THE EFFECTS OF TRYPTOPHAN METABOLITES ON VIRULENCE OF *SALMONELLA* TYPHIMURIUM

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

The Effects of Tryptophan Metabolites on Virulence of *Salmonella* Typhimurium. (May 2015)

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Salmonella is a foodborne pathogen that invades the human gastrointestinal (GI) tract from the intestinal lumen by targeting intestinal epithelial cells (IECs). It is able to breach the protective IEC barrier with the aid of needle-like complexes encoded by a cluster of genes known as the *Salmonella* pathogenicity island 1 (SPI1). The influence that molecules in the GI tract have on pathogenicity and virulence is not yet fully understood because of the complexity and abundance of metabolites present in the GI microenvironment. One source of metabolites is the amino acid tryptophan, which is transformed into indole and other indole-like metabolites by the microbiota in the GI tract. Roles for many of the gut metabolites are still unknown. Previous research in the Jayaraman laboratory has shown that indole, a tryptophan metabolite, decreases the virulence of *Salmonella.* In this project, we studied the effects of tryptophan derivatives which are present in the GI tract – indole-acetic acid, tryptamine, indole-pyruvic acid, and hydroxyindole – on the virulence of *Salmonella*. The motility of the pathogen in response to the different tryptophan derivatives was determined using the swimming motility assay (SMA). The expression of SPI1 genes was measured using the β-galactosidase reporter assay as a measure of virulence. The results of the SMA show that the tryptophan derivatives tested do not affect the motility of Salmonella significantly, whereas indole decreases motility by 40%. The effect of these

metabolites on the expression of SPI1 genes, *hilA* and *invF,* was observed to be much less compared with that of indole. We therefore conclude that not all tryptophan metabolites present in the GI tract decrease the virulence of *Salmonella* Typhimurium to the same extent. Further research needs to be done to determine the roles of these tryptophan derivatives as well as several other molecules present in the GI tract microenvironment.

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NOMENCLATURE

DMSO	dimethyl sulfoxide
HI	hydroxyindole
I	indole
IAA	indole-3-acetic acid
IPA	indole-3-pyruvic acid
IEC	intestinal epithelial cell
LB	lysogeny broth
MCT	microcentrifuge tubes
OD_{420nm}	optical density at 420nm wavelength of light
OD_{600nm}	optical density at 600nm wavelength of light
ONPG	ortho-nitrophenyl-β-galactoside
SCV	*Salmonella*-containing vacuole
SDS	sodium dodecyl sulfate
SPI	*Salmonella* pathogenicity island
T3SS	type 3 secretion system
TA	tryptamine
tet20	20 µg of tetracycline/ mL of 70% ethanol
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

The human gut is densely populated with microorganisms. It contains up to 10^{14} prokaryotic cells, which is 10 times more than the number of human body cells (Staib and Fuchs). The large number of metabolites produced in the gut is due to the constant metabolism of dietary compounds (i.e. carbon and nitrogen sources) by microbiota (Staib and Fuchs). A healthy balance of gut microbiota is vital for the maintenance of a strong immune system. The microbiota drain most of the nutrients in the gut before the pathogens can use them to colonize (Staib and Fuchs). Food particles entering the intestine are digested by gut microbiota and then, are converted into essential nutrients and other metabolic by-products. Metabolites can be absorbed into the intestinal lumen or can influence the activity of other gut microbes. Roles for many gut metabolites and their influence on pathogens are still unknown due to the complexity and abundance of metabolites present in the GI microenvironment.

Salmonella is a foodborne pathogen that will come into contact with gut metabolites upon oral ingestion with food particles. It invades the human gastrointestinal tract by targeting IECs. It uses a T3SS characterized by the formation of a needlelike complex responsible for invasion (Hegazy and Hensel). This complex injects proteins that alter the IEC cytoskeleton and manipulate the cell processes to provide favorable conditions for *Salmonella* growth (Basel). *Salmonella* can affect other types of cells downstream and eventually cause gastroenteritis (Hansen-Wester and Hensel). In the US alone, it has been associated with approximately 40,000

reported cases and 400 deaths per year. The actual number of infections is estimated to be more than thirty times greater than the number of reported cases (CDC).

