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Tissue-, age- and dose-dependent changes in avian β-defensin and LEAP2 mRNA abundance in the intestines of *Salmonella* Typhimurium challenged broilers

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ABSTRACT

Salmonella is a pathogen normally found in the gastrointestinal tract of poultry. The objective of this study was to determine changes in avian *β*-defensin (AvBD) and liver-enriched antimicrobial peptide 2 (LEAP2) mRNA following *Salmonella* challenge. Day of hatch chicks were challenged with 10^6, 10^7 or 10^8 colony-forming units (cfu) of *Salmonella typhimurium*. There were dose-, tissue- and age-specific changes in AvBD and LEAP2 mRNA. At 1-day post-infection (dpi) there was a transient upregulation of AvBD1, 8, 10 and 12 mRNA in the 10^8 cfu group. At 5 dpi, all seven AvBD mRNA were downregulated in the ileum, while only AvBD1, 6, 10 and 11 mRNA were downregulated in the jejunum and AvBD6, 8, 10, 12 and 13 were downregulated in the cecum. At 7 dpi, there was downregulation of all seven AvBD mRNA in the duodenum and downregulation of selected AvBD in the jejunum, ileum and cecum. LEAP2 mRNA was downregulated at all doses of *Salmonella* in the cecum at 1 dpi and in the ileum at 5 dpi. In summary, *Salmonella* infection caused an initial upregulation followed by a downregulation of AvBD mRNA.

KEYWORDS

Salmonella typhimurium; Avian *β*-defensin; LEAP2

Introduction

In the USA, *Salmonella* is the leading cause of bacterial foodborne illness.[1] *Salmonella* serovars, which cause illness in humans, are commonly found in the gastrointestinal tract of poultry, but usually do not cause illness in poultry. In most cases, birds may show little to no signs of being infected with *Salmonella*. Once the organism enters the bird via the oral route, it travels down the gastrointestinal tract and reaches the ileum and cecum. There it must outcompete the microflora, penetrate the mucosal epithelium and invade the epithelial cells with the use of proteins encoded within the *Salmonella* pathogenicity island 1.[2–4]

Salmonella colonization can induce an inflammatory response, which leads to an innate immune response by the host.[5,6] Avian *β*-defensin (AvBD) and liver-enriched antimicrobial peptide 2 (LEAP2) are two classes of host defense peptides (HDP), which are part of the innate immune system.[7] HDP are a diverse group of small peptides that are enriched with hydrophobic and cationic amino acid residues.[8]

A number of studies have examined HDP mRNA abundance in various tissues in response to a *Salmonella* challenge with varying results. This is likely due to a number of variables such as chicken line and sex, *Salmonella* serovar and dosage, age at infection and time post-infection of sample analysis. Challenge with various *Salmonella* serovars caused upregulation of AvBD mRNA in the cecal tonsils[9] and small intestine[10,11] of chickens. Similarly in geese, *Salmonella* challenge resulted in upregulation of AvBD mRNA in the small intestine.[12,13] AvBD mRNA has also been found to be upregulated in the reproductive tracts of *Salmonella*-infected chickens.[14] Furthermore, LEAP2 mRNA was upregulated in the intestines of *Salmonella* challenged chickens.[11,15] In contrast, Milona et al.[16] and Crhanova et al.[17] reported no changes in AvBD mRNA abundance in the small intestine and ceca of *Salmonella* challenged chickens.

The objective of this study was to examine changes in the mRNA abundance of AvBD and LEAP2 in the gastrointestinal tracts of young broiler chicks

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following a challenge with *Salmonella typhimurium* at 10^6, 10^7 or 10^8 cfu/chick.

Materials and methods

Salmonella challenge and tissue sampling

This study was approved by the Southern Plains Agricultural Research Center Animal Care and Use Committee and conducted at the Food and Feed Safety Research Laboratory (USDA Agricultural Research Service, College Station, TX, USA). Two hundred day of hatch chicks (Ross 308 cross broilers) were obtained from a local commercial hatchery and transported to the USDA-ARS facility. The paper chick tray liners were tested for the presence of *Salmonella*.[18,19] Chicks were housed in an environmentally-controlled room with four 1.8 × 1.4 m floor pens with fresh pine shavings. Fifty chicks were randomly assigned to each of four pens. To challenge the chicks with *Salmonella*, a stock strain of *S. typhimurium* (accession number 87-26541, case #4884) was obtained from the National Veterinary Services Laboratory (Ames, IA, USA). A novobiocin (NO)-nalidixic acid (NA) resistant strain was selected for use in these experiments and therefore all culture media used contained 25 µg/mL of NO and 20 µg/mL of NA. This strain was grown as an overnight culture and transferred every 24 h for 3 days in tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ, USA) containing NO and NA.[20] Bacterial titers were determined by dilution in phosphate-buffered saline (PBS) and incubation on XLT-4 plates containing NO and NA for 24 h at 37 °C. Day of hatch chicks ($n = 50$) were orally gavaged with 0.5 mL PBS containing 10^6, 10^7 or 10^8 cfu *S. typhimurium* or gavaged with 0.5 mL of sterile PBS (control). Chicks were placed into pens and provided feed and water *ad libitum*. The feed was a non-medicated corn-soybean meal starter diet that was formulated to meet or exceed the National Research Council nutrient requirements for poultry.[21] On 1-, 2-, 5- and 7-day post-infection (dpi, $n = 6$/group), chickens were euthanized by cervical dislocation and samples were collected. This represents an experimental design that includes: 4 challenge levels (control and 3 *Salmonella* doses) × 4 sampling times (1, 2, 5 and 7 dpi) for a total of 16 different treatments. The experimental unit was an individual chick. On sampling days, cecal contents were collected and tested for the presence of *Salmonella* by plating on XLT4 plates containing NO and NA. About 1–2 cm sections of duodenum, jejunum, ileum and cecum were collected, rinsed in PBS and stored in RNAlater (Qiagen, Germantown, MD, USA). Samples were frozen at −80 °C, until shipment to Virginia Tech.

