Serotonin modulates *Campylobacter jejuni* physiology and *in vitro* interaction with the gut epithelium

Joshua M. Lyte,*,1 Sandip Shrestha,† Basanta R. Wagle,† Rohana Liyanage,† Diego A. Martinez,† Annie M. Donoghue,*, Karrie M. Daniels,† and Mark Lyte ‡,*

*Poultry Production and Product Safety Research Unit, Agricultural Research Service, United States Department of Agriculture, Fayetteville, AR 72701, USA; †Department of Poultry Science, University of Arkansas, Fayetteville 72701, USA; and ‡Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames 50011, USA

ABSTRACT  Microbial endocrinology, which is the study of neurochemical-based host–microbe interaction, has demonstrated that neurochemicals affect bacterial pathogenicity. A variety of neurochemicals, including norepinephrine, were shown to enhance intestinal epithelial colonization by *Campylobacter jejuni*. Yet, little is known whether serotonin, an abundant neurochemical produced in the gut, affects the physiology of *C. jejuni* and its interaction with the host gut epithelium. Considering the avian gut produces serotonin and serves as a major reservoir of *C. jejuni*, we sought to investigate whether serotonin can affect *C. jejuni* physiology and gut epithelial colonization *in vitro*. We first determined the biogeo graphical distribution of serotonin concentrations in the serosa, mucosa, as well as the luminal contents of the broiler chicken ileum, cecum, and colon. Serotonin concentrations were greater (*P < 0.05*) in the mucosa and serosa compared to the luminal content in each gut region examined. Among the ileum, colon, and cecum, the colon was found to contain the greatest concentrations of serotonin. We then investigated whether serotonin may effect changes in *C. jejuni* growth and motility *in vitro*. The *C. jejuni* used in this study was previously isolated from the broiler chicken ceca. Serotonin at concentrations of 1mM or below did not elicit changes in growth (*P > 0.05*) or motility (*P > 0.05*) of *C. jejuni*. Next, we utilized liquid chromatography tandem mass spectrometry to investigate whether serotonin affected the proteome of *C. jejuni*. Serotonin caused (*P < 0.05*) the downregulation of a protein (CJJ81176_1037) previously identified to be essential in *C. jejuni* colonization. Based on our findings, we evaluated whether serotonin would cause a functional change in *C. jejuni* adhesion and invasion of the HT29MTX-E12 colonic epithelial cell line. Serotonin was found to cause a reduction in adhesion (*P < 0.05*) but not invasion (*P > 0.05*). Together, we have identified a potential role for serotonin in modulating *C. jejuni* colonization in the gut *in vitro*. Further studies are required to understand the practical implications of these findings for the control of *C. jejuni* enteric colonization *in vivo*.

Key words: serotonin, *Campylobacter jejuni*, gut, chicken, microbial endocrinology

2021 Poultry Science 100:100944
https://doi.org/10.1016/j.psj.2020.12.041

INTRODUCTION

The avian gut contains a repertoire of cellular machinery that synthesizes and releases neurochemicals into the gut lumen (Rawdon, 1984). Enteric concentrations of neurochemicals serve as interkingdom signaling molecules that can mediate a variety of changes in bacterial physiology that are often of consequence to interaction with the host (Neuman et al., 2015). Although investigations into microbial endocrinology, which is the study of neurochemical-based host–microbe interaction, have been carried out predominantly in mammalian models, an increasing number of studies have shown enteric neurochemicals to play key roles in affecting *Campylobacter jejuni* and other bacteria in the avian gut (Villageliu and Lyte, 2017). However, the majority of these studies have focused on the catecholamine neurochemicals (e.g. norepinephrine, epinephrine, and dopamine), which were demonstrated to affect *C. jejuni* gene expression (Xu et al., 2015), growth (Truccollo et al., 2020) as well as colonization of the broiler chicken gastrointestinal tract (Cogan et al., 2007; Aroori et al., 2014). It is unknown whether serotonin (5-hydroxytryptamine),
a monoamine neurochemical which is primarily produced in the gut, affects *C. jejuni* physiology or interaction with the gut epithelium. That *C. jejuni* from poultry remains a major cause of human campylobacteriosis and antibiotic alternative control strategies of this organism are needed in poultry (Allen et al., 2007; van Wagenberg et al., 2020), neurochemical-based targets in the gut may represent a novel antibiotic-alternative strategy to reduce prevalence of *C. jejuni* in preharvest poultry and therefore improve food safety.

Serotonin has been known for decades to be produced in the chicken intestinal tract and that its synthesis is affected by the presence of the microbiota (Phillips et al., 1961). Indeed, enterochromaffin cells, which are the type of gut neuroendocrine cell that synthesizes non-neuronal serotonin, were one of the first cells to be identified in the chicken gut epithelium (Rawdon, 1984). Recent investigations using mammalian models have shown enteric serotonin to affect the composition of the gut microbiota (Kwon et al., 2019) as well as modulate bacterial physiology (Knecht et al., 2016). Of particular note, immunocytochemical investigation into *Salmonella* Typhimurium invasion of porcine jejunal epithelial cells revealed very low invasion of enterochromaffin cells relative to other epithelial cell types (Schauser et al., 2004), potentially suggesting that areas of the epithelium where serotonin is concentrated may affect bacterial invasion. Although *C. jejuni* often colonizes the intestinal mucus layer (Van Deun et al., 2008), little is known about the distribution of serotonin in the chicken intestinal tract.