The SPI is the chromosomal region where T3SS genes are clustered. One loci, SPI1, contains more than 25 genes responsible for the formation of the needlelike complex (Hansen-Wester and Hensel). Here, we studied the effect of metabolites on the expression of two SPI1 genes — *hilA* and *invF*. They are both transcriptional factors that influence the expression of other SPI1 genes involved in the formation of T3SS needle proteins. *hilA* is known to be the master regulator of SPI1 and *invF* is downstream of it. Figure 1 below shows how *Salmonella* invades IECs (Hegazy and Hensel).

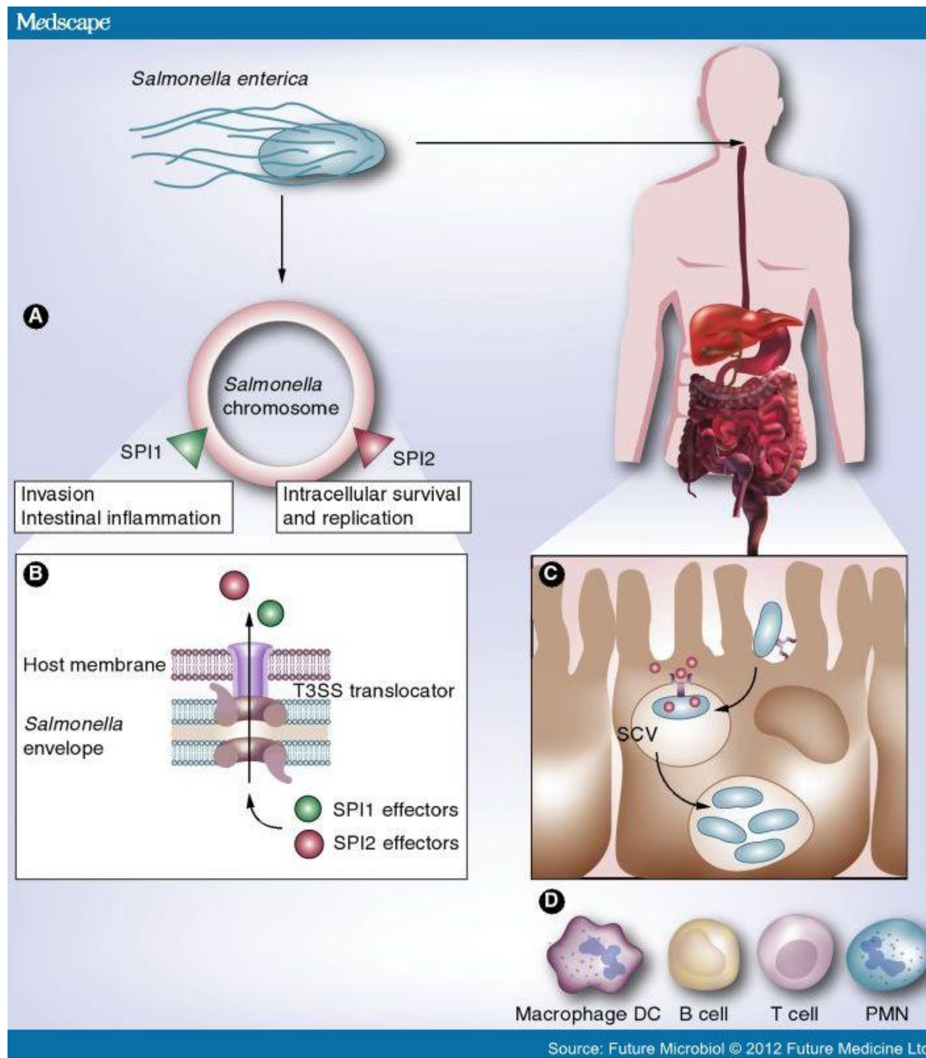

Figure 1. Key events in *Salmonella* pathogenesis. **(A)** Within the chromosome of *Salmonella enterica*, several virulence clusters known as *Salmonella* pathogenicity islands (SPI) are present. In particular, SPI1 and SPI2 play important roles in invasion and intracellular survival, respectively. **(B)** Genes within SPI1 and SPI2 of *Salmonella* encode two distinct virulence-associated T3SS that are important for different phases of pathogenesis. **(C)** Orally ingested *Salmonella* survive in the low pH of the stomach, enter the small intestine and invade epithelial cells. Upon invasion, *Salmonella* SPI1-T3SS delivers its effector proteins across the host cell plasma membrane, which leads to temporal reorganization of the host cell actin cytoskeleton and induces uptake of the bacteria by means of macropinocytosis and plays an important role in *Salmonella*-induced inflammatory responses. *Salmonella* remains in a specialized phagosome, the SCV, where multifunctional virulence system SPI2-T3SS plays its role in *Salmonella* survival inside the SCV and inducing systematic responses. **(D)** *Salmonella* preferentially enters microfold cells, which transport them to the lymphoid cells (T and B cells) in the underlying Peyer's patches. Nontyphoidal *Salmonella* strains induce an early local inflammatory response, which results in the infiltration of PMNs into the intestinal lumen and diarrhea. On the other hand, *Salmonella* serotypes that are associated with systemic illness enter intestinal macrophages and disseminate throughout the reticuloendothelial system. (Hegazy and Hensel)

As mentioned previously, gut metabolites can be absorbed into the intestinal lumen or influence the activity of other microbes. In the case of *Salmonella* food poisoning, metabolites that arise from the breakdown of food particles can affect the *Salmonella* invasion process. One main source of metabolites comes from the amino acid, tryptophan, a major constituent of the human diet. It can be catalyzed into indole and other indole-like metabolites by either the host or gut microbiota. Indole-3-pyruvic acid, tryptamine, 5-hydroxy-L-tryptophan, and indole have been detected in caecum luminal contents of mice (Sridharan et al.). This project focuses the following tryptophan metabolites: indole-3- acetic acid, indole-3-pyruvic acid, tryptamine, and 5-hydroxyindole. These metabolites, shown in Figure 2, were tested to determine their effects on virulence of *Salmonella* Typhimurium ATCC 14028s.