RNA extraction and real-time quantitative PCR

Total RNA was extracted using the Direct-zol RNA Miniprep protocol (Zymo Research Co., Irvine, CA, USA) from duodenal, jejunal, ileal and cecal samples ($n = 5$) that were collected at 1, 2, 5 and 7 dpi. These sampling days were selected to examine early and late responses to the *Salmonella* challenge and were based on results from preliminary trials. The RNA concentration for each sample was quantified using a Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA). The HDP analyzed included LEAP2 and the avian β-defensins AvBD1, AvBD6, AvBD8, AvBD10, AvBD11, AvBD12 and AvBD13. The cDNA was synthesized from 500 ng of total RNA using the High-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Each of the real-time quantitative PCR reactions contained 5 µL of Fast SYBR Green Master Mix (Applied Biosystems), 1 µL of forward primer (5 µM), 1 µL of reverse primer (5 µM), 2 µL of diethyl pyrocarbonate-treated water and 1 µL of diluted cDNA (1:30) and the reactions were run in duplicate in a 7500 Fast Real-time PCR instrument (Applied Biosystems) using the default program (95 °C for 20 s, 40 cycles of 95 °C for 3 s and 60 °C for 30 s). The primers for each of the genes are listed in Table 1. Primer efficiency (mean ± SD) was determined using 5 different RNA samples and the Applied Biosystems 7500 relative standard curve program. Fold change was calculated using the ΔΔCt method.[25] The geometric mean of the expression of cRPL4 (ribosomal protein L4) and cRPLP1 (ribosomal protein lateral stalk subunit P1) served as reference genes to calculate ΔCt. For each individual day, the average ΔCt of the control duodenum, jejunum, ileum and ceca was used as the calibrator to calculate ΔΔCt of corresponding treatment tissues.

Statistical analysis

Salmonella recovery levels (cfu/g of cecal contents, $n = 6$ per treatment) were analyzed by ANOVA using the JMP Pro 14 software (SAS Institute, Cary, NC, USA). The model included the main effect of log10 cfu, sorted by day. Significant differences ($p < 0.05$) were further separated using Tukey's test. Chi-Square analysis was performed to determine significant differences between groups in *S. typhimurium* recovery levels and ceca colonization. Changes in expression of

Table 1. Primers used for real-time quantitative PCR.

Gene	Description	Forward primer/Reverse primer	Amplicon size (bp)	Primer efficiency[a]	Accession No.
AvBD1[b]	Avian β-defensin 1	GAGTGGCTTCTGTGCATTTCTG/ TTGAGCATTTCCCACTGATGAG	62	98.9 ± 5.3	NM_204993.1
AvBD6[b]	Avian β-defensin 6	GCCCTACTTTTCCAGCCCTATT/ GGCCCAGGAATGCAGACA	63	96.2 ± 2.3	NM_001001193.1
AvBD8[b]	Avian β-defensin 8	ATGCGCGTACCTAACAACGA/ TGCCCAAAGGCTCTGGTATG	95	96.0 ± 3.1	NM_001001781.1
AvBD10[b]	Avian β-defensin 10	CAGACCCACTTTTCCCTGACA/ CCCAGCACGGCAGAAATT	64	92.2 ± 2.9	NM_001001609.2
AvBD11[b]	Avian β-defensin 11	GGTACTGCATCCGTTCCAAAG/ GCATGTTCCAAATGCAGCAA	56	95.1 ± 5.6	NM_001001779.1
AvBD12[b]	Avian β-defensin 12	TGTAACCACGACAGGGGATTG/ GGGAGTTGGTGACAGAGGTTT	114	88.9 ± 4.7	NM_001001607.2
AvBD13[b]	Avian β-defensin 13	CAGCTGTGCAGGAACAACCA/ CAGCTCTCCATGTGGAAGCA	59	96.5 ± 4.3	NM_001001780.1
LEAP2[c]	Liver-enriched antimicrobial peptide 2	CTCAGCCAGGTGTACTGTGCTT/ CGTCATCCGCTTCAGTCTCA	66	101.9 ± 3.7	NM_001001606.1
cRPL4[d]	Ribosomal protein L4	TCAAGGCGCCCATTCG/ TGCGCAGGTTGGTGTGAA	55	87.9 ± 5.1	NM_001007479.1
cRPLP1[d]	Ribosomal protein, lateral stalk subunit P1	TCTCCACGACGACGAAGTCA/ CCGCCGCCTTGATGAG	63	92.6 ± 5.6	NM_205322.1

