont, indoleproducer, and a native member of the microbiome, whereas the latter is a frank pathogen and does not produce indole. Hence, there are many reasons to believe one is not an approximate of the other, especially when it comes to causing disease.

We agree that much of what is known about indole taxis has come from excellent studies in well-behaved laboratory strains of E. coli, a powerful model. We believe that expanding this work to include clinically relevant pathogens is important for understanding its role in human disease. In this study, we contribute to that broader understanding by providing new mechanistic insights into Tsr-mediated indole taxis in S. Typhimurium, along with data demonstrating fecal attraction in other enteric pathogens and pathobionts. These findings help define a more general role for Tsr in enteric colonization and disease. While some of our results indeed confirm and extend prior findings, we respectfully believe that such confirmation in relevant pathogenic strains adds value to the field.

Regarding our ITC studies, to our knowledge no other study has investigated, using ITC whether indole does or does not bind the LBD (which we show it does not), nor investigated whether it interferes with L-Ser sensing (which we show it does not). Hence, these are not duplicate findings, although we do acknowledge this leaves the mechanism of indolesensing undiscovered. If we are incorrect in this regard, please provide us a citation and we will be happy to include it and revise our comments.

We now clarify in the text on lines 378-381: “While these leave the molecular mechanism of indole-sensing unresolved, it does eliminate two possibilities that have not, to our knowledge, been tested previously. Overall, our data add support to the hypothesis that a non-canonical sensing mechanism is employed by Tsr to respond to indole [8,18,69].”

Lastly, as noted by the reviewer, and which we mention in the text, essentially all prior studies on indole taxis were conducted in E. coli, and this is not what is new and novel about the work we present, which is focused on S. Typhimurium and testing the prediction that fecal indole protects against pathogen invasion. We have added in a few additional points of comparisons between our results and prior studies. While we appreciate that much understanding has come from E. coli as a model for indole taxis, we feel discussing prior work in extensive detail would be more suitable for a review and would occlude our new findings about Salmonella, and other enterics.

In an earlier version of the manuscript, we included more background on E. coli indole taxis. However, we found that the historical literature in this area was somewhat inconsistent, with different assays using varying time points and indole concentrations, often leading to results that were difficult to reconcile. Providing sufficient context to explain these discrepancies required considerable space and, ultimately, detracted from the focus of our current study. Hence, we have only brought in comparisons with E. coli where most relevant to the present work. Also, we provide new data that E. coli also exhibits fecal attraction, and so there is reason to believe the mechanisms we study here are also relevant to that system.

> *Some minor points*
>
> *(1) Hyphens are not needed with constructs like "naturally occurring" or "commonly used".*

Thank you. Revisions made throughout.

> *(2) The word "frank" as in "frank pathogen" seems odd. It seems "potent" would be better.*

Thank you for this comment. Per your recommendation, we have removed this term.

The term ‘frank pathogen’ is standard usage in the field of bacterial pathogenesis in reference to a microbe that always causes disease in its host (in this case humans) and causes disease in otherwise healthy hosts (example: https://www.sciencedirect.com/science/article/pii

/S1369527420300345). We actually used this specific term to distinguish an aspect of novelty of our study because E. coli can, sometimes, be a pathogen (i.e. a pathobiont) and of course E. coli indole taxis has been previously studied. Ours is the first study of indole taxis in a frank pathogen.

> *(3) It is unnecessary to coin a new word, chemohalation, to describe a phenomenon that is a simple consequence of repulsion by higher concentrations of a repellent and attraction by lower concentrations of attractant to generate a halo pattern of cell distribution.*

Thank you for your opinion on this. We have softened our statements on this point, and in the newly revised version of the text less space is devoted to this idea. We now state in line 304-307:

"There exists no consensus descriptor for taxis of this nature, and so we suggest expanding the lexicon with the term "chemohalation," in reference to the halo formed by the cell population, and which is congruent with the commonly-used terms chemoattraction and chemorepulsion."

We appreciate the reviewer's perspective and agree that the behavior we describe can be viewed as the result of competing attractant and repellent cues. However, we find that the traditional framework of "chemoattraction" and "chemorepulsion" is often insufficient to describe the spatial positioning behaviors we observe in our system. In our experience presenting and discussing this work, especially with audiences outside the chemotaxis field, it has been challenging to convey these dynamics clearly using only those two terms.