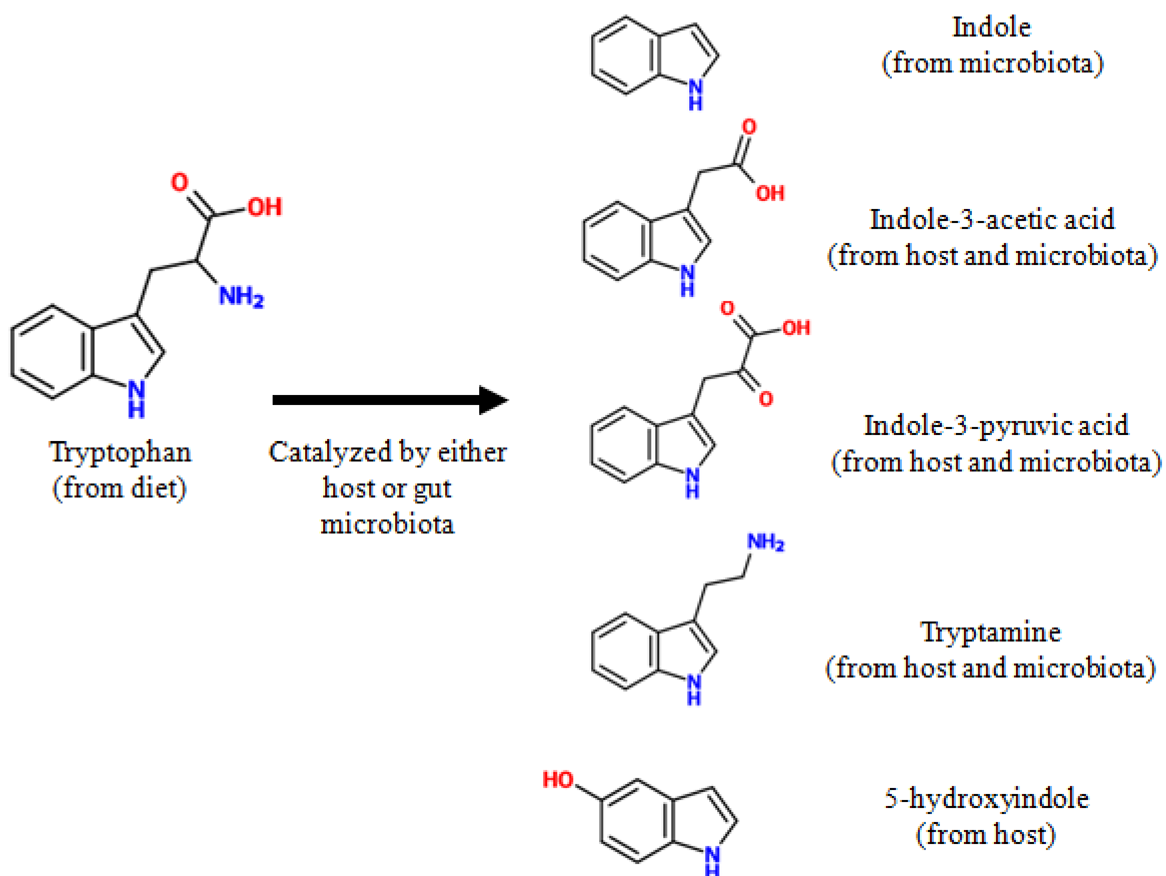

Figure 2. Tryptophan can be catalyzed by the host or microbiota to produce a range of other metabolites. These metabolites can also under chemical reactions with other metabolites to form indole derivatives.

Previous studies in the Jayaraman group have shown that indole, a tryptophan metabolite, decreases *Salmonella* virulence by downregulation of SPI1 genes (Kohli). The motility of *Salmonella* in response to the tryptophan metabolites in Figure 2 were tested using the swimming motility assay. The expression of SPI1 genes, *hilA* and *invF*, were measured using the β-galactosidase reporter assay as a measure of virulence. Studying these tryptophan metabolites will help determine their roles they play in the *Salmonella* invasion process within in the GI tract.

CHAPTER II

METHODS

Part A. Swimming Motility Assay

A WT strain of *S.* Typhimurium 14028s was grown on an LB agar plate. The strain was incubated overnight at 37°C. A single isolated colony of the WT strain was inoculated in 5 mL of LB media. The tube was incubated overnight for 16 hours at 37°C, 250 rpm. The OD_{600nm} was recorded by diluting sample of the overnight culture in 1:10 total (100 µL cell culture + 900 µL LB media). The overnight culture was diluted to an OD_{600nm} of 0.05 in fresh 5 mL LB media. The culture tubes were incubated at 37°C, 250 rpm , and the incubation was stopped once the culture turbidity was about 1.0 (approximately 2 hours).