[a]Primer efficiency is shown as mean ± SD of five different RNA samples.
[b–d]Primer sequences are from Casterlow et al.,[22] Su et al.[23], and Zhang and Wong.[24]

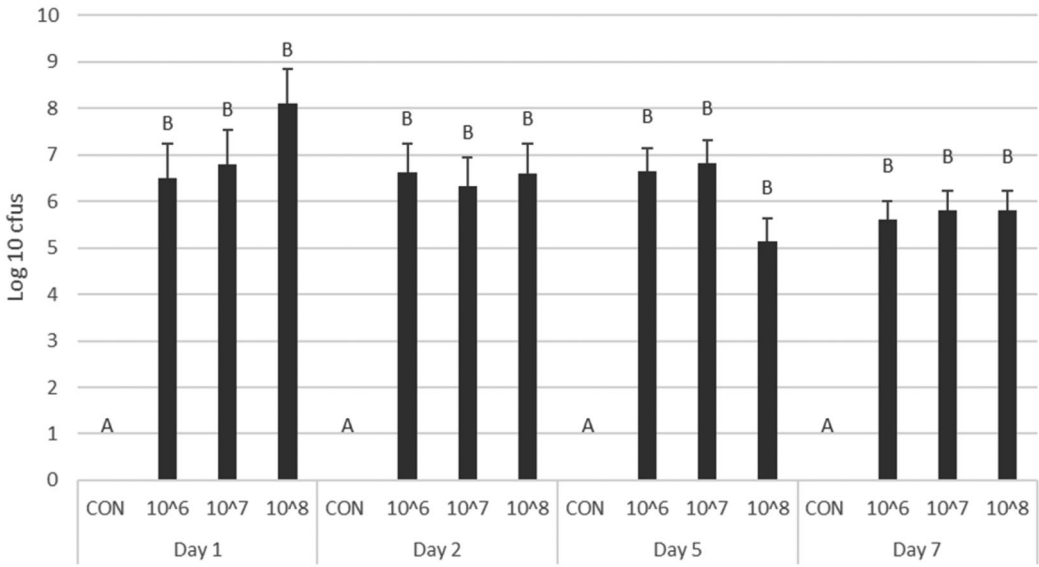

Figure 1. Recovery levels of *Salmonella* from the ceca of broilers 1-, 2-, 5- and 7-day post-infection. Chicks were challenged with 10^6 (10^6), 10^7 (10^7) and 10^8 (10^8) cfu of *Salmonella*. CON are control chicks that were not challenged. *Salmonella* were titered on XLT-4 plates containing 25 μg/mL of novobiocin and 20 μg/mL of nalidixic acid for 24 h at 37 °C. Bars with different letters are significantly different ($p < 0.05$).

HDP ($n = 5$ per treatment) were analyzed by ANOVA in the JMP Pro 14 software (SAS Institute). The pooled standard error was obtained from ANOVA. The model included the main effect of treatment, sorted by tissue and genes. Significant differences ($p < 0.05$) were further separated using Tukey's test.

Results

Salmonella challenge of broilers

The cecal contents were collected from broilers challenged with 10^6, 10^7 or 10^8 cfu of *S. typhimurium* at 1, 2, 5 and 7 dpi and the titer of *S. typhimurium* was determined (Fig. 1). At 1, 2, 5 and 7 dpi, there were no colonies of *S. typhimurium* detected in the control chicks. All three treatment groups at 1, 2, 5 and 7 dpi had greater than 10^5 colonies recovered with no difference among these groups.

AvBD and LEAP2 mRNA abundance – 1-day post-infection with S. typhimurium

Changes in the mRNA abundance of AvBD and LEAP2 at 1 dpi with *S. typhimurium* are shown in Table 2. AvBD1 mRNA was upregulated in the 10^8

Table 2. Avian β-defensin and LEAP2 mRNA abundance in *Salmonella typhimurium*-infected and non-infected broilers at 1-day post-infection.