For this reason, we introduced the term chemohalation to describe this more nuanced behavior, which appears to reflect a balance of signals rather than a simple unidirectional response. More bacteria enter the field of view, but they are clearly positioned differently than regular 'chemoattraction.' We also note that Reviewers 2 and 3 did not raise concerns about the term, and after careful consideration, we have opted to retain it in the revised manuscript.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *Lines 143-156 seem somewhat overcomplicated and may be confusing. For example: in line 143: "However, when colonic tissue was treated with purified indole at the same concentration, the competitive advantage of WT over the chemotactic mutants was abolished compared to fecaltreated tissue...". But indole was tested alone, so it did not abolish the response; rather the absence of fecal material did.*

We appreciate your point. We have made revisions throughout to help improve the clarity of how we discuss the explant infection data and provide new visuals to help explain the experiment and data (Fig. 1C, Fig. S5).

> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) Line 46 - Are references 9-11 really about topography?*

Thank you. You are correct. Revised and eliminated this statement.

> *(2) Lines 87-89 - It seems to me that a bit more information on this would be helpful to the reader.*

In our revision of the text, to make it more centered on our primary findings of the differences between indole taxis when indole is the sole effector versus amidst other

effectors, we have removed this section.

> *(3) Line 112 - When mentioning the infection of the cecum and colon, authors should specify that this is in mice.*

Thank you for this comment. In our revised version we provide references both for animal model infections and work in human patients (ex: https://www.sciencedirect.com/science/article/abs/pii/S0140673676921000)

We have revised our statement to be (Line 99-100: “Salmonella Typhimurium preferentially invades tissue of the distal ileum but also infects the cecum and colon in humans and animal models [42–46].”

> *(4) Lines 122-123 - Authors state that "This experimental setup simulates a biological gradient in which the effector concentration is initially highest near the tissue and diffuses outward into the buffer solution.". Was this experimentally demonstrated? If not, authors should tone this down.*

We have removed this comment and instead present a conceptual diagram illustrating this idea (Fig. 1C). Also, addressed by above.

> *(5) When looking at the results in Figure 1, I wonder what the results of this experiment would be if the authors tested an invasion mutant of Salmonella. In a strain that is able to perform chemotaxis (attraction and repulsion) but unable to actively invade, would there be a phenotype here? Is it possible that the fecal material affects cellular uptake of Salmonella, independently of active invasion? I don't think the authors necessarily need to perform this experiment, but I think it could be informative and this possibility should at least be discussed.*

Thank you for your comments and suggestions. We have included new data of an explant co-infection experiment with WT and an invasion-deficient mutant invA (Fig. S1). Under these conditions, WT exhibits an advantage in the gentamicin-treated homogenate, but not the untreated homogenate, suggestive of an advantage in cellular invasion.

However, we did not repeat all experiments with this genetic background. We felt that would be outside the scope of this work, and would probably require dual chemotaxis/invA deletions to assess the impact of each, which also could be difficult to interpret. The hypothesis mentioned by the Reviewer is possible, but we were not able to devise a way to test this idea, as it seems we would need to deactivate all other mechanisms of Salmonella invasion.

> *(6) Lines 137-140 - Because this is a competition experiment and results are plotted as CI, the reader can't readily assess the impact of human feces on invasion by WT Salmonella.*

Thank you for pointing this out. We want to mention that the data are plotted as CI in the main text, but the supplemental contains the disaggregated CFU data (Fig. S1-2) and the numerical values (Data S1).

> *Please include the magnitude of induction in this sentence, compared to the buffer control.*

The text of this section has been changed to account for new data.

> *Additionally, although unlikely, the presence of the chemotaxis mutants in the same infection may be a confounding factor. In order to irrefutably ascertain that feces*

> *induces invasion, I suggest authors perform this experiment with the wildtype strain (and mutant) alone in different conditions.*

Thank you for this suggestion, although after careful consideration we have decided not to repeat these explant studies with monoinfections. Coinfections are a common tool in *Salmonella* pathogenesis studies, including prior chemotaxis studies which our work builds upon (ex: https://pmc.ncbi.nlm.nih.gov/articles/PMC3630101/). The explant experiments, even controlling as many aspects as we did, still show lots of variability and one way to mitigate this is through competition experiments so that each strain experiences the same environment.

We agree that a cost of this approach is that one strain may affect the other, or may alter the environment in a way that impacts the other. Thus, the resulting data must also be understood through this lens. We have revised the text to stay closer to the competitive advantage phenotype.