Swimming motility agar plates (25 mL per plate) were prepared with the composition (1% tryptone, 0.25% NaCl, and 0.3% agar, with or without additives to be tested) The 6 conditions tested were as follows:

1. DMSO (solvent control)
2. 1 mM IAA
3. 1 mM TA
4. 1 mM IPA
5. 1 mM HI
6. 1 mM I

1 M stock of the treatment chemical was added to SMA media to achieve a final concentration of 1 mM prior to pouring. 1 µL of the culture was spotted on each plate, which were incubated

upright at 37°C. The diameters of the motility halos were measured after 8 hours using Vernier calipers. (Bansal et al.)

Part B. β-galactosidase Reporter Assay for SPI1 Gene Expression

SPI1 gene reporter strains were used to determine the effect of tryptophan metabolites on virulence gene expression. A *lacZ* gene was fused to both the *hilA* and *invF* genes to form *hilA*::*lacZ* and *invF*::*lacZ* (Altier, Suyemoto and Lawhon). The *hilA* and *invF* reporter strains were grown on LB plates containing tetracycline (tet) and X-gal at a concentration of 20 µg/mL and 40 µg/mL, respectively. Overnight cultures were started from a single colony in a tube containing 5 mL of LB media (1% tryptone, 0.5% NaCl, and 0.5% yeast extract) containing tet20 and 1 mM of respective signals at 37°C with shaking.

