Research Thesis: Enteric proteomic endocrine pathway changes to day of hatch chicks after *in ovo* bacterial inoculation

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Expected Graduation: Autumn 2019

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Abstract

Microbial populations within the gastrointestinal tract (GIT) have potential to alter the physiological performance of a variety of systems within poultry. From a production standpoint, the ability to manipulate this population can provide an invaluable way to influence overall health and performance. The objective of this study was to compare the effects of *in ovo* inoculation with a commercially available LAB-probiotic (L) or two different Gram-negative strains for their influence on the endocrinological profile of day of hatch chicks. At d18 of embryogenesis, eggs were inoculated with sterile saline (S), 10_2 CFU of one of two species of *Citrobacter* (C1 or C2), or L. Eggs were hatched by treatment and, on day of hatch, the GIT, duodenum to rectum, was collected from 10 chicks. The extracted protein was subjected to separation by fusion mass spectrometry and identified via Sequest with a *Gallus gallus* reference in Uniprot. Data was then entered into Ingenuity Pathway Analysis (IPA) and analyzed for differences. All experimental treatments were compared to S in IPA to determine effects on canonical pathway activation and inhibition at $p<0.05$, with a minimum z-score of +/-1.5. The C1 group showed inhibition of pathways related to insulin and cholesterol regulation and production including HNF1A (z-score -1.969) and HNF4A (z-score -2.000). This is in contrast to C2 which expressed opposite results of these pathways, with activation of insulin and cholesterol regulation (ex: HNF1A z-score +1.982), plus activation of pathways and receptors for sex hormones (Progesterone receptor z-score +1.982) and the precursor to the hormones related to angiogenesis, angiotensinogen (z-score +1.898), among other endocrine-related proteins. The L group showed activation of sex and steroid hormone pathways including corticosterone (z-score +1.949) and steroid hormone receptor ERR1 (z-score +1.948) as well as Insulin-1 (z-score +1.49), and inhibition of calcitriol (z-score -1.709). These changes suggest that neonatal colonization of poultry can affect significant physiological pathways, especially those related to sex and steroid hormones, insulin, and other glucose-regulating pathways, and emphasize the role of pioneer colonizing bacteria in growth and development of perinatal poultry.

Introduction

The gastrointestinal tract (GIT) serves as both a major gateway and barrier to the rest of the body (Furness, 1999). By allowing nutrients to selectively flow into the body and preventing infiltration by bacteria. The GIT is an essential part of every living organism. Besides being a gatekeeper to the rest of the body, the GIT serves as an optimal environment for microbial growth (Tellez, 2006). A mature gastrointestinal tract will house more bacterial cells in itself than there are native cells in the entire host system. In birds, the development of the GIT microbiome starts after the chick's hatch. In a commercial setting, this means the only sources for bacterial development are from residual bacteria on the surface of the eggs, presumably from the mother, and from the bacteria that live in the hatchery, which may not always be the most beneficial. Despite good biosafety techniques, bacteria such as *Salmonella* (Cox, 1990) and *Campylobacter* (Byrd, 2007) have been recovered from broiler hatcheries. This poses a huge risk, both to the health of the birds and to the people that could be infected should these birds enter into the food chain. Through the use of *in ovo* pioneer colonizers, we can prime the GIT's microbiome prior to hatch, reducing the incidence of infectious

disease entering the chick and taking hold due to the chick's naïve immune system (Wilson, 2019). Specifically, the use of probiotics composed of lactic acid producing bacteria (LAB) are very helpful for prevention *Salmonella* in neonatal broilers. (Higgins, 2010)

The perinatal period, which is a transitional period where chicks undergo a shift from dependence on the lipid-rich yolk to exogenous feed for nutrition, is recognized as a crucial time for GIT development in poultry. Numerous publications have shown the last three days of embryogenesis are crucial for intestinal growth, including rapid weight gain, morphological changes, increases in enzyme activity, and development of the brush border (Noy and Sklan, 1997; Uni et al., 2003; Christensen et al., 2004; Ferket, 2012). The immediate post-hatch period is crucial for enterocyte proliferation, villi development, and mucin production (Uni et al., 2000, 2003). Ferket (Ferket, 2012) proposed that the first meal may affect which genes in the chick are activated and could potentially influence life-long performance. This is supported by research showing that mineral deficiency early in life led to permanently altered broilers ability to use those minerals later on (Yan et al., 2005; Ashwell and Angel, 2010). However, most hatchery systems delay deliverance to farms for up to 24 hours (or more), inevitably delaying access to feed at such a crucial time for GIT development. This delay may also extend to delayed or significantly altered development of the GIT microbiota.