		Gene[b]							
Tissue	Challenge[a]	AvBD1	AvBD6	AvBD8	AvBD10	AvBD11	AvBD12	AvBD13	LEAP2
Duodenum	CON	1.18^{B}	1.43	1.15^{B}	1.39^{B}	1.18	1.19^{B}	1.18^{AB}	1.78
	10^6 cfu	1.71^{AB}	1.05	1.78^{AB}	1.43^{B}	2.37	2.09^{AB}	1.72^{AB}	0.25
	10^7 cfu	0.59^{B}	0.47	0.67^{B}	0.51^{B}	0.58	0.56^{B}	0.54^{B}	0.27
	10^8 cfu	2.99^{A}	2.12	3.24^{A}	5.02^{A}	3.19	3.86^{A}	2.66^{A}	0.18
p value		**0.003**	0.08	**0.002**	**0.01**	0.09	**0.001**	**0.03**	0.17
Pooled SEM		0.38	0.42	0.39	0.84	0.73	0.49	0.46	0.56
Jejunum	CON	1.49^{B}	1.62	1.35	1.93	1.77	1.59	2.12	2.65
	10^6 cfu	2.89^{AB}	2.79	3.84	8.23	4.23	5.12	4.02	0.28
	10^7 cfu	0.59^{B}	0.59	0.92	1.52	1.91	1.79	1.24	0.35
	10^8 cfu	5.19^{A}	4.29	7.53	8.21	7.45	7.94	6.03	0.29
p value		**0.01**	0.06	0.09	0.24	0.27	0.21	0.24	0.29
Pooled SEM		0.90	0.90	1.91	3.02	2.21	2.34	1.71	1.01
Ileum	CON	1.57	1.39	1.24	1.28^{B}	1.30	1.65	2.65	2.42
	10^6 cfu	1.67	1.99	2.87	0.66^{B}	2.06	4.94	2.25	0.54
	10^7 cfu	0.53	0.40	0.69	0.17^{B}	0.46	0.55	0.56	0.16
	10^8 cfu	3.74	4.16	6.73	6.89^{A}	5.22	2.62	3.69	0.06
p value		0.11	0.16	0.12	**0.0002**	0.10	0.31	0.39	0.15
Pooled SEM		0.88	1.14	1.83	0.89	1.33	1.63	1.27	0.78
Cecum	CON	1.56	1.43	1.10	1.69^{AB}	1.45	1.83	1.76	1.11^{A}
	10^6 cfu	1.66	1.73	1.82	0.68^{AB}	1.84	2.08	1.89	0.02^{B}
	10^7 cfu	0.62	0.52	0.67	0.24^{B}	0.56	0.61	0.64	0.07^{B}
	10^8 cfu	1.78	2.15	3.18	2.35^{A}	1.93	2.99	2.49	0.03^{B}
p value		0.46	0.35	0.27	**0.04**	0.38	0.38	0.41	**<0.0001**
Pooled SEM		0.56	0.64	0.92	0.50	0.59	0.94	0.77	0.14

[a]Broilers were infected with 0 (CON), 10^6, 10^7 or 10^8 cfu of *Salmonella typhimurium*.
[b]Values with a different letter (A–B) within a gene and a tissue differ significantly ($p < 0.05$). $p < 0.05$ is highlighted in bold type AvBD1 = Avian β-defensin 1; AvBD6= Avian β-defensin 6; AvBD8 = Avian β-defensin 8; AvBD10 = Avian β-defensin 10; AvBD11 = Avian β-defensin 11; AvBD12 = Avian β-defensin 12; AvBD13 = Avian β-defensin 13; LEAP2 = liver-enriched antimicrobial peptide 2.

cfu group compared to the 10^7 cfu and the control groups in the duodenum ($p = 0.003$) and jejunum ($p = 0.01$). AvBD8 mRNA was upregulated in the duodenum of the 10^8 cfu group compared to the 10^7 cfu and the control groups ($p = 0.002$). AvBD10 mRNA was upregulated in the 10^8 cfu group compared to the control, 10^6 and 10^7 cfu groups in the duodenum ($p = 0.01$) and in the ileum ($p = 0.0002$). In the cecum, AvBD10 mRNA was upregulated in the 10^8 cfu group compared to the 10^7 cfu group ($p = 0.04$), but was not different from the control. AvBD12 mRNA was upregulated in the 10^8 cfu group compared to the control and 10^7 cfu group ($p = 0.001$). AvBD13 mRNA was upregulated in the 10^8 cfu group compared to the 10^7 cfu group ($p = 0.03$).

LEAP2 mRNA was downregulated in the cecum of the 10^6, 10^7 and 10^8 cfu groups compared to the control ($p < 0.0001$) (Table 2).

AvBD and LEAP2 mRNA abundance – 2-day post-infection with S. typhimurium

Changes in the mRNA abundance of AvBD and LEAP2 at 2 dpi with *S. typhimurium* are shown in Table 3. AvBD10 mRNA was upregulated in the duodenum of the 10^8 cfu group compared to the 10^6 cfu group ($p = 0.02$). There were no changes in mRNA abundance of AvBD1, AvBD6, AvBD8, AvBD11, AvBD12, AvBD13 and LEAP2. In the duodenum, AvBD11 and AvBD13 mRNA showed significant differences by ANOVA but not by Tukey's test.

AvBD and LEAP2 mRNA abundance – 5-day post-infection with S. typhimurium

Changes in the mRNA abundance of AvBD and LEAP2 at 5 dpi with *S. typhimurium* are shown in Table 4. There was downregulation in all *Salmonella* challenged groups (10^6, 10^7 and 10^8 cfu) compared to the control for AvBD1 mRNA in the jejunum ($p = 0.02$) and ileum ($p = 0.001$), for AvBD6 mRNA in the jejunum ($p = 0.01$), ileum ($p = 0.01$) and cecum ($p = 0.01$), and for AvBD8 mRNA in the ileum ($p = 0.01$) and cecum ($p = 0.02$). For AvBD10 mRNA, there was downregulation in the 10^6, 10^7 and 10^8 cfu groups compared to the control in the jejunum ($p = 0.01$), ileum ($p = 0.001$) and cecum ($p = 0.002$). In the duodenum, AvBD10 mRNA was downregulated only in the 10^8 cfu group compared to the control ($p = 0.03$). There was downregulation in all *Salmonella* challenged groups (10^6, 10^7 and 10^8 cfu) compared to the control for AvBD11 mRNA in the jejunum ($p = 0.02$) and ileum ($p = 0.0001$), for AvBD12 mRNA in the jejunum ($p = 0.01$), ileum ($p = 0.001$) and cecum ($p = 0.001$) and for AvBD13 mRNA in the ileum ($p = 0.005$) and cecum ($p = 0.004$). In the

Table 3. Avian β-defensin and LEAP2 mRNA abundance in *Salmonella typhimurium*-infected and non-infected broilers at 2-day post-infection.