The OD_{600nm} was recorded by diluting a sample of the overnight culture in 1:4 total (250 µL cell culture + 750 µL media). For the β-galactosidase assay, the following chemicals were added in a 2 mL microcentrifuge tube (MCT): 900 µL Z buffer, 100 µL of cell suspension, 5 µL SDS, and 10 µL chloroform. The tubes were vortexed for 10 seconds and then placed on ice. The procedure was repeated for the blank by using 100 µL of LB media in place of the cell culture. The tubes were incubated at 28°C for 5 minutes. The reaction was started by adding 200 µL of 4 mg/mL ONPG substrate. Once the solution turned yellow, the reaction was stopped by adding 500 µL of 1 M Na_2CO_3. The total time of reaction in minutes was recorded. The tubes were centrifuged for 2 minutes at 10,000 g, 4°C and then placed on ice. The OD_{420nm} was measured for the supernatant, and the Miller β-galactosidase units were calculated by Equation 1.

$$\text{Miller } \beta - \text{Gal Units} = \frac{(1000)\times(\text{OD}_{420\text{nm}})}{(\text{t}_{(\text{minutes})})\times(0.1 \text{ mL cell culture})\times(\text{OD}_{600\text{nm}})} \quad (1)$$

The fold decrease of Miller β-galactosidase units produced for each treatment was compared with that of DMSO. Fold decrease was calculated by Equation 2.

$$\text{Fold Decrease} = \frac{\text{Miller } \beta\text{−Gal Units produced for DMSO}}{\text{Miller } \beta\text{−Gal Units produced for treatment}} \quad (2)$$

The assay was performed for three biological replicates (Miller).

CHAPTER III

RESULTS

Part A. Swimming Motility Assay

Swimming motility halos form due to outward migration of bacteria from the center where they were spotted. For each condition — DMSO (solvent control), IAA, TA, IPA, HI, and I — plate images were taken after eight hours of incubation as shown in Figure 3a-f below.

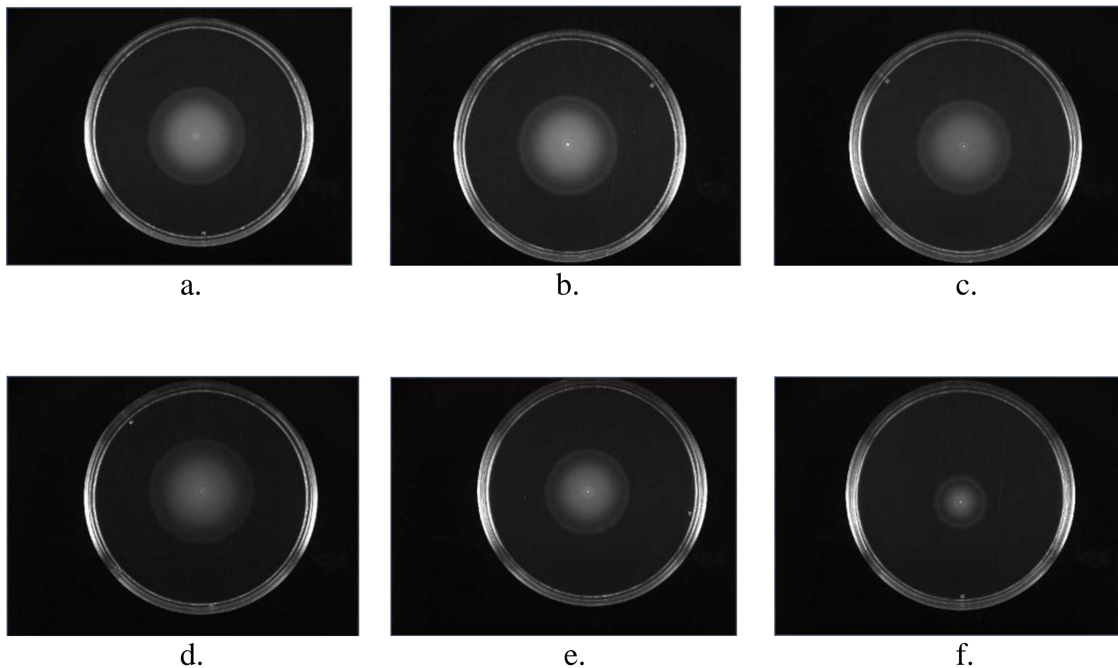

Figure 3. Swimming motility assay plate images representative of each treatment. The outermost halo diameter was recorded. a.) DMSO control, b) 1 mM IAA, c) 1 mM TA, d) 1 mM IPA, e) 1 mM HI, and f) 1 mM I.