Previous studies have identified serotonin concentrations in full thickness sections of the intestinal tract of various chicken breeds (Phillips et al., 1961; Beaver and Wostmann, 1962; Rawdon, 1984). However, a literature search (PubMed, key terms used “chicken,” “serotonin,” “lumen,” “content,” “gut,” and/or “intestine”) revealed only a single study (Redweik et al., 2019) to have determined serotonin concentrations in the luminal content of any gut region. Considering evidence in rodents reporting serotonin concentrations to differ according tissue type (Thompson, 1966), further investigation into the biogeography of serotonin distribution in the chicken intestinal tract is warranted. Despite the occurrence of peristalsis and anti-peristalsis in the chicken gut (Clench and Mathias, 1992; Godwin and Russell, 1997), regional differences along the intestinal tract present unique physical and biochemical challenges that affect host–microbe interaction (Woodward et al., 2019). Although some *C. jejuni* strains can colonize each region of the intestinal tract (Chaloner et al., 2014), the highest numbers of this organism are typically found in the cecal mucosal crypts with lower prevalence in the small intestine, thereby indicating a role for gut biogeography in mediating *C. jejuni* colonization of the poultry gut. Production and concentration differences of serotonin in different gut regions may represent a potential neurochemical-based route of host interaction with *C. jejuni* and therefore a possible target to affect *C. jejuni* throughout the gut.

As such, we sought to determine serotonin concentrations in different regions of the broiler chicken intestinal tract and to investigate whether serotonin affects functional changes in *C. jejuni* physiology and interaction with an *in vitro* model of the gut epithelium.

**MATERIALS AND METHODS**

**Chickens and Tissue Collection**

All procedures and management practices were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC; protocol #20054) before the start of the study. We obtained 250 Cobb500 fertile eggs from a local hatchery, which were then incubated at the University of Arkansas Hatchery (Fayetteville, AR, United States of America) following the genetic line standard procedures (Cobb-Vantress, 2013). After hatching, 100 first-quality unsexed chicks were randomly selected, transferred to an experimental broiler house, and distributed in 4-floor pens, at a density of 25 birds per pen (1.5 × 3.0 m) on one-inch deep fresh wood-shaving top dressing over reused nontreated litter material. Birds were continuously provided ad libitum a crumbled pellet diet that exceeded the National Research Council (1994) requirements and was formulated according to the genetic line guidelines (Cobb-Vantress, 2018). The diet consisted of 3,035 kcal metabolizable energy per kg diet and 21% crude protein, 1.18% digestible lysine, 0.85% calcium, and 0.43% nonphytic phosphorus. Birds received 2 h of light during the first 3 d and then 6 h dark until 42 d. Light intensity was maintained between 27 and 44 lux throughout the whole period. Ventilation and temperature were automatically controlled to ensure a progressively changing temperature curve with 34°C, 32°C, 29°C, and 25°C at 0, 7, 14, and 42 d, respectively. Through the whole growing period, relative humidity was between 30 and 50%. As *C. jejuni* colonization in broiler chickens has been reported to occur between approximately 3 and 5 wk of age (Evans and Sayers, 2000; Van Deun et al., 2008; Connerton et al., 2018), we randomly selected 10 birds at 3 wk and then again at 5 wk of age for the determination of intestinal concentrations of serotonin. An n = 10 birds per age group was chosen as this number was previously demonstrated as sufficient to detect a significant (*P < 0.05*) change in chicken intestinal serotonin concentrations (Redweik et al., 2019). Birds were not challenged with *C. jejuni* in the present study. Birds were humanely euthanized by cervical dislocation.

To reduce variation in sample collection, the same researcher performed all mucosal scrapings and tissue collection. Intestinal segments (i.e., proximal ileum, cecum, and proximal colon) were dissected on petri dish was first removed, then ice was placed into the dish, after which the lid was replaced, and the tissue was dissected on the cold surface of the petri dish lid. A fresh petri dish and lid were used for each intestinal segment and each bird in order to eliminate cross-contamination between samples. A 2 cm long section of...
ileum, cecum, and colon was collected as this consistently gave the required 100–200 mg needed for ultrahigh-performance liquid chromatography with electrochemical detection (UHPLC-ECD) analysis. Each intestinal segment was carefully opened longitudinally using a ball-tipped scissor to avoid damaging the mucosa. Luminal content was collected without disturbing the mucosal layer. To collect mucosal scrapings, the section immediately below the collected luminal content was gently scraped a single time using a glass slide. Following the mucosal scraping, the entire underlying tissue (muscularis externa) was collected. All tissues were weighed, weight recorded, and then immediately submerged in 2 mL reinforced tubes containing 6 ceramic beads (Catalog #s: 19-648 and 19-646, Omni International, Kennesaw, GA) and 1 mL of 0.2 N perchloric acid (0.2 N perchloric acid consisted of HPLC-grade water (Catalog # 7732-18-5, VWR Life Science, Radnor, PA), and perchloric acid (Catalog #: AAA44464-AP, VWR Life Science)), then snap-frozen on dry ice, and stored at −80°C until analysis.

Ultrahigh-Performance Liquid Chromatography With Electrochemical Detection

Tissues were thawed and then homogenized twice in a Bead Ruptor (Catalog #: 19-040E, Omni International) for 30 s at 5 m per second, with samples allowed to rest for 10 s in between each 30 s cycle. Immediately following homogenization, samples were centrifuged at 3,000 × g and 4°C for 15 min. Sample supernatant was collected and placed into 2-3 kDA molecular weight cutoff spin filters (Catalog #: 89.132-006, VWR). Flow-through was stored at −80°C until UHPLC-ECD analysis as previously described (Villageliu et al., 2018). The UHPLC-ECD consisted of a Dionex Ultimate 3,000 autosampler, a Dionex Ultimate 3,000 pump, and a Dionex Ultimate 3,000 RS electrochemical detector (Thermo Fisher Scientific, Sunnyvale, CA). Buffered 10% acetonitrile (Catalog #: NC9777698, Thermo Fisher Scientific) was used as the mobile phases, and the flow rate was 0.6 mL/min on a 150 mm (length) 3 mm (internal diameter) 3 μm (particle size) Hypersil BDS C18 column (Catalog #: 28,103-153030, Thermo Fisher Scientific). All samples were kept at 4°C in the autosampler prior to injection. A 6041RS glassy carbon electrode set at 400 mV was used for electrochemical detection. Data was analyzed using the Chromeleon software package (version 7.2, Thermo Fisher Scientific), and serotonin identification was confirmed using the relative retention time of the corresponding analytical standard from Millipore-Sigma (serotonin, Catalog #: 61-47-2).