		Gene[b]							
Tissue	Challenge[a]	AvBD1	AvBD6	AvBD8	AvBD10	AvBD11	AvBD12	AvBD13	LEAP2
Duodenum	CON	1.92	1.25	1.07	1.12^{AB}	1.07	1.19	1.09	1.10
	10^6 cfu	1.86	1.26	0.88	0.77^{B}	0.80	1.43	1.09	0.78
	10^7 cfu	3.28	1.31	0.97	2.03^{AB}	2.25	2.24	2.14	1.61
	10^8 cfu	0.34	1.42	1.24	2.36^{A}	2.87	2.88	1.98	1.83
p Value		0.09	0.26	0.54	**0.02**	0.04*	0.051	0.03*	0.19
Pooled SEM		0.73	0.26	0.18	0.35	0.52	0.43	0.28	0.36
Jejunum	CON	1.59	1.08	1.10	1.08	1.05	1.16	1.05	1.05
	10^6 cfu	1.65	1.20	0.87	0.89	0.64	1.31	0.55	0.95
	10^7 cfu	2.12	1.61	1.16	1.41	1.01	1.26	0.73	0.78
	10^8 cfu	0.58	2.38	1.91	3.75	3.11	3.34	1.79	1.86
p Value		0.19	0.25	0.35	0.08	0.08	0.11	0.11	0.46
Pooled SEM		0.48	0.48	0.42	0.80	0.69	0.69	0.36	0.50
Ileum	CON	2.15	1.21	1.03	1.11	1.13	1.26	1.17	1.09
	10^6 cfu	1.03	0.81	0.59	1.18	0.97	0.78	0.79	0.40
	10^7 cfu	2.02	1.05	0.86	1.69	1.54	1.18	1.36	1.09
	10^8 cfu	0.42	1.50	1.35	3.60	3.81	1.99	2.74	0.67
p Value		0.07	0.38	0.39	0.26	0.27	0.51	0.35	0.42
Pooled SEM		0.49	0.27	0.31	0.96	1.11	0.56	0.79	0.34
Cecum	CON	2.14	2.53	1.06	1.18	1.11	1.22	1.15	1.11
	10^6 cfu	1.02	1.66	0.49	1.19	1.22	1.12	1.32	0.60
	10^7 cfu	1.34	2.34	0.57	0.92	1.23	1.04	1.11	1.04
	10^8 cfu	0.17	1.57	0.52	1.39	1.58	0.77	1.04	1.19
p Value		0.23	0.43	0.06	0.77	0.73	0.78	0.91	0.47
Pooled SEM		0.65	0.48	0.16	0.31	0.31	0.31	0.29	0.28

[a]Broilers were infected with 0 (CON), 10^6, 10^7 or 10^8 cfu of *Salmonella typhimurium*.
[b]Values with a different letter (A–B) within a gene and a tissue differ significantly ($p < 0.05$). $p < 0.05$ is highlighted in bold type *indicates significance by ANOVA but not by Tukey's test. AvBD1 = Avian β-defensin 1; AvBD6 = Avian β-defensin 6; AvBD8 = Avian β-defensin 8; AvBD10 = Avian β-defensin 10; AvBD11 = Avian β-defensin 11; AvBD12 = Avian β-defensin 12; AvBD13 = Avian β-defensin 13; LEAP2 = liver-enriched antimicrobial peptide 2.

cecum, AvBD1 mRNA showed a significant difference by ANOVA but not by Tukey's test.

LEAP2 mRNA was downregulated in the ileum of all *Salmonella*-challenged groups (10^6, 10^7 and 10^8 cfu) compared to the control ($p = 0.02$) (Table 4). In the cecum, LEAP2 mRNA showed a significant difference by ANOVA but not by Tukey's test.

AvBD and LEAP2 mRNA abundance – 7-day post-infection with S. typhimurium

Changes in the mRNA abundance of AvBD and LEAP2 at 7 dpi with *S. typhimurium* are shown in Table 5. AvBD1 mRNA was downregulated in the duodenum of the 10^7 and 10^8 cfu groups compared to the control ($p = 0.01$). AvBD6 mRNA was downregulated in the 10^7 and 10^8 cfu groups compared to the control in the duodenum ($p = 0.003$) and ileum ($p = 0.03$). In the jejunum, AvBD6 mRNA was downregulated in the 10^6, 10^7 and 10^8 cfu groups compared to the control ($p = 0.005$). AvBD8 mRNA was downregulated in the 10^7 and 10^8 cfu groups compared to the control in the duodenum ($p = 0.01$) and jejunum ($p = 0.02$). AvBD10 mRNA was downregulated in the 10^7 and 10^8 cfu groups compared to the control in the duodenum ($p = 0.002$), jejunum ($p = 0.02$) and cecum ($p = 0.004$). AvBD11 mRNA was downregulated in the 10^7 and 10^8 cfu groups compared to the control in the duodenum ($p = 0.01$), but downregulated in only the 10^8 cfu group compared to the control in the cecum ($p = 0.02$). AvBD12 mRNA was downregulated in the duodenum of the 10^7 and 10^8 cfu groups compared to the 10^6 cfu group ($p = 0.02$) and downregulated in the cecum of the 10^8 cfu group compared to the 10^6 cfu group and the control ($p = 0.01$). AvBD13 mRNA was downregulated in the duodenum of the 10^7 and 10^8 cfu groups compared to the 10^6 cfu group ($p = 0.01$) and downregulated in the cecum of the 10^8 cfu group compared to the control ($p = 0.02$).