Diameters of the motility halos formed by the swimming bacteria were recorded in millimeters as shown in Table 1 and then plotted in Figure 4. Indole decreased halo diameters by 40% compared to the solvent control whereas the change was not significant for other treatments.

Table 1. Swimming motility assay halo diameters (mm)

Replicate	DMSO	IPA	IAA	TA	HI	I
1	35.39	38.91	37.63	35.98	32.08	22.14
2	32.72	36.51	34.26	34.54	31.50	20.31
3	29.19	32.75	31.34	31.01	27.84	18.24
Average	32.43	36.06	34.41	33.84	30.47	20.23
Std. Dev.	3.11	3.10	3.15	2.56	2.30	1.95

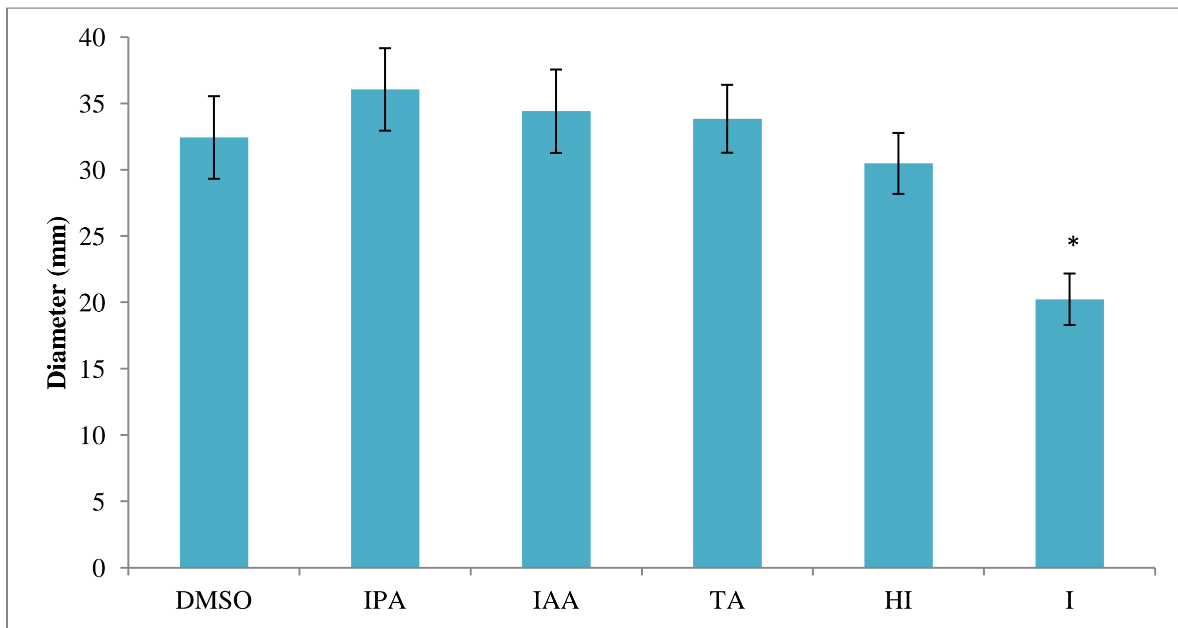

Figure 4. Average halo diameters for treatment plotted with error bars of one standard deviation. (Student T-tests, *p<0.05)

Part B. β-galactosidase Reporter Assay for SPI1 Gene Expression

Miller β-galactosidase units are a measure of gene expression. They were recorded for the reporter strains under the following treatments: DMSO (solvent control), IAA, TA, IPA, HI, and I.

Miller β-galactosidase units produced by each treatment were recorded for the *hilA*::*lacZ* as shown in Table 2 and then plotted in Figure 5. The same was done for the *invF*::*lacZ* strain in Table 3 and then plotted in Figure 6.