> *(7) Line 150 - Authors state that bacterial loads are similar. However, authors should perform and report statistical analyses of these comparisons, at least in the supplementary data.*

We have removed this statement as requested. We do note, however, that the mean CFU values across treatments at identical time points appear qualitatively similar, which is an observation that does not require statistical testing.

> *(8) Lines 154-154 - This seems incorrect, as the effect observed with the mixture of indole and serine is very similar to the addition of serine alone. Therefore, there was no "neutralization" of their individual effects.*

We have revised this statement.

> *(9) Line 159-161 - I strongly suggest authors reword this sentence. I don't think this is the best way to describe these results. The stronger phenotype observed was with the fecal material. Therefore, it is the indole (alone) condition that does not "elicit a response". Focusing on indole too much here ignores everything else that is present in feces and also the fact that there was a drastic phenotype when feces were used.*

Thank you for your opinion on this. We believe this is one of the ways in which our earlier draft was unclear. It was actually a primary motivation of this work to test whether there were differences in pathogen infection, mediated by chemotaxis, in the presence of indole as a singular effector or in its near-native context in fecal material, and our revised text centers our study around this question. We believe this distinction is important for the reasons mentioned earlier.

Relative to buffer treatment, indole changes the behavior of the system, eliminating the WT advantage, and this is the effect we refer to. We have made many revisions to the text of these sections and hope it better conveys this idea. We expect we may still have differences regarding the interpretation of these results, but regardless, thank you for your suggestions and we have tried to implement them to improve the clarity of the text.

> *(10) Line 162 - Again, I disagree with this. Indole does not have an effect to be cancelled out by serine.*

Addressed above, and this text has been changed. Also, we provide new chemotaxis data that at fecal-relevant concentrations of indole and L-Ser, indole chemorepulsion is overridden (Fig. 5).

> *(11) Lines 166-168 - Again, this is a skewed analysis. Indole and serine could not possibly provide an "additive effect" since they do not provide an effect alone. There is nothing to be added.*

This text has been deleted.

> *(12) Lines 168-170 - Most of the citations provided to this sentence are inadequate. Our group has previously shown that the mammalian gut harbors thousands of small molecules (Antunes LC et al. Antimicrob Agents Chemother 2011). You obviously do not have to cite our work, but there is significant literature out there about the complexity of the gut metabolome.*

Thank you for this comment. We have revised this particular text, but do make mention of potential other effectors driving these effects, which was also requested by the other reviewers.

Your work and others indeed support there being thousands of molecules in the gut, but our work centers on chemotaxis, and bacteria have a small number of chemoreceptors and only sense a very tiny fraction of these molecules as effectors. Since the impacts of infection of the explants depends on chemotaxis, we keep our comments restricted to those, but agree that there are likely many interactions involved, such as those impacting gene expression.

Please note our more detailed description of the explant infection assay (and shown in Fig. 1C) that may change your view on the significance of non-chemotaxis effects. The bacteria only experience the effectors at low concentration, not the high concentration that is used to soak and prepare the tissue prior to infection.

> *(13) Figure 2 - The letter 'B' from panel B is missing.*

Thank you very much for bringing this oversite to our attention. We have fixed this.

> *(14) Legend of Figure 3 - Panel J is missing a proper description. Figure legends need improvement in general, to increase clarity.*

Thank you for noting this. This is now Fig. 6E. We have provided an additional description of what this panel shows. We have edited the legend text to read: “E. Shows a quantification of the relative number of cells in the field of view over time following treatment with 5 mM indole for a competition experiment with WT and tsr (representative image shown in F).”

We also have made other edits to figure legends to improve their clarity and add additional experimental details and context. By breaking up larger figures into smaller figures, we also hope to have improved the clarity of our data presentation.

> *(15) Lines 264-265 - Maybe I am missing something, but I do not see the ITC data for serine alone.*

We have clarified in the text that this was measured in our previous study https://elifesciences.org/articles/93178). The present study is a ‘Research Advance’ article format, and so builds on our prior observation.

We have revised the text to read: “To address these possibilities, we performed ITC of 50 μM Tsr LBD with L-Ser in the presence of 500 μM indole and observed a robust exothermic binding curve and KD of 5 μM, identical to the binding of L-Ser alone, which we reported previously (Fig. 6H) [36].”

