Chapter 10
Applications of "Omics" Technologies to Study Gut Health in Poultry

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10.1 Introduction

Over the past 50 years, the United States poultry industry has transformed itself from a backyard business into one of the most advanced sectors of agriculture, supplying products to customers globally. At present, the United States is the largest poultry producer and second largest exporter of poultry meat in the world (USDA-ERS 2012), with its total value exceeding $20 billion, primarily from broiler production, followed by eggs, turkey, and other poultry products (USDA-ERS 2012). Despite these improvements, the microbiological safety of poultry products remains a challenge for the industry. Between 1998 and 2008, contaminated poultry products were responsible for the majority (18.9% of total) of foodborne outbreaks (MMWR, Centers for Disease Control and Prevention 2013). Since 2008, the consumption of contaminated poultry products accounted for at least 14 major foodborne outbreaks resulting in illnesses to over a million people (Centers for Disease Control and Prevention 2014; Scallan et al. 2011). *Salmonella* and *Campylobacter* are two common foodborne pathogens that are responsible for the majority of these illnesses. Chickens act as the reservoir host for these pathogens (Bakshi et al. 2003; Anonymous 2005), wherein the bacteria colonize the chicken gut (especially the ceca), thereby leading to contamination of carcass during slaughter and subsequent

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K. Venkitanarayanan et al. (eds.), *Food Safety in Poultry Meat Production*, Food Microbiology and Food Safety, https://doi.org/10.1007/978-3-030-05011-5_10

human infections (Keller et al. 1995; Cagri et al. 2004). The most commonly detected *Salmonella* serovars in chicken that are associated with human infections include *Salmonella enterica* serovar Enteritidis, *Salmonella* Typhimurium, and *Salmonella* Heidelberg (Foley et al. 2011). In the case of *Campylobacter* spp., humans are most frequently sickened by *Campylobacter jejuni* (~90%), followed by *Campylobacter coli* (~10%) (Gillespie et al. 2003; Taboada et al. 2013). The healthcare cost associated with poultry-related foodborne pathogens is greater than $5 billion each year (USDA-ERS 2012). Therefore, it is important to develop strategies for reducing pathogen colonization in chickens as a first step toward food safety.

The poultry industry has implemented various genetic selection strategies, management approaches, and dietary modifications with an aim to improve performance or disease resistance in chickens (Emmerson 1997; Lumpkins et al. 2010). With recent advancements in next-generation sequencing, the chicken gut has become the focus of extensive research both for improving productivity (better feed utilization, feed conversion ratio) and developing resistance against enteric diseases caused by pathogenic microbes (Park et al. 2016; Roto et al. 2015; Waite and Taylor 2015). Gut health encompasses a plethora of interrelated factors such as nutrient digestion, absorption, epithelial barrier function, gut microbiome, and mucosal immune responses (Kogut and Arsenault 2016). A comprehensive understanding of how these factors interact to bring about overall health and productivity in chickens is still elusive; however, significant scientific endeavors are currently underway to delineate the biochemical cross talk and critical pathways responsible for maintaining gut homeostasis and function. Several research groups have attempted to characterize the gut microbiome of chickens in health and disease (Brisbin et al. 2008; Stanley et al. 2014), and the major bacterial groups that constitute the chicken microbiome along with their potential role in gut metabolism have been identified (Qu et al. 2008; Wei et al. 2013; Oakley et al. 2014). Moreover, the role of microbiota-derived metabolites that signal the host's immune and endocrine system potentially altering host physiology is also under extensive scientific investigations (Belkaid and Hand 2014; Schroeder and Bäckhed 2016).

This book chapter summarizes the current research employing "omics" approaches to study gut health in chickens. In addition, the effect of various feed additives on modulating microbiome/metabolome parameters as they relate to pathogen colonization in chickens is discussed.

10.2 Intestinal Microbiota of Poultry

The gut microbiota represents a stable and specific association between a host and microorganisms, which has developed through a long series of selection, competition, and coevolution (Angelakis et al. 2012; Ley et al. 2008; Yeoman et al. 2011). The spatial heterogeneity and distribution of bacterial communities in the gut are governed by several factors such as microenvironment (pH, redox potential, oxygen levels), nutrient selection, and immune activation (Donaldson et al. 2016; Belkaid and Hand 2014). The host benefits from receiving nutrients catabolized by resident

microbiota from otherwise poorly utilized dietary substrates (Sekirov et al. 2010), whereas the microbiota gains from the availability of an ecosystem that provides a niche and facilitates their survival. In the case of food animals and poultry, gut health is critical for nutrient assimilation, maintenance, growth, and productivity.