Essential colonization of GIT bacterial populations starts at hatch and is followed by progressive assembly of a complex and dynamic microbial society. Establishment of a healthy microbiome in poultry has been recognized as pivotal for growth performance and health of poultry (Brisbin et al., 2008; Kohl, 2012; Oakley et al., 2013; Pan and Yu, 2014). This would suggest that early manipulation of the microbiome may be an ideal route for optimizing flock health and performance. Modern poultry production practices have separated the chick from the hen and, by association, from the complex maternal microbiota she could have transmitted. Chicks are now hatched in sanitized hatching cabinets where the first gut colonizers are often pathogens such as *Salmonella* or *E. coli. In ovo* deliverance of probiotics may allow colonization of beneficial bacteria before the chick is ever exposed to pathogens within the hatching cabinets.

Microbial populations within the GIT have the potential to alter physiological performance of a variety of systems within poultry. From a production standpoint, the ability to manipulate this population can provide an invaluable way to influence health and performance of poultry. Depending on the bacteria or probiotic introduced, we can potentially reduce disease incidence and improve growth performance, as well as influence a variety of physiologic pathways that do not have a direct connection to the GIT. As a large neuroendocrine organ, via the gut-brain-axis, the GIT can influence systems across the entire body. The objective of this study was to compare the effects of *in ovo* inoculation with a commercially available LAB-probiotic (L), or one of two different Gram-negative strains for their influence on endocrinological profile in day of hatch (DOH) chicks.

Materials and Methods

Inoculation, tissue collection and processing: At day 18 of embryogenesis, eggs were randomly divided into 4 different treatment groups and inoculated *in ovo* with 200

µL of sterile saline (S) or 10_2 CFU of either a commercially available LAB probiotic, or one of two strains of a Gram negative bacteria *Citrobacter freundii* (C1) or *Citrobacter sp.* (C2). On DOH, 10 chicks from each group were euthanized and GIT aseptically removed, including duodenum to rectum, placed in 2mL tubes, and flash frozen in liquid nitrogen, followed by storage at -80°C. When ready for processing, samples were removed from the -80°C freezer and kept on ice while approximately 0.1g of equal parts of DOH GIT were placed into 5mL of urea-thiourea with dithiothreitol (UTU-DTT). The samples were initially homogenized for 5 seconds and then additionally homogenized with a bead beater. A total of 0.1g of stainless-steel beads (0.9-2mm diameter) were placed into 1mL of sample and homogenized for 3 minutes total in 30 second intervals, placing samples on ice between for 30 seconds to prevent heating. The bead beater was kept in a 4°C walk-in cooler as an additional precaution against heating. Samples were centrifuged at 4°C and 14,000 rpm for 20 minutes. The supernatant was collected and aliquoted. The concentration of proteins collected was quantified using a Bradford assay against a standard BSA curve and ranged between 1.56-1.86 µg/µL. Samples were pooled, according to treatment group, at even concentrations into 3 samples per treatment. To ensure protein extraction an SDS-PAGE was performed on each sample with a 10% acrylamide gel; 4% stacking gel at 100V for 2 hours.

In solution digestion: Samples were precipitated in TCA and resuspended in 50 mM ammonium bicarbonate. 5µL of DTT was added and the samples were incubated at 56°C for 15 min. After incubation, 5µL of iodoacetamide was added and the sample kept in the dark at room temperature for 30 min. Sequencing grade-modified trypsin (Promega, Madison WI) prepared in 50mM ammonium bicarbonate was added to the sample with an estimated 1:20/1:100 enzyme-substrate ratio and the reaction was carried on at 37°C overnight. The reaction was quenched the following morning by adding acetic acid. The sample was concentrated, and the peptide concentration measured by nanodrop.