LEAP2 mRNA abundance was not affected by *Salmonella* challenge in any intestinal segment with any of the three doses tested (Table 5).

Discussion

The expression of AvBD mRNA in various intestinal tissues was altered by challenge with various *Salmonella* serovars. In studies where older terminology, such as gallinacins, was reported, the current avian β-defensin names are substituted.[26] Akbari et al.[9] examined abundance of AvBD1, 2, 4 and 6 mRNA in the cecal tonsils following challenge of 1-day-old female broilers with 10^6 cfu of *S. typhimurium*. There were no changes in any of the AvBD mRNA at 1 dpi, but all four AvBD mRNA were

Table 4. Avian β-defensin and LEAP2 mRNA abundance in *Salmonella typhimurium*-infected and non-infected broilers at 5-day post-infection.

		Gene[b]							
Tissue	Challenge[a]	AvBD1	AvBD6	AvBD8	AvBD10	AvBD11	AvBD12	AvBD13	LEAP2
Duodenum	CON	1.51	1.33	1.34	1.26^{A}	1.90	1.40	1.18	1.19
	10^6 cfu	0.31	0.32	0.35	0.36^{AB}	0.53	0.41	0.44	0.73
	10^7 cfu	0.47	0.53	0.63	0.51^{AB}	0.88	0.70	0.60	0.96
	10^8 cfu	0.47	0.47	0.72	0.31^{B}	0.57	0.44	0.57	1.01
p Value		0.18	0.10	0.19	**0.03**	0.09	0.09	0.09	0.45
Pooled SEM		0.41	0.29	0.31	0.23	0.40	0.29	0.21	0.20
Jejunum	CON	1.36^{A}	1.26^{A}	1.17	1.30^{A}	1.38^{A}	1.25^{A}	1.41	1.13
	10^6 cfu	0.28^{B}	0.34^{B}	0.41	0.22^{B}	0.24^{B}	0.34^{B}	0.37	0.78
	10^7 cfu	0.25^{B}	0.29^{B}	0.36	0.31^{B}	0.26^{B}	0.27^{B}	0.37	1.03
	10^8 cfu	0.29^{B}	0.31^{B}	0.46	0.17^{B}	0.25^{B}	0.30^{B}	0.43	0.99
p Value		**0.02**	**0.01**	0.04*	**0.01**	**0.02**	**0.01**	0.07	0.34
Pooled SEM		0.25	0.22	0.20	0.24	0.26	0.21	0.30	0.13
Ileum	CON	1.30^{A}	1.51^{A}	1.50^{A}	1.38^{A}	1.21^{A}	1.21^{A}	1.54^{A}	1.41^{A}
	10^6 cfu	0.20^{B}	0.20^{B}	0.20^{B}	0.13^{B}	0.16^{B}	0.24^{B}	0.18^{B}	0.36^{B}
	10^7 cfu	0.24^{B}	0.24^{B}	0.27^{B}	0.19^{B}	0.21^{B}	0.28^{B}	0.22^{B}	0.41^{B}
	10^8 cfu	0.12^{B}	0.12^{B}	0.17^{B}	0.11^{B}	0.09^{B}	0.13^{B}	0.13^{B}	0.34^{B}
p Value		**0.001**	**0.01**	**0.01**	**0.001**	**0.0001**	**0.001**	**0.005**	**0.02**
Pooled SEM		0.18	0.27	0.27	0.19	0.14	0.16	0.27	0.25
Cecum	CON	1.36	1.57^{A}	1.39^{A}	1.18^{A}	1.42	1.14^{A}	1.17^{A}	1.32
	10^6 cfu	0.28	0.26^{B}	0.22^{B}	0.16^{B}	0.22	0.29^{B}	0.28^{B}	0.36
	10^7 cfu	0.19	0.23^{B}	0.19^{B}	0.14^{B}	0.18	0.22^{B}	0.16^{B}	0.36
	10^8 cfu	0.23	0.15^{B}	0.14^{B}	0.08^{B}	0.10	0.10^{B}	0.12^{B}	0.35
p vaLue		0.047*	**0.01**	**0.02**	**0.002**	0.057	**0.001**	**0.004**	0.03*
Pooled SEM		0.31	0.30	0.28	0.19	0.36	0.16	0.19	0.25

[a]Broilers were infected with 0 (CON), 10^6, 10^7 or 10^8 cfu of *Salmonella typhimurium*.

[b]Values with a different letter (A–B) within a gene and a tissue differ significantly ($p < 0.05$). $p < 0.05$ is highlighted in bold type *indicates significance by ANOVA but not by Tukey's test. AvBD1 = Avian β-defensin 1; AvBD6 = Avian β-defensin 6; AvBD8 = Avian β-defensin 8; AvBD10 = Avian β-defensin 10; AvBD11 = Avian β-defensin 11; AvBD12 = Avian β-defensin 12; AvBD13 = Avian β-defensin 13; LEAP2 = liver-enriched antimicrobial peptide 2.