Bacterial Strain

The wild-type KADAMBIS8 strain of C. jejuni was isolated from the broiler chicken cecum in our laboratory and was used for all the experiments in this manuscript. The genome sequence of this strain was reported in GenBank under the accession number SFCH00000000 (Wagle et al., 2020b). C. jejuni inoculum was prepared as previously described (Shrestha et al., 2019). In brief, one loopful of C. jejuni glycerol stock was inoculated into 5 mL of sterile Campylobacter enrichment broth (CEB: Catalog # 7526A, Neogen Corp., Lansing, MI) and incubated at 42°C for 48 h in a microaerophilic atmosphere (5% O2, 10% CO2, and 85% N2). C. jejuni was subcultured under the same temperature and atmospheric conditions for 24 h. Cultures were then centrifuged at 3,500 × g for 10 min. The supernatant was discarded, and the cell pellet was reconstituted in CEB. C. jejuni inoculum was 10-fold diluted in CEB and then surface plated onto Campylobacter Line Agar (CLA) plates (Line, 2001) to enumerate the inoculum concentration.

C. jejuni Growth Assay

To determine the effect of serotonin on C. jejuni growth, we utilized a dose response curve of serotonin concentrations. These concentrations were based on broiler intestinal mucosa values (Figure 1) and those previously reported to assess an in vivo relevant effect on gut microbial species (Lopes and Sourjik, 2018; Kwon et al., 2019). The CEB was prepared with or without serotonin (Catalog #W224502, MilliporeSigma., St. Louis, MO) and added (100 μL/well) to sterile 96-well polystyrene plates ( Falcon, Corning Incorporated, Corning, NY). To determine an effect of inocula size, wells were inoculated with either 3 or 5 log CFU in a total volume of 100 μL of CEB. The final concentrations of serotonin in each well were 0, 0.01, 0.1, 1, and 6.25 mM. All plates were incubated at 42°C for 24 h under microaerophilic conditions. Bacterial growth was determined by culturing on CLA plates.

C. jejuni Motility Assay

Campylobacter jejuni motility was determined as previously described (Upadhyay et al., 2017) with slight modifications to assess an effect of serotonin. Motility medium (0.4% agar) was prepared alone or with serotonin concentrations of 0, 0.01, 0.1, or 1 mM, and 25 mM of motility medium was added to sterile petri dishes (VWR, Catalog #25384-342). A mid-log culture (10 h) of C. jejuni was centrifuged at 3,500 × g for 10 min, then the supernatant was discarded, and the cell pellet was washed twice in Butterfield’s phosphate diluent (BPD: 0.625 mM potassium dihydrogen phosphate, pH 6.7) before being reconstituted in BPD. Then, 5 μL of culture was stab inoculated at the center of the plate containing motility medium. The inoculum size of 7 log CFU/mL was used as this provided a visible zone of motility. All plates were incubated under microaerophilic condition at 42°C for 24 h, at which time the zone of motility was measured.
Liquid Chromatography Tandem Mass Spectrometry Proteomic Analysis

To determine an effect of serotonin on the proteome of \textit{C. jejuni}, we utilized liquid chromatography with tandem mass spectrometry (LC-MS/MS) as previously described (Miyamoto et al., 2015; Wagle et al., 2019; Wagle et al., 2020a). In brief, \textit{C. jejuni} (6 log CFU/mL) was incubated in the presence or absence of serotonin (1 mM) at 42°C for 12 h. This concentration of serotonin was used in several previous publications investigating an effect of serotonin on gut bacteria (Lopes and Sourjik, 2018; Kwon et al., 2019). \textit{C. jejuni} cultures were then centrifuged at 3,500 × g for 10 min, the supernatant was discarded, and cell pellets were washed with nuclease-free water (Catalog # AM9937, Thermo Fisher Scientific, Carlsbad, CA). Proteins were extracted from the cell pellets using B-Per bacterial protein extraction reagent (Catalog # 90,084 Thermo Fisher Scientific) and then subjected to SDS-PAGE (Catalog # NP0327BOX, NuPage 4-12% Bis-Tris protein gel, Thermo Fisher) at 220 V for 35 min. Gel lanes were sliced into 5 sections, and then each subsection was further excised (piece size of 1 mm²). Gel pieces from each subsection were processed and analyzed by LC-MS/MS separately, but data were combined before comparing different conditions. Gel pieces from each section were destained with 50% acetonitrile (Catalog # AX0145; Sigma-Aldrich) in ammonium bicarbonate (Catalog # A6141 Sigma-Aldrich) for 45 min. Dehydration of the gel pieces was accomplished by the addition of acetonitrile followed by vacuum drying for 10 min. Dehydrated gels were treated with dithiothreitol (Catalog #D9779, Sigma-Aldrich; 15 mg/mL in 25 mmol/L ammonium bicarbonate), followed by iodoacetamide (Catalog # 11,149, Sigma-Aldrich; 37 mg/mL in ammonium bicarbonate) for 1 h. An ammonium bicarbonate buffer wash was used to remove residual iodoacetamide before the gel pieces were again dehydrated using acetonitrile. Trypsin (Catalog #: 786-690 Biosciences St. Louis, MO; 20 ng/µL in 25 mmol/L ammonium bicarbonate) was used to digest (overnight, 37°C) proteins in the gel pieces. Peptides were analyzed by LC-MS/MS, which consisted of an Agilent 1,200 series micro flow HPLC coupled to a Bruker AmaZon-SL quadrupole ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, MA) with a captive spray ionization source. Protein identification was performed by matching MS/MS spectra to protein sequences of \textit{C. jejuni} NCTC 81-176 available at the Universal Protein Resource online database of annotation information and protein sequences (www.uniprot.org) using in house software (Matrix Science Inc., Boston, MA) MASCOT (Perkins et al., 1999). Proteins were identified based on <5% false discovery rate and 1 unique peptide from a protein.