> *(16) Lines 296-297 - What is the effect of these combinations of treatments on bacterial cells? I commend the authors for performing the careful growth assays, but I wonder if bacterial lysis could be a factor here. I am not doubting the effect of chemotaxis, but I am wondering if toxic effects could be a confounding factor. For instance, could it be that the "avoidance" close to the compound source and subsequent formation of a halo suggest bacterial death and lysis? I suggest the authors perform a very simple experiment, where bacteria are exposed to the compounds at various concentrations and combinations, and cells are observed over time to ensure that no bacterial lysis occurs.*

Thank you for mentioning this possibility. If we understand correctly, the Reviewer is asking if the chemohalation effect we report could be from the bacteria lysing near the source. Our data actually argue against this possibility through a few lines of evidence.

First, if this were the case in experiments with the cheY mutant, we would also see an effect near the source. But actually, in experiments with either the cheY mutant or the tsr mutant, neither of which can sense indole, the bacteria just ignore the stimulus and show an even distribution (see current Fig. 6F).

Second, our calculations suggest that in the chemotaxis assay (CIRA), the bacteria only experience rather low local concentration of indole, mostly I the nM concentration range, because as soon as the effector treatment is injected into the greater volume, it is immediately diluted. This means the local concentration is far below what we see inhibits growth of the cells in the long run and may not be toxic (Fig. 7, Fig. S3).

Lastly, in the representative video presented we can observe individual cells approach and exit the treatment (Movie 11). Due to the above we have not performed additional experiments to test for lysis.

> *(17) Lines 310-311 - Isn't this the opposite of the model you propose in Figure 5? The higher the concentration of indole in the lumen the more likely Salmonella is to swim away from it and towards the epithelium, favoring invasion, no?*

We appreciate the opportunity to clarify this point and apologize for any confusion caused. In response, we have revised the text to place less emphasis on chemohalation, and the specific statement and model in question have now been removed. Instead, we provide a summary of our explant data in light of the other analyses in the study (Fig. S5).

What we meant here was in relation to the microscopic level, not whether or not a host/intestine is colonized. To put it another way, we think our data supports that the pathogen colonizes and infects the host regardless of indole presence, but it uses indole as a means to prioritize which tissues are optimal for colonization at the microscopic level. The prediction made by others was that bacteria swim away from indole source and therefor this could prevent or inhibit pathogen colonization of the intestines, which our data does not support.

> *(18) Lines 325-326 - Maybe, but feces also contain several compounds with antibacterial activity, as well as other compounds that could elicit chemorepulsion. This should be stated and discussed.*

We have removed this statement since we did not explicitly test the growth of the bacteria with fecal treatments. We have refrained from speculating further in the text since we do not have direct knowledge of how that relationship with differing effectors could play out.

We agree with the reviewer that the growth assays are reductionist and give insight only into the two effectors studied. We provide evidence from several different types of enterics that they all exhibit fecal attraction, and it seems unlikely the bacteria would be attracted to something deleterious, but we have not confirmed.

> *(19) Lines 371-374 - How preserved (or not) is the mucus layer in this model? The presence of an inhibitory molecule in the lumen does not necessarily mean that it will protect against invasion. It is possible that by sensing indole in the lumen Salmonella preferentially swims towards the epithelium, thus resulting in enhanced evasion.*

The text in question has been removed. However, we acknowledge the reviewer's point, and that these explant tissues do not fully model an in vivo intestinal environment. Other than a gentle washing with PBS to remove debris prior to the experiment the tissue is not otherwise manipulated, and feasibly the mucus layer is similar to its in vivo state.

In mentioning this hypothesis about indole, which our data do not support, we were echoing a prediction from the field, proposed in the studies we cite. We agree with the reviewer that there were other potential outcomes of indole impacting chemotaxis and invasion, and indeed our data supports that.

> *(20) Lines 394-395 - The authors need to remember that the ability to invade the intestinal epithelium is not only a product of chemoattraction and repulsion forces. Several compounds in the gut are used by Salmonella as cues to alter invasion gene expression. See PMID: 25073640, 28754707, 31847278, and many others.*

Thank for you for this point, and we now include these citations. We have revised the text in question, stating:

"In addition to the factors we have investigated, it is already well-established in the literature that the vast metabolome in the gut contains a complex repertoire of chemicals that modulate Salmonella cellular invasion, virulence, growth, and pathogenicity [79–81]."

Our intent is not to diminish the role of other intestinal chemicals but rather to put our new findings into the context of bacterial pathogenesis. We do provide evidence that specific chemoeffectors present in fecal material alter where bacteria localize through chemotaxis, which is one method of control over colonization.