Table 5. Avian β-defensin and LEAP2 mRNA abundance in *Salmonella typhimurium*-infected and non-infected broilers at 7-day post-infection.

		Gene[b]							
Tissue	Challenge[a]	AvBD1	AvBD6	AvBD8	AvBD10	AvBD11	AvBD12	AvBD13	LEAP2
Duodenum	CON	1.12^{A}	1.13^{A}	1.25^{A}	1.06^{A}	1.06^{A}	1.14^{AB}	1.07^{AB}	1.13
	10^6 cfu	0.50^{AB}	0.51^{AB}	0.44^{AB}	0.74^{AB}	0.90^{AB}	1.76^{A}	1.28^{A}	1.64
	10^7 cfu	0.14^{B}	0.05^{B}	0.03^{B}	0.11^{B}	0.14^{B}	0.17^{B}	0.14^{B}	0.97
	10^8 cfu	0.21^{B}	0.13^{B}	0.06^{B}	0.17^{B}	0.20^{B}	0.24^{B}	0.21^{B}	1.05
p Value		**0.01**	**0.003**	**0.01**	**0.002**	**0.01**	**0.02**	**0.01**	0.60
Pooled SEM		0.18	0.18	0.24	0.17	0.19	0.35	0.26	0.38
Jejunum	CON	1.40	1.20^{A}	1.34^{A}	1.20^{A}	1.25	1.34	1.19	1.11
	10^6 cfu	0.53	0.35^{B}	0.27^{AB}	0.42^{AB}	0.60	0.76	0.64	1.29
	10^7 cfu	0.24	0.17^{B}	0.08^{B}	0.20^{B}	0.26	0.33	0.33	0.95
	10^8 cfu	0.23	0.14^{B}	0.03^{B}	0.19^{B}	0.25	0.29	0.26	1.12
p Value		0.11	**0.005**	**0.02**	**0.02**	0.10	0.14	0.10	0.80
Pooled SEM		0.36	0.20	0.29	0.22	0.30	0.34	0.27	0.24
Ileum	CON	1.92	1.32^{A}	1.49	1.76	1.82	1.45	1.70	1.30
	10^6 cfu	0.38	0.33^{AB}	0.50	0.35	0.48	0.89		0.86
	10^7 cfu	0.10	0.10^{B}	0.06	0.13	0.15	0.18	0.15	0.20
	10^8 cfu	0.22	0.07^{B}	0.04	0.15	0.13	0.15	0.14	0.23
p Value		0.21	**0.03**	0.07	0.13	0.15	0.10	0.14	0.07
Pooled SEM		0.66	0.30	0.40	0.53	0.56	0.40	0.50	0.31
Cecum	CON	1.27	1.74	1.48	1.31^{A}	1.31^{A}	1.36^{A}	1.34^{A}	1.68
	10^6 cfu	0.95	1.14	0.78	0.83^{AB}	1.04^{AB}	1.31^{A}	0.93^{AB}	1.43
	10^7 cfu	0.31	0.39	0.18	0.28^{B}	0.45^{AB}	0.51^{AB}	0.47^{AB}	0.70
	10^8 cfu	0.22	0.18	0.06	0.14^{B}	0.17^{B}	0.19^{B}	0.19^{B}	0.31
p Value		0.08	0.09	0.08	**0.004**	**0.02**	**0.01**	**0.02**	0.10
Pooled SEM		0.31	0.44	0.40	0.21	0.26	0.25	0.24	0.41

[a]Broilers were infected with 0 (CON), 10^6, 10^7 or 10^8 cfu of *Salmonella typhimurium*.

[b]Values with a different letter (A–B) within a gene and a tissue differ significantly ($p < 0.05$). $p < 0.05$ is highlighted in bold type AvBD1 = Avian β-defensin 1; AvBD6 = Avian β-defensin 6; AvBD8 = Avian β-defensin 8; AvBD10 = Avian β-defensin 10; AvBD11 = Avian β-defensin 11; AvBD12 = Avian β-defensin 12; AvBD13 = Avian β-defensin 13; LEAP2 = liver-enriched antimicrobial peptide 2.

upregulated at 3 dpi. At 5 dpi, only AvBD2 and 6 mRNA were still upregulated. Shao et al.[11] examined the abundance of AvBD1, 4 and 10 mRNA in the jejunum of 7- to 9-day-old male Cobb broilers challenged with 10^9 cfu *Salmonella* Enteriditis. *Salmonella* infection caused an upregulation of AvBD1 and AvBD4 mRNA at both 7 and 15 dpi. In contrast, *Salmonella* infection caused an increase in AvBD10 mRNA at 7 dpi but not 15 dpi. Ramasamy et al.[10] examined abundance of 14 AvBD (AvBD1 to AvBD14) mRNA in the duodenum, jejunum, ileum and ceca of 3-day-old Punjab broilers challenged with 5×10^7 cfu *Salmonella* Pullorum. At 24 h post-infection (hpi), there was upregulation of AvBD3 in the cecum, AvBD4 in the duodenum, AvBD5 in the duodenum, jejunum and ileum and AvBD12 in the duodenum and jejunum. In contrast, there was down-regulation of mRNA abundance for AvBD6 in the ileum, AvBD10 in the cecum, AvBD11 in the duodenum and ileum, AvBD13 in the ileum and AvBD14 in the ileum and cecum. Because *S.* Pullorum is pathogenic to poultry, it may induce a different immune response than *Salmonella* serotypes that are not pathogenic to poultry but cause human food-borne illnesses.