\textbf{C. jejuni Adhesion and Invasion Assay}

\textit{C. jejuni} adhesion and invasion of the gut epithelium were determined using an \textit{in vitro} model as previously reported (Upadhyay et al., 2017) with limited modifications. We used a human colonic epithelial cell line as an immortalized chicken gut epithelial cell line has not yet

![Figure 1](https://example.com/figure1.png)

Figure 1. Biogeographical distribution of serotonin in the luminal content, mucosa, and muscularis externa of the broiler chicken ileum (A), cecum (B), and colon (C) at 3 and 5 wk of age. Significant difference (\(P < 0.05\)) was indicated as: *denotes comparison to luminal content of the same age group; # denotes comparison to mucosa of opposing age group; ^ denotes comparison to muscularis externa of the same age group. Values are µg of serotonin per g of tissue. All values are expressed as mean ± SEM (\(n = 10\) chickens/group).
been established. Prior published research has demonstrated relevance and utility of human gut epithelial cells in the study of interactions between the avian gut epithelium and C. jejuni as well as other bacteria (Byrne et al., 2007; Wagle et al., 2017; Yang et al., 2018). We selected the HT29MTX-E12 cell line (Millipore-Sigma, Catalog #12040401-1VL) as it has been reported to more closely approximate the in vivo epithelial environment and has been used extensively in the assessment of adhesion and invasion assays of C. jejuni and other enteric bacteria (Álemka et al., 2010; Gagnon et al., 2013). The HT29MTX-E12 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and L-glutamine (Corning, Catalog #10-013-CV) supplemented with (v/v) 10% fetal bovine serum (FBS; VWR, Catalog #10018-828). Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. Cells were seeded at a density of ~1 × 10^5 cells/well in 24 well culture plates (1.9 cm² per well; VWR, Catalog #29442-048), and culture media was changed every 2 d. Experiments were performed when cells were observed to be 90% confluent.

C. jejuni was inoculated at a multiplicity of infection 10:1 (6 log CFU/well) either alone (control) or with the subinhibitory concentration (SIC; 1 mM) of serotonin. The SIC is the highest concentration identified in the present study that did not cause growth inhibition of C. jejuni. Monolayers were incubated at 37°C for 1.5 h in a microaerophilic atmosphere. Immediately following incubation, monolayers were rinsed 3 times in DMEM to remove unattached C. jejuni cells and then incubated with 1 mL of 0.1% Triton X-100 (catalog no. 171315-01, Invitrogen, Carlsbad, CA) for 15 min. The contents of each well were serially diluted in BPD, plated onto CLA plates, and incubated for 48 h at 37°C under microaerophilic conditions before bacterial enumeration. The invasion assay was conducted in an identical manner to the adhesion assay except that following monolayer incubation of 1.5 h, monolayers were washed 3 times in minimal media (DMEM) and then incubated with DMEM containing gentamicin (100 µg/mL) for 2 h at 37°C to kill non-internalized bacteria. The DMEM containing gentamicin was then removed, and the monolayers were washed in DMEM. As done in the adhesion assay, cells were then lysed using Triton X-100, serially diluted, and plated on CLA before bacterial enumeration.

C. jejuni Quorum Sensing Assay

The effect of SIC of serotonin on C. jejuni autoinducer (AI)-2 production was determined using a Vibrio harveyi bioluminescence assay as per the standard published protocol with limited modifications (Bassler et al., 1994; Castillo et al., 2014). This protocol was previously validated for use with the C. jejuni strain used in the present study (Wagle et al., 2020a). In brief, C. jejuni was grown to mid-log phase (10 h) in the presence or absence of serotonin (1 mM) and then centrifuged at 3,500 × g for 10 min. Following centrifugation, the cell-free supernatant (CFS) was harvested and then filtered using a 0.2 µm syringe filter. The V. harveyi strain BB152 was grown overnight in Luria Bertani (LB) broth (Catalog #M1245, HiMedia Laboratories Pvt. Ltd., Mumbai, India) at 30°C before collection and 0.2 µm filtration to obtain CFS. Then, V. harveyi (BB170), the reporter strain in this assay, was grown in LB at 30°C for 24 h and diluted 1:5,000 with autoinducer assay (AA) medium (Inoculum ≈3 log CFU/mL). Next, 90 µL of the diluted reported strain was first dispensed into a 96-well white microtiter plate, and then followed by the addition of 10 µL of CFS of C. jejuni grown in the presence or absence of serotonin, or the CFS of V. harveyi strain BB152 (positive control) or AA medium (negative control). The luminescence of each well was then determined every 20 min over a total period of 8 h at 30°C using a Cytation 5 multimode reader (BioTek Instruments, Inc. Winooski, VT). Before the data analysis, the self-induction of luminescence in negative controls due to the growth of V. harveyi BB170 was deducted from treatments and positive controls.