> *(21) Line 408 - I think it could be hard to observe this using your experimental approach.*
>
> *Because you need to observe individual cells, the number of cells you observe is relatively small. If, in a bet-hedging strategy, the proportion of cells that were chemoattracted to indole was relatively low you likely would not be able to distinguish it from an occasional distribution close to the repellent source. You may or may not want to discuss this.*

Thank you for this observation. It is indeed challenging to both observe large scale population behaviors and also the behaviors of individual cells in the same experiment. Our ability to make this distinction is similar to the approach used in the study we cite, so that is our comparison.

But, if there was a subpopulation that was attracted we would predict a 'bull's-eye' population structure, with some cells attracted and other avoiding the source, which we do not see - we see the halo. So, we find no evidence of the bet-hedging response seen in a different study using E. coli and using different time scales than we have.

> *(22) Lines 410-411 - What could the other attractants be? Would it be possible/desirable to speculate on this?*

We have changed the text here, but we present new data that examines some of these other attractants (Fig. 5).

> *(23) Line 431 - What exactly do you mean by "running phenotype"? Please, provide a brief explanation.*

We have removed this text, but a running phenotype means the swimming bacteria rarely make direction changes (i.e. tumbles), which has been associated with promoting contact with the epithelium, described in the references we cite. Hence, this type of swimming behavior could contribute to the effects we observe in the explant studies, potentially explaining some of the Tsr-mediated advantage that was not dependent on L-Ser/indole.

> *(24) Line 441 - Other work has shown that feces contain inhibitors of invasion gene expression. The authors should integrate this knowledge into their model. In fact, indole has been shown to repress host cell invasion by Salmonella, so it is important that authors understand and discuss the fact that the impact of indole is multifaceted and not only a reflection of its action as a chemorepellent. PMID: 29342189, 22632036.*

We agree with the reviewer about this point, and mention this in the text (lines 55-57): "Indole is amphipathic and can transit bacterial membranes to regulate biofilm formation and motility, suppress virulence programs, and exert bacteriostatic and bactericidal effects at high concentrations [16–18,20–22]."

We have added in the references suggested.

What we test here is the specific hypothesis made by others in the field about indole chemorepulsion serving to dissuade pathogens from colonizing.

For instance, the statement from: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0190613

"Since indole is also a chemorepellent for EHEC [23], it is intriguing to speculate that in addition to attenuating Salmonella virulence, indole also attenuates the recruitment and directed migration of Salmonella to its infection niche in the GI tract."

And from: https://doi.org/10.1073/pnas.1916974117

"We propose that indole spatially segregates cells based on their state of adaptation to repel invaders while recruiting beneficial resident bacteria to growing microbial communities within the GI tract."

And

"Thus, foreign ingested bacteria, including invading pathogens such as E. coli O157:H7 and S. enterica, are likely to be prevented by indole from gaining a foothold in the mucosa."

As shown by others, indole certainly does have many roles in controlling pathogenesis, and there are other chemicals we do not investigate that control invasion and bacterial growth, but we keep our statements here restricted to chemotaxis since that is what are experiments and data show.

> *(25) Line 472 - "until fully motile". How long did this take, how variable was it, and how was it determined?*

Thank you for asking for this clarification. We have added that the time was between 1-2 h, and confirmed visually. Our methods are similar to those described in earlier chemotaxis studies (ex: 10.1128/jb.182.15.4337-4342.2000).

> *(26) Line 487 - I worry that the fact fecal samples were obtained commercially means that compound stability/degradation may be a factor to consider here. How long had the sample been in storage? Is this information available?*

Thank you for this question. We agree that the fecal sample we used serves as a model system and we cannot rule out that handling by the supplier could potentially alter its contents in some way that would impact bacterial chemosensing. However, we note that the measurements of L-Ser and indole we obtained are in the appropriate range for what other studies have shown.

The fecal sample used for all work in the study were from a single healthy human donor, obtained from Lee Biosolutions (https://www.leebio.com/product/395/fecal-stool-samplehuman-donor-991-18). The supplier did not state the explicit date of collection, nor indicated any specific handline or storage methods that would obviously degrade its native metabolites, but we cannot rule that out. In our hands, the fecal sample was collected and kept frozen at -20 C. For research purposes, portions were extracted and thawed as needed, maintaining the frozen state of the original sample to limit degradation from freeze-thaws.

https://doi.org/10.7554/eLife.106261.2.sa0