Table 2. Miller β-galactosidase units produced by *hilA*::*lacZ* strain

Replicate	DMSO	TA	HI	IAA	IPA	I
1	1586	1288	762	767	495	37
2	1774	1798	1318	1401	710	90
3	2118	2009	1608	817	414	53
Average	1826	1699	1229	995	540	60
Std. Dev.	270	371	430	353	153	27
Fold Decrease	—	1.1	1.5	1.8	3.4	30.4

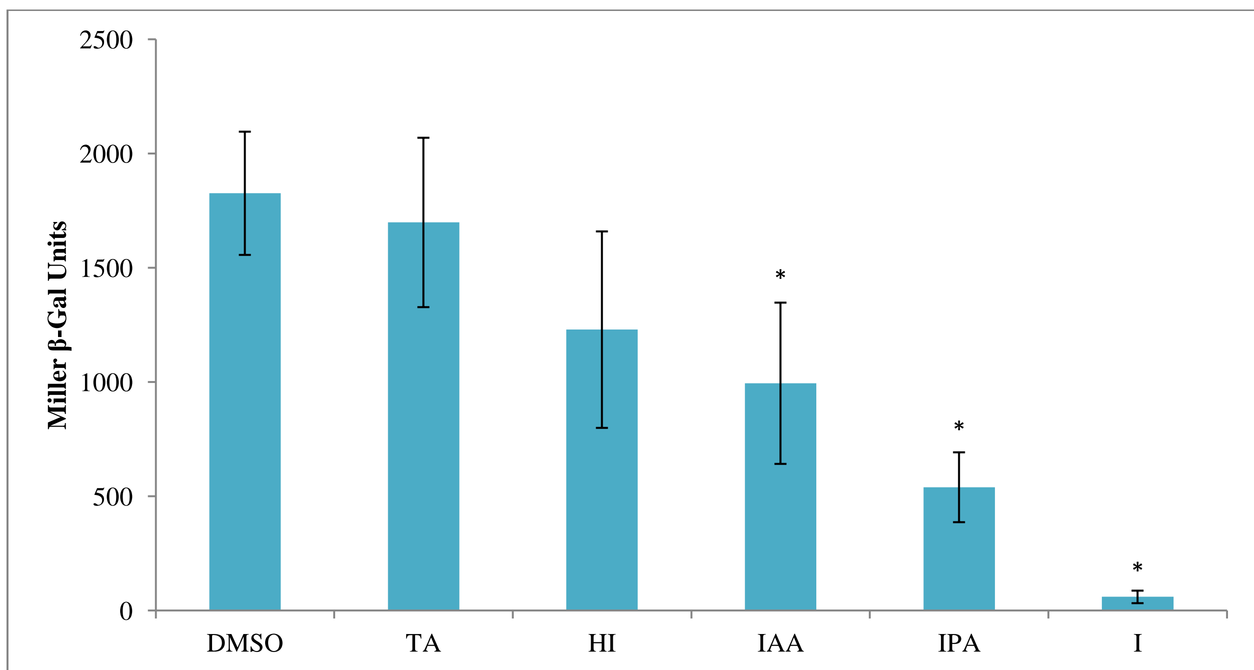

Figure 5. Miller β-galactosidase units for each treatment for the *hilA::lacZ* strain were plotted along with error bars of one standard deviation. (Student T-tests, *$p<0.05$)

Table 3. Miller β-galactosidase units produced by *invF*::*lacZ* strain

Replicate	DMSO	TA	HI	IAA	IPA	I
1	1240	1131	625	451	399	11
2	1393	1178	750	520	363	9
3	848	—	—	447	321	4
Average	1316	1154	688	486	381	10
Std. Dev.	243	19	51	34	33	3
Fold Decrease	—	1.1	1.9	2.7	3.5	128.2