**Statistical Analysis**

Bacterial counts were log transformed before analysis to achieve a normal distribution. All bacterial experiments had triplicate samples and were replicated 2 times (n = 6). Data from independent trials were pooled and analyzed using ANOVA with Tukey post hoc test for multiple comparisons on GraphPad Prism (version 8.4.3; La Jolla, CA). For the proteomic analysis, Scaffold Proteome Software version 4.8 (Proteome Software Inc, Portland, OR) was used to analyze MASCOT files. Differentially expressed proteins between serotonin-treated and untreated samples were determined using two-tailed unpaired Student’s t test. Intestinal serotonin data (n = 10 chickens per each age group) were checked for normality using the D’Agostino and Pearson test and analyzed using two-way ANOVA with Sidak post hoc test. Differences were considered significant at the threshold of P < 0.05.

**RESULTS**

**Determination of Serotonin Concentrations in the Broiler Chicken Gut**

Broiler chicken intestinal serotonin concentrations (µg of serotonin per g of tissue) in the ileum (Figure 1A) and the cecum (Figure 1B) were greater in the mucosa and muscularis externa (P < 0.05) compared to respective luminal content. Concentrations did not significantly differ (P > 0.05) between mucosa and muscularis externa in either ileum or cecum. Broiler age did not significantly (P > 0.05) alter serotonin concentrations in the mucosa, luminal content, or muscularis externa of either the ileum or the cecum. In the colon (Figure 1C), 3 wk of age broilers had greater (P < 0.05) mucosal serotonin concentration compared to 5 wk old broilers. At 3 wk of age, colonic mucosal serotonin
was higher \((P < 0.05)\) compared to that in the muscularis externa. At 5 wk of age, colonic mucosa was not different \((P > 0.05)\) compared to the muscularis externa. At both 3 and 5 wk of age, serotonin in the mucosa and the muscularis externa exceeded \((P < 0.05)\) that found in the colonic luminal content.

**The Effect of Serotonin on C. jejuni Growth**

The inoculum size of 3 log CFU/mL (Figure 2A) or 5 log CFU/mL (Figure 2B) was used to determine the effect of different concentrations of serotonin on C. jejuni growth *in vitro*. The growth in the control group, which was grown in the absence of serotonin, was 9.3 log CFU/mL (Figure 2A), which was not significantly different \((P > 0.05)\) compared to C. jejuni grown in the presence of 0.01, 0.1, or 1 mM serotonin. However, at the dose of 6.25 mM, the growth of C. jejuni was significantly \((P < 0.05)\) reduced when compared to the control group. A similar dose-dependent impact of serotonin concentration on C. jejuni growth at the inocula size of 5 log CFU/mL was observed where growth was unaffected \((P > 0.05)\) at 0.01, 0.1, or 1 mM but was significantly \((P < 0.05)\) reduced at 6.25 mM (Figure 2B).

**The Effect of Serotonin on C. jejuni Motility**

We determined the effect of subinhibitory concentrations of serotonin on C. jejuni motility (Figure 3). The control group, in which the motility medium did not contain serotonin, had a motility halo of 1.8 cm. Serotonin concentrations (0.01, 0.1, or 1 mM) did not significantly affect \((P > 0.05)\) C. jejuni motility compared to the control group. Motility did not differ between any of the serotonin treated groups.

**Proteomic Profile of C. jejuni Exposed to Serotonin**

Multiple proteins that are involved in intestinal colonization by C. jejuni were downregulated \((P < 0.05)\) by exposure to serotonin (Table 1). The exposure of C. jejuni to serotonin (1 mM) caused the downregulation of the high-affinity branched-chain amino acid ABC transporter periplasmic amino acid binding protein (gene name Cjj81176_1037; 2.32-fold downregulation), proteins with oxidoreductase activity and involved in microbial respiration (gene names’ PetC and OorA; -0.74-fold downregulation), as well as Enolase, a protein involved in carbohydrate metabolism (gene name Eno; -1.32-fold downregulation).

**The Effect of Serotonin on C. jejuni Adhesion/Invasion to HT29MTX-E12 cells**

In the absence of serotonin, 3.9 log CFU/well of C. jejuni was adhered to (Figure 4A) and 2.3 log CFU/well of C. jejuni had invaded (Figure 4B) HT29MTX-E12 cells. Serotonin was observed to decrease the adhesion of C. jejuni by 0.5 log CFU/well as compared to the control \((P < 0.05; \text{Figure 4A})\). Serotonin was not found to affect C. jejuni invasion of HT29MTX-E12 cells \((P > 0.05; \text{Figure 4B})\).

**The Effect of Serotonin on C. jejuni Quorum Sensing Activity**

The AI-2 concentration in the supernatant of C. jejuni (control) not exposed to serotonin was assayed using luminescence measurement and was observed to be 1,610 relative light units (RLU) at the completion of the assay. The value of 1,610 RLU corresponds to 17% of the RLU generated by the positive control, V. harveyi BB152. Treatment of C. jejuni with serotonin caused the reduction \((P < 0.05)\) of AI-2 concentrations (Figure 4). Luminescence measurement of AI-2 concentrations produced by serotonin-treated C. jejuni was 884 RLU (this was a 45% reduction compared to the positive control).

**DISCUSSION**

Neurochemicals, including serotonin, have long been demonstrated to serve as interkingdom signaling molecules between host and bacteria, often playing critical roles at principal sites of host–microbe interaction such
as the gut epithelium (Neuman et al., 2015). Despite the abundant production of serotonin in the chicken intestinal tract, the catecholamines (e.g., norepinephrine, dopamine, and epinephrine) have so far received the greatest attention in relation to their ability to affect the physiology and ability of bacteria, including *C. jejuni*, to colonize the chicken gut (Cogan et al., 2007; Aroori et al., 2014). As *C. jejuni* is a major foodborne pathogen in which the chicken gut serves as a primary reservoir, and serotonin is found throughout the chicken intestinal tract, we sought to determine enteric spatial concentrations of serotonin in the broiler gut and whether serotonin can affect *C. jejuni* physiology and colonization in vitro.