As compared to other food animal species (e.g., cattle), the gastrointestinal tract of poultry is an anatomically simple yet physiologically efficient system that helps in feed digestion and nutrient assimilation. Unlike mammalian hosts, the digesta pass through the poultry gut faster with an average transit time of less than 3.5 h (Hughes 2008). This fast passage rate selects for a microbiota with high affinity for mucosal binding and colonization (Pan and Yu 2014; Crhanova et al. 2011). However, the ceca (intestinal out pocketing at the junction of small intestine and colon) have a slow passage rate (~12–20 h) potentially facilitating a longer interaction time for digestion/nutrient assimilation. Several critical physiological functions have been attributed to the ceca including nitrogen cycling, water absorption, carbohydrate fermentation, and production of short-chain fatty acids (SCFA) (Mead 1989; Józefiak et al. 2004; van der Wielen et al. 2000). These anatomic features and physiological demands drive the development of a complex microbiome in the ceca. In fact, most densely populated (10^9 to 10^{11} bacteria/g) microbial community within the chicken gut is found in the ceca (Clench and Mathias 1995; Gong et al. 2006), with more than 2200 operational taxonomic units (Danzeisen et al. 2011) and 3500 genotypes (Qu et al. 2008). Microbial colonization of the gastrointestinal tract in poultry begins immediately post-hatch and establishes by 2 weeks in the small intestine (Amit-Romach et al. 2004; Lu et al. 2003). Thereafter, the microbiota modulates and alters itself based on environmental factors, age, and dietary patterns of the birds (Stanley et al. 2013). The mature chicken gut microbiota consists primarily of bacteria (Wei et al. 2013), followed by archaea (Saengkerdsub et al. 2007a, b) and viruses (Qu et al. 2008). The microbial community is highly diverse with over 1000 bacterial species (Chambers and Gong 2011). Through phylogenetic profiling and 16S rRNA-based sequencing of the intestinal microbiome of poultry, a global census was developed (Wei et al. 2013), which serves as the working framework for describing bacterial diversity in the poultry gut. In total, 13 bacterial phyla were found, including *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* constituting greater than 90% of the intestinal bacteria, with *Firmicutes* being the predominant phyla in the small intestine and cecum of chickens (Rehman et al. 2007; Qu et al. 2008; Danzeisen et al. 2011). The most predominant genera found in poultry (both chickens and turkey) are *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides* with *Lactobacillus* spp. dominating the crop and small intestinal niches (Lu et al. 2003; Apajalahti and Kettunen 2006; Abbas Hilmi et al. 2007). The ceca of poultry consist strictly of anaerobic bacteria (many of unknown bacterial genus) primarily dominated by order *Clostridiales* and families *Lachnospiraceae* and *Ruminococcaceae*. A significant portion of the cecal bacteria also belong to the families *Bifidobacteriaceae* and *Coriobacteriaceceae* (Lu et al. 2003; Bjerrum et al. 2006). These groups of bacteria are known for their ability to utilize dietary polysaccharides (especially components unavailable to the host) for producing SCFA, thereby expanding the overall energy capture from the feed. A positive correlation was reported between cecal *Lachnospiraceae* spp. and feed conversion efficiency in

commercial broilers (Torok et al. 2008; Rinttilä and Apajalahti 2013). However, despite these commonalities in poultry, chickens and turkeys share only 16% similarity at species-equivalent level (Pan and Yu 2014).

10.3 Modulation of Chicken Intestinal Microbiota in Response to Nutritional, Physiological, or Microbial Cues

Extensive microbiota-host cross talk occurs in the poultry gut through nutritional, physiological, and immunological signaling. Scientists are in the beginning stage in deciphering these molecular communications, their effect on the gut microbiome, and their relevance for poultry health and productivity. Gene-based metagenomic studies provide a measure of such metabolic capabilities of the microbiome. Recent findings in this area are presented in this section.