Mass Spectrometry: Tandem mass spectrometry of global protein identification was performed on a Thermo Fisher Fusion mass spectrometer in positive ion mode. Samples were separated on a Thermo Nano C18 column. Each sample was injected into the Micro Precolumn Cartridge and desalted with 50mM acetic acid for 5 minutes. The peptides were eluted off onto the column. MS/MS data was acquired with a spray voltage pf 1.7 KV and a capillary temperature of 275°C; S-Lens RF level was set at 60%. To achieve high mass accuracy MS determination, the full scan was performed at FT mode and the resolution was set at 120,00. Dynamic exclusion is enabled with a repeat count of 1, exclusion duration of 60s and a low mass width and high mass width of 10ppm.

Quantification and Analysis: Only Proteins with a false discovery rate of less than 1% were included. Significance was set at $p<0.10$. Proteins with a Mascot score of 50 or higher with a minimum of two unique peptides from one protein with a sequence tag of five residues or better were accepted. A Student-T test was performed by scaffold to determine significance ($p<0.10$). The data was imported into Ingenuity Pathway Analysis to determine up and downstream regulators of protein expression. A P value of overlap was established at $p<0.05$ with a minimum z score of +/-1.5.

Results and Discussion

The C1 treatment showed the least amount of GIT-associated endocrinological changes, but some major pathways were still altered (Table 1). Major observed

changes were inhibitions in relation to insulin production and glucose regulation. Decreases in HNF4A (z-score=-1.969) and HNF1A (z-score=-2.000) affect insulin tolerance and production, respectively. HNF1A can also affect cell growth and differentiation, but the associated changes in D-glucose (z-score=-1.662) and HNF4A presented here, suggest changes in blood glucose regulation as the primary altered pathway. Through the inhibition of HNF1A, HNF4A, and D-glucose there are alterations in pathways relating to the enzymes involved in glycolysis, gluconeogenesis, and other forms of carbohydrate digestion, protein synthesis, muscle contractions, extracellular matrix proteins, and thyroid hormone binding proteins (Figure 1).

Contrary to the C1 treatment group, C2 had treatment the greatest amount of significantly activated and inhibited pathways (Table 2). There was activation of pathways related to insulin production with significant increase in HNF1A (z-score= 1.982) associated with changes in D-glucose (z-score= 2.356), which suggests changes in blood glucose regulation. Along with this, pathways and receptors for sex hormones, such as PGR (z-score= 1.982) were activated. There was also activation of the precursor to angiogenesis, AGT (z-score= 1.898) along with activation of STAT3 (z-score= 1.501) and FGF2 (z-score= 1.922), which have various positive angiogenetic effects, and inhibition of PPARA (z-score= -1.949) which is a steroid receptor thought to affect cell proliferation and inflammation. Through the activation of HNF1A, PGR, AGT, STAT3, FGF2, and D-glucose and the inhibition of PPARA we see a broad spectrum of downstream pathways altered (Figure 2). Alterations in fibrin matrixes, embryonic lens development, enzymes involved in carbohydrate and protein digestion, and nucleotide maintenance are just some of the changes observed following the changes seen by the treatment in the upstream regulators.

The LAB group was included in the experiment to show “normal” positive effects, as the probiotic is known to have beneficial effects in birds. Activation of corticosterone (z-score= 1.949), ESRRA (z-score= 1.948), and 15-deoxy-delta-12,14-PGJ 2 (z-score= 1.982) suggested a significant effect on the sex and steroid hormone pathways. Activation of HNF4A (z-score=1.942) indicated some mild blood glucose regulatory affects and the inhibition on calcitriol (z-score= -1.709) shows downregulation of vitamin D production. Following the activation of corticosterone, 15-deoxy-delta-12,14-PGJ 2, ESRRA, and HNF4A and the inhibition of calcitriol, the downstream pathways associated with sugar breakdown and interconversion, mitochondrial enzymes for amino acid interconversion, thyroid hormone binding proteins, muscle contractions, and transfers along the electron transport chain were all affected by the upstream changes. The overall results of the LAB group showed overlap with the C2 more than C1, which could be an indicator that Gram-negative bacteria have varying effects on the GIT.