In the present study, we used Cobb500 chickens and serotonin concentrations identified in cecal tissue and luminal content (Figure 1) fell in the same approximate range as those previously reported in the White Leghorn cecum (Redweik et al., 2019). Likewise, the ileal tissue concentrations here reported (Figure 1) were near the same concentrations previously reported in the White Leghorn ileum (Phillips et al., 1961; Beaver and Wostmann, 1962). Attention to gross regional differences in serotonin concentrations under physiologically constitutive conditions has been reported in the duodenum, ileum (Phillips et al., 1961), and cecum (Beaver and Wostmann, 1962; Redweik et al., 2019) of White Leghorn chickens. Yet, little attention has been directed to the highly crossbred broiler breeds (e.g., Cobb500) that are widely used in the modern poultry industry. Our findings suggest that serotonin concentrations in the chicken gut may remain relatively unchanged despite genetic differences, which separate purebred (e.g., White Leghorn) and highly crossbred (e.g., Cobb500) chickens. This may lend feasibility to the development of neurochemical-based strategies within the framework of microbial endocrinology in targeting enteric colonization of food safety-related bacteria across various breeds of chickens involved in meat and egg production.

In the context of enteric host–microbe interaction, a critical aspect is the distribution of neurochemicals in the gut. This is particularly important for *C. jejuni* as this organism has been reported to proliferate predominantly in the mucosa (Van Deun et al., 2008) of the cecum, and, to a lesser extent, the colon and regions of the upper intestinal tract. We identified that in the Cobb500 ileum and cecum, mucosal concentrations of serotonin were relatively similar to the underlying muscularis externa. Conversely, mucosal concentrations of the colon were greater compared to the muscularis externa. These findings demonstrate that serotonin is present at sites in the chicken gut typically colonized by *C. jejuni*, thereby setting the foundation for *in vivo* chicken studies that investigate how these serotonin concentrations change in response to *C. jejuni* challenge and potentially modulate enteric colonization. An important aspect for examination in future studies is the impact of stress on chicken gut serotonin concentrations, as it has been previously demonstrated that stress can cause rapid increases in serotonin in the gut (Lyte et al., 2020).

As such, we examined a range of serotonin concentrations in affecting *C. jejuni* physiology. In agreement with Kwon et al. (2019), who investigated a dose-dependent effect of serotonin on several gut bacterial species but did not include *C. jejuni*, concentrations above 1 mM caused an inhibition in growth. Changes in the concentration of serotonin in the gut occur under different

Table 1. Differentially expressed proteins of *C. jejuni* among serotonin and control samples.

| Protein description                                      | Gene name             | Function               | Fold change in expression (Log2 transformed) |
|----------------------------------------------------------|-----------------------|------------------------|---------------------------------------------|
| Downregulated expression                                 |                       |                        | Serotonin                                    |
| High-affinity branched-chain amino acid ABC transporter   | CIJ81176_1037         | Adherence              | $-2.32^{b}$                                  |
| periplasmic amino acid-binding protein                   |                       |                        | 0*                                         |
| Ubiquinol—cytochrome c reductase                         | petC                  | Anaerobic respiration   | $-0.74^{b}$                                  |
| cytochrome c1 subunit                                    |                       |                        | 0*                                         |
| 2-oxoglutarate:acceptor oxidoreductase, alpha subunit OS | ooaA                  | Anaerobic respiration   | $-0.74^{b}$                                  |
| Enolase OS                                               | Eno                   | Metabolism             | $-1.32^{b}$                                  |

Different letters within row indicate significant change in expression ($P < 0.05$).
physiological conditions, such as restraint stress (Lyte et al., 2020). Indeed, enterochromafﬁn cells release serotonin directly into the lumen and stress can elicit increased serotonin production in the gut, thereby warranting future investigations to assess if stress may raise mucosal serotonin concentrations to affect C. jejuni growth in vivo. If stress is found to increase avian enteric serotonin in vivo, the results of the present study may hold implications linking bird welfare and susceptibility to C. jejuni colonization. Microbial endocrinology-based mechanisms have already been proposed to mediate host–microbe dynamics, including C. jejuni, in the poultry gut following production-relevant types of stress (Humphrey, 2006; Lara and Rostagno, 2013; Villageliu and Lyte, 2017). Moreover, the manipulation of serotonin concentrations in the gut is likely possible via the microbiota as serotonin is an interkingdom signaling molecule between host and microbe that can be sensed (Lyte and Brown, 2018) and metabolized (Nzakizwanayo et al., 2015) by speciﬁc gut bacteria, including probiotic strains.

As motility is an important factor in the ability of C. jejuni to colonize the gut, we examined whether serotonin could influence motility in vitro. While we did not find that serotonin impacted C. jejuni motility, this is perhaps unsurprising as Lopez and Sourjik (2018) had previously reported that serotonin at the same concentration used in the present study did not affect gut bacterial chemotaxis. Nevertheless, examination of serotonin on C. jejuni motility should be studied in other environments that more closely mimic the in vivo gut environment. Such a consideration is important as the effects of other neurochemicals, for example, the augmentation of growth of Gram-negative species by norepinephrine, are typically observed in iron-limited environments, which more closely resemble the conditions of the gut (Freestone et al., 2000).