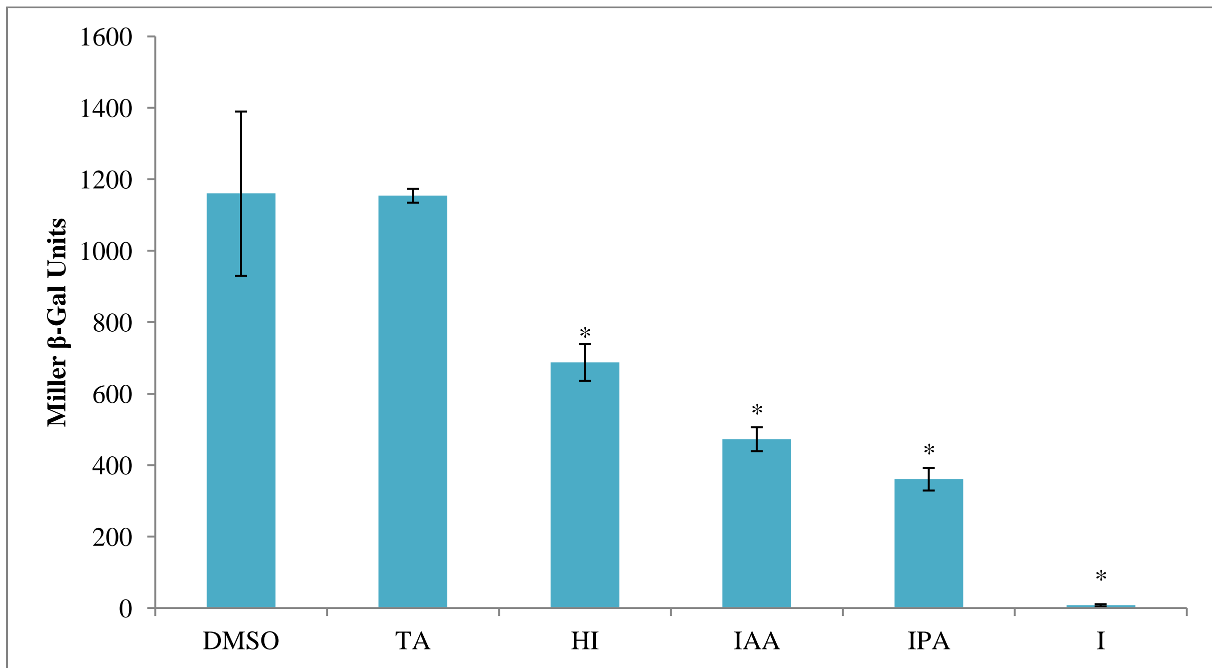

Figure 6. Miller β-galactosidase units for each treatment for the *hilA*::*lacZ* strain were plotted with error bars of one standard deviation. (Student T-tests, *p<0.05) (only two replicates were tested for TA and HI)

Summary of Results

The results of the swimming motility assay and β-galactosidase assay are summarized below in Table 4.

Table 4. Summary of results for swimming motility assay and β-galactosidase assay

<table>
<tr><th colspan="2">Assay</th><th>Observations</th></tr>
<tr><td colspan="2">Swimming Motility Assay</td><td>Diameter changes were not significant for all treatments except for I, which resulted in a 40% decrease</td></tr>
<tr><td rowspan="3">β-galactosidase Assay</td><td>hilA</td><td>Decrease in Miller β-galactosidase units were significant for all treatments except for TA and HI</td></tr>
<tr><td>invF</td><td>Decrease in Miller β-galactosidase units were significant for all treatments except for TA</td></tr>
<tr><td>Overall</td><td>All metabolites had a lower fold decrease compared with that of indole</td></tr>
</table>

CHAPTER IV

CONCLUSIONS

The swimming motility assay showed that for all treatments except for indole, the change in motility was not significant. The β-galactosidase assay showed that the decrease in *hilA* gene expression was significant for indole-3-acectic acid, indole-3-pyruvic acid, and indole treatments. Decrease in *invF* gene expression was significant for hydroxyindole, indole-3-acetic acid, indole-3-pyruvic acid, and indole treatments. However, the small magnitudes of fold decrease in gene expression for all treatments were all incomparable to the large magnitude of fold decrease observed for indole. Therefore, we can conclude that not all tryptophan metabolites have the same effect on *Salmonella* virulence. The metabolites that caused a significant decrease in gene expression did not behave the same for the motility assay, suggesting a mutiple-pathway interaction between metabolites and *Salmonella*.

Future studies include testing other tryptophan derivatives as well as other gut metabolites in varying concentrations. The relationship between the chemical structures of metabolites and their effect on *Salmonella* virulence can also be explored. The pharmaceutical industry can potentially use the results of future studies to develop medical drugs for preventing *Salmonella* food poisoning.

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