Conclusion

The neonatal colonization of poultry was shown to affect upstream regulators of endocrine pathways with a variety of downstream effects. After evaluating the data, the LAB-probiotic activated pathways relating to sex and other steroid hormones, steroid hormone receptors, and glucose metabolism while inhibiting pathways relating to the hormone calcitriol. The experimental groups C1 and C2, despite being of the same genus, exhibited nearly opposite results, with C1 only displaying inhibition of metabolic glucose pathways while the C2 group activated glucose metabolic pathways as well as

activated a variety of steroid hormones and receptors. This emphasizes the importance not only of specific species but possibly even the strain of bacteria used when providing *in ovo* pioneer colonizers in perinatal poultry development, as well as the need to promote appropriate colonization of the GIT at the hatchery level. Potential future studies show should investigate the ability of the microbiome to alter endocrine aspects of poultry, especially related to gluconeogenesis, as shown here.

References

V. C., ., M. W., ., I. Y., ., D. O., & ., K. M. (2004). Incubator Temperature and Oxygen Concentration at the Plateau Stage Affects Intestinal Maturation of Turkey Embryos. *International Journal of Poultry Science*, *3*(6), 378–385. doi: 10.3923/ijps.2004.378.385

Ashwell, C. M., & Angel, R. (2010). Nutritional genomics: a practical approach by early life conditioning with dietary phosphorus. *Revista Brasileira De Zootecnia*, *39*(suppl spe), 268–278. doi: 10.1590/s1516-35982010001300030

Brisbin, J. T., Gong, J., & Sharif, S. (2008). Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Animal Health Research Reviews*, *9*(1), 101–110. doi: 10.1017/s146625230800145x

Byrd, J., Bailey, R. H., Wills, R., & Nisbet, D. (2007). Recovery of Campylobacter from Commercial Broiler Hatchery Trayliners. *Poultry Science*, *86*(1), 26–29. doi: 10.1093/ps/86.1.26

Cox, N. A., Bailey, J. S., Mauldin, J. M., & Blankenship, L. C. (1990). Research Note: Presence and Impact of Salmonella Contamination in Commercial Broiler Hatcheries. *Poultry Science*, *69*(9), 1606–1609. doi: 10.3382/ps.0691606

Ferket, P. R. (2012). Embryo epigenetic response to breeder management and nutrition. *World's Poult Congress, Salvador Proceedings*.

Furness, J. B., Kunze, W. A. A., & Clerc, N. (1999). II. The intestine as a sensory organ: neural, endocrine, and immune responses. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *277*(5). doi: 10.1152/ajpgi.1999.277.5.g922

Higgins, J. P., Higgins, S. E., Wolfenden, A. D., Henderson, S. N., Torres-Rodriguez, A., Vicente, J. L., … Tellez, G. (2010). Effect of lactic acid bacteria probiotic culture treatment timing on Salmonella Enteritidis in neonatal broilers. *Poultry Science*, *89*(2), 243–247. doi: 10.3382/ps.2009-00436

Noy, Y., & Sklan, D. (1997). Posthatch Development in Poultry. *The Journal of Applied Poultry Research*, *6*(3), 344–354. doi: 10.1093/japr/6.3.344

Oakley, B. B., Morales, C. A., Line, J., Berrang, M. E., Meinersmann, R. J., Tillman, G. E., … Seal, B. S. (2013). The Poultry-Associated Microbiome: Network Analysis and Farm-to-Fork Characterizations. *PLoS ONE*, *8*(2). doi: 10.1371/journal.pone.0057190

Pan, D., & Yu, Z. (2013). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes*, *5*(1), 108–119. doi: 10.4161/gmic.26945