In other avian species, such as geese, the effect of *Salmonella* challenge has been examined.[12,13] showed that AvBD2 and AvBD3 mRNA were upregulated at 48 and 72 hpi, while AvBD5 and AvBD10 mRNA were upregulated at 72 hpi in the small intestine of 15-day-old female Chinese geese challenged with 5×10^6 cfu *Salmonella* Enteriditis.

In contrast, some studies showed that expression of AvBD mRNA was unaltered by a *Salmonella* challenge. Milona et al.[16] reported that AvBD4 mRNA (Gal 7) in 5-day-old female Goldline chickens was not changed in the small intestine following challenged with 5×10^6 cfu *Salmonella* Enteriditis or *S. typhimurium*. Crhanova et al.[17] reported no changes in AvBD1, 2, 4 and 6 (named Gal1, 2, 4 and 6) mRNA abundance at 3, 10 or 42 dpi in the ceca following challenge of 1-, 4- and 16-day-old male chickens with 10^6 cfu *Salmonella* Enteritidis.

Our results show that any changes in AvBD mRNA abundance were dependent upon tissue, time post-infection and dose. There was an initial upregulation of AvBD1, 8, 10 and 12 mRNA mainly in the duodenum at 1 dpi, but only at the highest dose tested (10^8 cfu). Although not statistically significant, there was a 10-fold increase in the amount of recovered *Salmonella* from the ceca of the 10^8 cfu group compared to the 10^6 and 10^7 cfu groups at 1 dpi, which could explain the observed differential gene expression. This upregulation was transient, since by 2 dpi there were very few differences in AvBD mRNA, and by 5 and 7 dpi there was downregulation of AvBD mRNA. In general, all AvBD mRNA were downregulated in the lower gastrointestinal tract at 5 dpi with all three doses tested (10^6, 10^7 and 10^8 cfu). At 7 dpi, there was continued downregulation of a number of AvBD mRNA mainly in the duodenum and ileum at the higher 10^7 and 10^8 doses. The downregulation of AvBD at 5 and 7 dpi suggests that *Salmonella* may be altering intestinal AvBD mRNA abundance as part of its immune evasion strategy. Our results also demonstrate that AvBD mRNA can be upregulated, downregulated or unchanged depending upon a number of variables. This could help explain the apparent discrepancy in the reported changes in AvBD mRNA abundance in response to *Salmonella* infection.

Changes in mRNA abundance of LEAP2 have also been examined following *Salmonella* challenge. Townes et al.[15] showed that at 3 dpi there was upregulation of LEAP2 mRNA in the small intestine of chickens challenged with *Salmonella* Enteriditis and *S. typhimurium*. Shao et al.[11] reported that LEAP2 mRNA was upregulated at 15 dpi but not 7 dpi in the jejunum of 7- to 9-day-old male Cobb broilers challenged with 10^9 cfu *Salmonella* Enteriditis. In contrast, in our study, LEAP2 mRNA was downregulated in the ileum at 1 dpi and the jejunum at 5 dpi. It is not clear why there is a difference in results for LEAP2 mRNA between our and the other studies.

Host defense peptides play an important role in enhancing the intestinal barrier function in multiple species by inducing expression of mucins and tight junction proteins (reviewed in Robinson et al.[27]) In chickens, supplementation of feed with HDP has been shown to affect growth performance, nutrient utilization, the intestinal microbiome and intestinal morphology. The antimicrobial peptide cecropin, which was originally isolated from insects, improved BW gain and feed:gain ratio, increased nutrient utilization, increased villus height and decreased aerobic bacterial counts in intestinal digesta in a dose-dependent manner.[28] In a similar manner, the antimicrobial peptide A3 improved BW gain, increased retention of dry matter, gross energy, and crude protein, increased villus height and decreased excreta coliforms in a dose-dependent manner.[29] Although dietary supplementation of HDPs was effective, it is currently not cost-effective to implement on an industrial scale. Thus, the identification of compounds that can induce HDPs is a preferred alternative. Sunkara et al.[30]

showed that butyrate induced HDP gene expression in the small intestine, which resulted in a 10-fold reduction in the cecal titer of *S.* Enteriditis. A better understanding of the mechanism of gene expression and antimicrobial actions of HDP would enhance their potential role as an alternative to antibiotics.

In summary, the mRNA abundance of AvBD and LEAP2 displayed dose-, tissue- and age-dependent changes following a challenge with *S. typhimurium*. The initial response to Salmonella was an upregulation of AvBD mRNA at 1 dpi. This effect disappeared at 2 dpi. At Day 5 and Day 7 dpi, there was downregulation of AvBD mRNA, which suggests that *S. typhimurium* may be able to suppress the HDP response to evade the immune system once it has colonized the lower gastrointestinal tract.

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