Proteomic analysis revealed that serotonin caused the downregulation of several proteins that play roles in C. jejuni colonization. Proteins with oxidoreductase activity, including those here identiﬁed to be downregulated by serotonin, have been demonstrated to be involved in C. jejuni colonization of the gut (Weingarten et al., 2008). Likewise, the amino acid binding cassette (AA-ABC) transporter system has been identiﬁed as critical in the colonization of the chicken gut (Hendrixson and DiRita, 2004). Serotonin was found to downregulate an AA-ABC protein (Table 1) that is part of a transporter system of functional importance in C. jejuni colonization (Ribardo and Hendrixson, 2011).

Following on our proteomic results, we sought to assess if serotonin would inﬂuence C. jejuni interaction with the gut epithelium in vitro. Serotonin caused a reduction in adhesion but not invasion of a gut epithelial monolayer in vitro (Figure 4). Adhesion is a critical step of several bacterial colonization strategies, including C. jejuni in the gut mucosa (Beery et al., 1988; Hermans

---

**Figure 4.** The effect of SIC of serotonin (1 mM) on adhesion (A) and invasion (B) of C. jejuni wild-type S-8 to HT29MTX-E12 cells. Results are averages of 2 independent experiments, each containing triplicate samples (mean ± SEM). Different letters above bars indicate statistical difference at \( P < 0.05 \). Abbreviation: SIC, subinhibitory concentration.
et al., 2011). Relatedly, the ability of *C. jejuni* to invade human cell lines *in vitro* has been positively correlated with the ability to successfully colonize the chicken gut (Hanel et al., 2004; Pope et al., 2007). Considering the gut mucosa is a milieu of neurochemicals, not solely serotonin, further studies should assess whether the effect of serotonin on *C. jejuni* adhesion occurs *in vivo*. Indeed, catecholamine neurochemicals that are present in the gut have been reported to affect bacterial adhesion (Cambronel et al., 2019; Cambronel et al., 2020).

Similarly, catecholamines were reported to affect gut bacterial quorum sensing, which itself has been demonstrated critical in the regulation of host colonization (Koutsoudis et al., 2006). Although limited attention has been directed toward serotonin in this regard, Knecht et al. (2016) demonstrated serotonin affected quorum sensing activity in *Pseudomonas aeruginosa*. As such, we investigated if serotonin affected *C. jejuni* AI-2 production. We selected AI-2 as this quorum sensing molecule has been identified as important in the colonization of the chicken gut (Quinones et al., 2009). Serotonin was found to cause a reduction in *C. jejuni* AI-2 production (Figure 5), indicating a novel role of this gut neurochemical in the regulation of *C. jejuni* quorum sensing function. This finding should also be examined in the context of bacterial–bacterial cross-communication as AI-2 is produced by several other gut bacteria, including those relevant as food safety-related organisms in poultry such as *Salmonella* spp. (Cloak et al., 2002). It would therefore be of interest to examine how serotonin in the chicken gut affects overall microbial dynamics in relation to foodborne pathogen signaling and colonization.

Together, we have shown that serotonin concentrations in the broiler chicken gut exhibit distinct spatial distribution and that serotonin can affect the physiology of *C. jejuni* and its ability to colonize the gut epithelium *in vitro*. Our findings suggest a novel role for microbial endocrinology-based neurochemical host–microbe interaction in the chicken gut, potentially identifying serotonin as a novel target that may affect *C. jejuni* colonization. As other neurochemicals, such as norepinephrine, have been shown to affect *C. jejuni* colonization *in vivo*, further studies are warranted to evaluate a role of serotonin on *C. jejuni* colonization of the chicken gut *in vivo*.

**ACKNOWLEDGMENTS**

The USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. This research was supported, in part, by the U.S. Department of Agriculture, Agricultural Research Service.

This study was, in part, supported by internal Iowa State University funds provided by the W. Eugene Lloyd Chair in Toxicology to Mark Lyte. Rohana Liyanage would like to acknowledge the statewide mass spectrometry facility and the COBRE grant NIH P30 GM103450.

**DISCLOSURES**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

**REFERENCES**

Alemka, A., M. Clyne, F. Shanshan, T. Tompkins, N. Caccioniovoschi, and B. Bourke. 2010. Probiotic colonization of the adherent mucus layer of HT29MTXE12 cells attenuates Campylobacter jejuni virulence properties. Infect. Immun. 78:2812–2822.
Allen, V. M., S. A. Bull, J. E. Corry, G. Domingue, F. Jorgensen, J. A. Frost, R. Whyte, A. Gonzalez, N. Elviss, and T. J. Humphrey. 2007. Campylobacter spp. contamination of chicken carcasses during processing in relation to flock colonisation. Int. J. Food Microbiol. 113:54–61.

Aroori, S. V., T. A. Cogan, and T. J. Humphrey. 2014. Effect of noradrenaline on the virulence properties of Campylobacter species. Int. J. Microbiol. 2014:279075.

Bowell, M. A., T. S. Cogan, and L. E. Rees. 2014. Multiple signalling systems controlling expression of luminescence in Vibrio harveyi: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. 13:273–286.

Beaver, M. H., and B. S. Wostmann. 1962. Histamine and 5-hydroxytryptamine in the intestinal tract of germ-free animals, animals harbouring one microbial species and conventional animals. Br. J. Pharmacol. Chemother. 19:385–393.

Beery, J. T., M. B. Hugdahl, and M. P. Doyle. 1988. Colonization of gastrointestinal tracts of chicks by Campylobacter jejuni. Appl. Environ. Microbiol. 54:2365–2370.

Bezy, C. M., M. Clyne, and B. Bourke. 2007. Campylobacter jejuni adhere to and invade chicken intestinal epithelial cells in vitro. Microbiol 153:561–569.

Cambronel, M., F. Nilly, O. Mesguida, A. M. Boukerb, P. J. Racine, Evans, S. J., and A. R. Sayers. 2000. A longitudinal study of Campylobacter jejuni in the chicken gut. Vet. Microbiol. 101:75–82.