10.3.1 Nutritional/Physiological Signaling

The different sections of poultry gut are inhabited by specialist microbiota adapted to available nutrients and physicochemical conditions of the niche. Most easily digested dietary carbohydrates are absorbed in the proximal gut by the host and the indigestible/residual carbohydrates, and dietary fibers are metabolized by the distal gut bacteria (especially from the ceca) by production of polysaccharide-specific enzymes (Hooper et al. 2002; Rehman et al. 2007). Chickens lack the genes for glycoside hydrolase, polysaccharide lyase, and carbohydrate esterase (Morris 2003) required for carbohydrate metabolism in the gut. Metagenomic studies have shown that genes coding for aforementioned enzymes and various other proteins involved in carbohydrate metabolism are abundantly found (~20% of the gene pool) in the cecal microbiome (Qu et al. 2008; Danzeisen et al. 2011). Breakdown of dietary polysaccharides leads to the production of SCFA in the gut, primarily acetate, followed by propionate and butyrate (Topping and Clifton 2001; Dunkley et al. 2007). The ratio and amount of SCFAs produced depend on the microbial composition and fiber component in poultry diet (Topping and Clifton 2001). The SCFAs are utilized as energy source by the host epithelium and contribute to the development of villus morphology (Panda et al. 2009; Donohoe et al. 2011). They also represent a major source of carbon to the host facilitated by the microbiome (Koutsos and Arias 2006; Tellez et al. 2006). The gut microbiome also contributes to nitrogen metabolism. Genes involved in the metabolism of proteins (9–10% of gene pool), amino acids (8–9%), and nitrogen (1%) have been identified (Qu et al. 2008; Danzeisen et al. 2011). Many of the microbes (e.g., *Lactobacilli*) with fastidious nutritional requirements are usually found in the proximal part of the small intestine, where availability of amino acids, vitamins, and carbohydrates is abundant. These microbes compete

with the host for available nutrients. This leads to the induction of compensatory host mechanisms (secretion of acids, bile salts, antibodies) to balance the growth of microbiota. In the distal part of small intestine, these microbes face strong competition from microbes with lower requirements for easily digested nutrients (e.g., *E. coli*, which does not depend on external amino acids). In this part of the intestine, the microbial metabolism of dietary proteins provides amino acids for host's growth and production (Latshaw and Zhao 2011). However, a high level of bypass protein to the ceca could lead to an increase in protein-fermenting bacteria that negatively affect poultry health by the production of putrefactive protein by-products such as branched chain fatty acids, 3-methyl-indole (skatole), etc. (Smith and Macfarlane 1998).

Several predisposing factors could lead to high protein bypass in poultry. Apajalahti and Bedford (1999) showed that an *Eimeria maxima* challenge to poultry caused an elevation in total biogenic amine levels in the cecum potentially by reducing the integrity and absorptive capacity of the small intestinal epithelium. Similar effects have been observed when birds were administered with feed, high in dietary proteins (Shojadoost et al. 2012). Metabolic processing of proteins to ammonia or urea is another nutritionally inefficient process that leads to their losses in excretion. This also exerts negative effects on the health and productivity of poultry along with environmental and public health concerns (Xin et al. 2011). Several nutritional strategies (e.g., addition of proteolytic enzymes) have been developed to counter these losses (Bregendahl and Roberts 2006); however, microbiome-based strategies to reduce ammonia-associated losses are yet to be explored.

Gene repertoire associated with fatty acid and lipid metabolism has also been identified (Qu et al. 2008). Ding et al. (2016) sequenced the whole gut microbial genomes of two chicken lines (fat and lean) that had undergone long-term divergent selection for abdominal fat pad weight. Results revealed that proportions of *Fusobacteria* (8 vs 18%) and *Proteobacteria* (33 vs 24%) differed significantly between the two lines. Microbial genome functional analysis showed that the gut microbiota was involved in lipid and glycan formation pathways. Citrate cycle and peroxisome proliferator-activated receptor (PPAR) signaling pathways that play crucial roles in lipid storage and metabolism were found to be more prevalent in the fat line than in the lean line of poultry. In another study, Hou et al. (2016) analyzed the gut microbiome of divergently selected lean and fat broiler chicken lines. A significant difference was observed between the lean and fat chicken fecal microbiota structure. Significantly more *Bacteroidetes* was observed in lean broilers. At the genus level, butyrate-producing bacteria (*Subdoligranulum*, *Butyricicoccus*, *Eubacterium*), propionate-producing bacteria (*Bacteroides*), and acetate-producing bacteria (*Blautia*) were reduced in fat line broiler chickens. Since these SCFAs improve barrier function and reduce low-grade inflammation, a precursor for obesity (Costa et al. 2017) and a reduction in these microbiota/compounds could predispose poultry to increased pathogen load and fat accumulation. Follow-up fecal functional metagenomic analysis (KEGG module level) showed that two methanogenesis modules (M00357 and M00567) and pyridoxal biosynthesis (M00124) module were enriched in the fat line broiler chickens which may contribute to fat accumulation. The experimental design of aforementioned studies allows

only correlation-based analysis between modified host physiology and shifts in gut microbiota and does not confirm whether it is the microbiota or the physiology that drives such a selection. However, once a microbiota is selected for a certain physiological phenotype, it could modulate the health/physiology when transplanted into a recipient host (Ridaura et al. 2013; Ley et al. 2005).