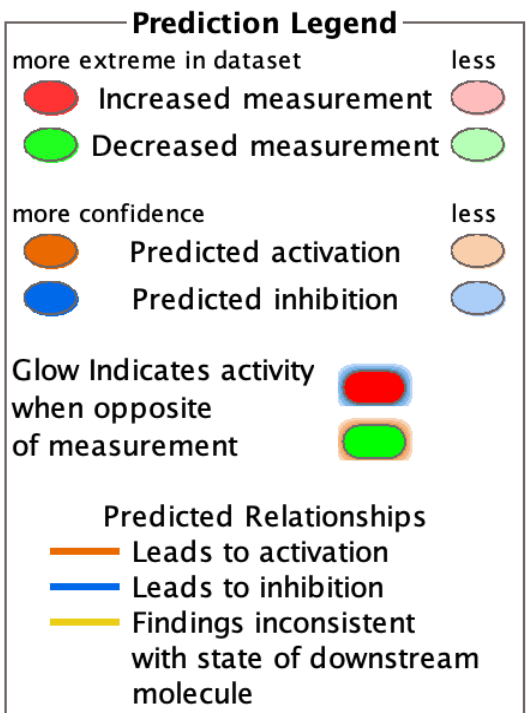
Tellez, Higgins, Donoghue, Hargis, & M., B. (2006, March 1). Digestive Physiology and the Role of Microorganisms 1. Retrieved from https://academic.oup.com/japr/article/15/1/136/755232.

Uni, Z., Geyra, A., Ben-Hur, H., & Sklan, D. (2000). Small intestinal development in the young chick: Crypt formation and enterocyte proliferation and migration. *British Poultry Science*, *41*(5), 544–551. doi: 10.1080/00071660020009054

Uni, Z., Tako, E., Gal-Garber, O., & Sklan, D. (2003). Morphological, molecular, and functional changes in the chicken small intestine of the late-term embryo. *Poultry Science*, *82*(11), 1747–1754. doi: 10.1093/ps/82.11.1747

Wilson, K. M., Rodrigues, D. R., Briggs, W. N., Duff, A. F., Chasser, K. M., & Bielke, L. R. (2019). Evaluation of the impact of in ovo administered bacteria on microbiome of chicks through 10 days of age. *Poultry Science*, *98*(11), 5949–5960. doi: 10.3382/ps/pez388

Yan, F., Angel, R., Ashwell, C., Mitchell, A., & Christman, M. (2005). Evaluation of the broilers ability to adapt to an early moderate deficiency of phosphorus and calcium. *Poultry Science*, *84*(8), 1232–1241. doi: 10.1093/ps/84.8.1232



The key to the left should be used as a guide for interpreting the figures attached. The figures are a visual representation of the data in the corresponding table as well as a guide to the molecules up and downstream that are affected by the pathways altered by the selected treatment.

Table 1: Endocrinological upstream regulators affected by *in ovo* inoculation with the *Citrobacter fruendii* (C1) treatment, compared to S, at d18 of embryogenesis.

	Gene	Z-score	P-value
D-glucose	D-glucose	-1.662	<0.001
HNF4A	Hepatocyte nuclear factor 4-alpha	-1.969	0.004
HNF1A	Hepatocyte nuclear factor 1-alpha	-2.000	0.005

Figure 1: Upstream analysis of D-glucose, HNF4A, and HNF1A showing activation and inhibition following *in ovo* inoculation with C1 compared to saline.

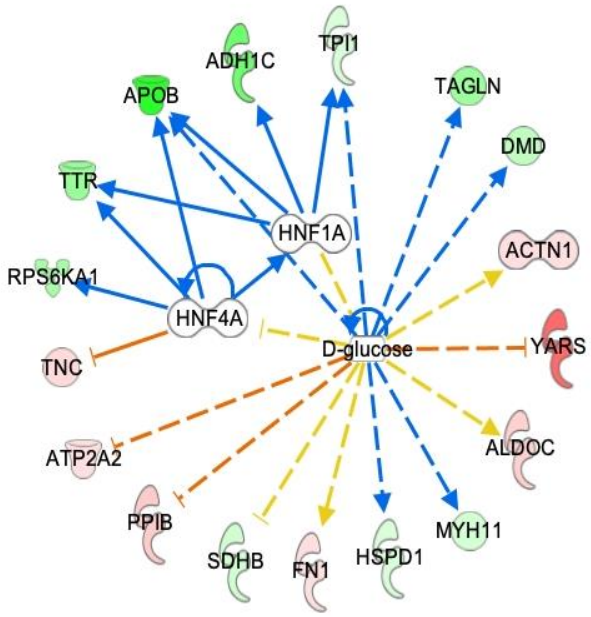


Table 2: The endocrinological upstream regulators affected by *in ovo* inoculation with the C2 treatment, as compared to S.