Cobb-Vantress. 2013. Hatchery management guide. Accessed May 833e29.pdf.

Cobb-Vantress, Spring 9th rev. ed. Natl. Acad. Press, Washington, DC.

Colebatch, J. H. Nash, K. Rahn, and G. W. Tannock. 2007. Epidemiology, relative invasive ability, molecular characterization, and competitive performance of Campylobacter jejuni strains in the chicken gut. Appl. Environ. Microbiol. 73:7959–7966.

Quinones, B. W., G. Miller, A. H. Bates, and R. E. Mandrell. 2009. Autoinducer-2 production in Campylobacter jejuni contributes to chicken colonization. Appl. Environ. Microbiol. 75:281–285.

Rawdon, B. V. 1984. Gastrointestinal hormones in birds: morphological, chemical, and developmental aspects. J. Exp. Zool. 232:659–670.
Redweik, G. A. J., K. Daniels, A. J. Severin, M. Lyte, and M. Mellata. 2019. Oral treatments with probiotics and live Salmonella vaccine induce unique changes in gut neurochemicals and microbiome in chickens. Front. Microbiol. 10:3064.

Ribardo, D. A., and D. R. Hendrixson. 2011. Analysis of the LIV system of Campylobacter jejuni reveals alternative roles for LivJ and LivK in commensalism beyond branched-chain amino acid transport. J. Bacteriol. 193:6233–6243.

Schauser, K., J. E. Olsen, and L. I. Larsson. 2004. Immunocytochemical studies of Salmonella Typhimurium invasion of porcine jejunal epithelial cells. J. Med. Microbiol. 53:691–695.

Shrestha, S., B. R. Wagle, A. Upadhyay, K. Arsi, I. Upadhyaya, D. J. Donoghue, and A. M. Donoghue. 2019. Edible coatings fortified with carvacrol reduce Campylobacter jejuni on chicken wingettes and modulate expression of select virulence genes. Front. Microbiol. 10:583.

Thompson, J. H. 1966. The distribution of serotonin (5-hydroxytryptamine) in the gastrointestinal mucosa of the rat: a comprehensive study. Irish J. Med. Sci. 41:411.

Truccollo, B., P. Whyte, and D. J. Bolton. 2020. An investigation of the effect of catecholamines and glucocorticoids on the growth and pathogenicity of Campylobacter jejuni. Pathogens 9.

Upadhyay, A., K. Arsi, B. R. Wagle, I. Upadhyaya, S. Shrestha, A. M. Donoghue, and D. J. Donoghue. 2017. Trans-cinnamaldehyde, carvacrol, and eugenol reduce Campylobacter jejuni colonization factors and expression of virulence genes in vitro. Front. Microb. 8:713.

Van Deun, K., F. Pasmans, R. Ducatelle, B. Flahou, K. Vissenberg, A. Martel, W. Van den Broeck, F. Van Immerseel, and F. Haesebrouck. 2008. Colonization strategy of Campylobacter jejuni results in persistent infection of the chicken gut. Vet. Microbiol. 130:285–297.

van Wagening, C. P. A., P. L. M. van Horne, and M. van Asseldonk. 2020. Cost-effectiveness analysis of using probiotics, prebiotics, or synbiotics to control Campylobacter in broilers. Poult. Sci. 99:4077–4084.

Villagei, D. N., and M. Lyte. 2017. Microbial endocrinology: Why the intersection of microbiology and neurobiology matters to poultry health. Poult. Sci. 96:2501–2508.

Villagei, D. N., S. Rasmussen, and M. Lyte. 2018. A microbial endocrinology-based simulated small intestinal medium for the evaluation of neurochemical production by gut microbiota. FEMS Microbiol. Ecol. 94.

Wagle, B. R., A. M. Donoghue, S. Shrestha, I. Upadhyaya, K. Arsi, A. Gupta, R. Liyanage, N. C. Rath, D. J. Donoghue, and A. Upadhyay. 2020a. Carvacrol attenuates Campylobacter jejuni colonization factors and promotes critical for persistence in the chicken gut. Poult. Sci. 99:4566–4577.

Wagle, B. R., D. Marasini, I. Upadhyaya, S. Shrestha, K. Arsi, A. M. Donoghue, F. Carbonero, D. J. Donoghue, K. Maas, and A. Upadhyay. 2020b. Draft genome sequences of Campylobacter jejuni strains isolated from poultry. Microbiol. Resour. Announc. 9.

Wagle, B. R., A. Upadhyay, K. Arsi, S. Shrestha, K. Venkitanarayanan, A. M. Donoghue, and D. J. Donoghue. 2017. Application of beta-resorcylic acid as potential antimicrobial feed additive to reduce Campylobacter colonization in broiler chickens. Front. Microbiol. 8:599.

Woodward, S. E., Z. Krekhno, and B. B. Finlay. 2019. Here, there, and everywhere: how pathogenic Escherichia coli sense and respond to gastrointestinal biogeography. Cell. Microbiol. 21:e13107.

Xu, F., C. Wu, F. Guo, G. Cui, X. Zong, B. Yang, and J. Lin. 2015. Transcriptomic analysis of Campylobacter jejuni NCTC 11168 in response to epinephrine and norepinephrine. Front. Microbiol. 6.

Yang, Y., J. D. Latorre, B. Khatri, Y. M. Kwon, B. W. Kong, K. D. Teague, L. E. Graham, A. D. Wollfenden, B. D. Mahaffey, M. Baxter, X. Hernandez-Velasco, R. Merino-Guzman, B. M. Hargis, and G. Tellez. 2018. Characterization and evaluation of lactic acid bacteria candidates for intestinal epithelial permeability and Salmonella Typhimurium colonization in neonatal Turkey pouls. Poult. Sci. 97:724.