	Gene	Z-score	P-value
D-glucose	D-glucose	2.356	0.004
PGR	Progesterone receptor	1.982	0.006
HNF1A	Hepatocyte nuclear factor 1-alpha	1.982	0.027
FGF2	Fibroblast growth factor 2	1.922	0.014
AGT	Angiotensinogen	1.898	0.028
STAT3	Signal transducer and activator of transcription 3	1.501	<0.001
PPARA	Peroxisome proliferator-activated receptor alpha	-1.949	0.001

Figure 2: Upstream analysis of D-glucose, PGR, HNF1A, FGF2, AGT, STAT3, and PPARA showing activation and inhibition following *in ovo* inoculation with C2 compared to saline.

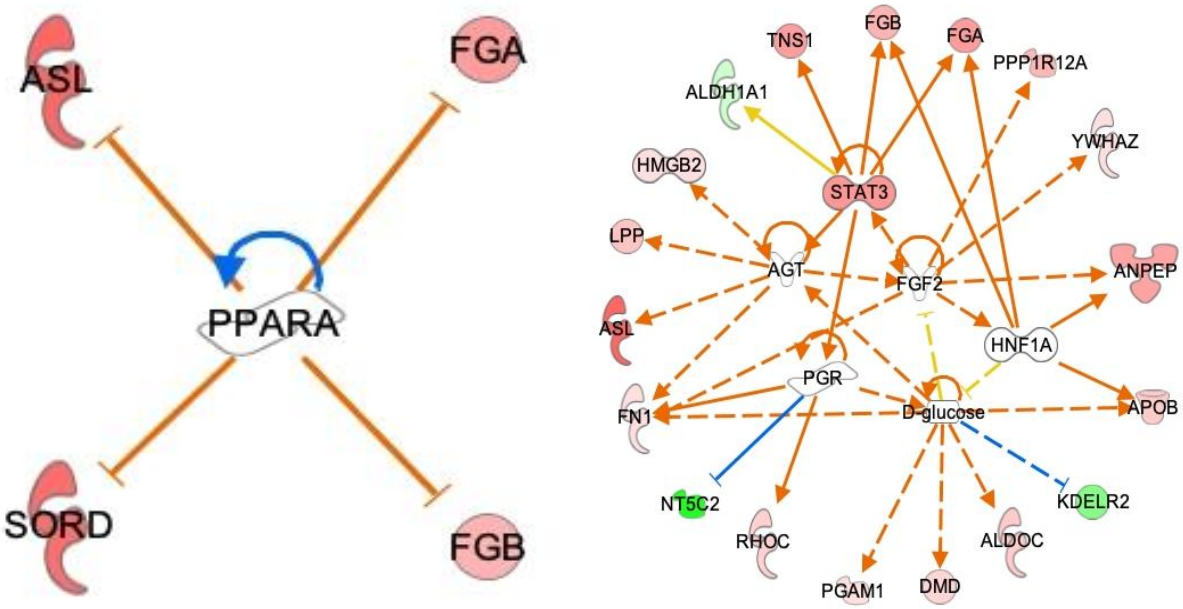


Table 3: Endocrinological upstream regulators affected by *in ovo* inoculation with the LAB treatment, compared to S.

	Gene	Z-score	P-value
15-deoxy-delta-12,14 -PGJ 2	15-deoxy-delta-12,14 -PGJ 2	1.982	0.005
corticosterone	corticosterone	1.949	<0.001
ESRRA	Steroid hormone receptor ERR1	1.948	0.004
HNF4A	Hepatocyte nuclear factor 4-alpha	1.942	<0.001
calcitriol	calcitriol	-1.709	0.001

Figure 3: Upstream analysis of 15-deoxy-delta-12,14-PGJ 2, corticosterone, ESRRA, HNF4A, and calcitriol showing activation and inhibition following *in ovo* inoculation with the LAB treatment compared to saline.