10.3.2 Antibiotic Growth Promoters (AGPs)

The food industry has engaged for decades in developing nutritional strategies for improving weight gain in food animals. One such strategy is the use of low-dose antibiotics as feed additives (Frost and Woolcock 1991). Although the precise mechanisms behind the growth-promoting effects of AGPs are unknown, recent evidence suggests that the interactions of AGPs with gut microbiota is a major contributor (Chapman and Johnson 2002; Dibner and Richards 2005; Lin 2011). Dumonceaux et al. (2006) studied changes in the gut microbiota in response to virginiamycin supplementation in broiler chickens using chaperonin 60 (*cpn60*) gene as the target sequence. Virginiamycin increased the abundance of many bacterial targets in the proximal gastrointestinal tract, including lactobacilli (*Lactobacillus crispatus*, *Lactobacillus johnsonii*, *Lactobacillus aviarius*), *Clostridium nexile*, and *Enterococcus cecorum*. In a recent study, Costa et al. (2017) investigated the impact of zinc bacitracin, enramycin, halquinol, virginiamycin, and avilamycin on the cecal microbiota of broiler chickens. Several bacterial genera were identified as representative of usage of each drug. Treatment with enramycin decreased richness and relative abundance of unclassified *Firmicutes*, *Clostridium* XI, and unclassified *Peptostreptococcaceae*, whereas increased abundance of *Clostridium* XIVb and *Anaerosporobacter* spp. occurred. Similar results were observed by Torok et al. (2008) when broiler diets were supplemented with avilamycin, zinc bacitracin, and flavophospholipol. The antimicrobial treatments modulated the composition of gut microbiota. Groups such as *Lachnospiraceae*, *Lactobacillus johnsonii*, *Ruminococcaceae*, *Clostridiales*, and *Oxalobacteraceae* were less prevalent in the guts of chicks fed with antimicrobial supplemented diets. Overall, these studies suggest that AGPs modulate the diversity and structure of microbial population in the poultry gut ultimately resulting in an optimal microbiota that potentially facilitates in more efficient energy harvestation and better growth performance.

10.3.3 Host Immune Interaction with Microbiota

As discussed in the previous section, the association between gut microbiota and host has developed through a long series of selection, competition, and coevolution. Through these bidirectional interactions, the immune system has learnt to respond appropriately to commensal microbiota or pathogens. In turn the microbiota

participates in educating the immune system to function properly (Chow and Mazmanian 2010), and perturbations in early microbiota could affect intestinal immune development (Schokker et al. 2015). Extensive studies in germ-free (GF) mice, in the past decades, have demonstrated the critical role played by gut microbiota in shaping the host intestine immune system (Macpherson and Harris 2004; Grover and Kashyap 2014). Colonization of the poultry gut with microorganisms begins immediately after hatch, eventually leading to the establishment of a complex microbiota (Brisbin et al. 2008). As a first line of defense against pathogens, the avian gut is coated with mucus layer (Forder et al. 2012) consisting of a loose outer layer that harbors microbiota and an inner compact layer which repels most bacteria (Hansson and Johansson 2010). Recent studies suggest that components of the avian mucus modulate the expression of critical virulence traits of pathogens. *C. jejuni* colonizes the chicken gut in high numbers yet does not cause any disease in birds. In vitro data suggest that the presence of chicken mucin reduces the attachment and invasion efficiency of *C. jejuni* to intestinal epithelial cells (Byrne et al. 2007; Alemka et al. 2010, 2012). Struwe et al. (2015) used liquid chromatography mass spectrometry to perform structural analysis of O-glycans released in chicken intestinal mucin. The O-glycans were abundantly sulfated compared with the human intestinal mucus samples. In addition, alpha 1–2 linked fucose residues, which have high binding affinity to *C. jejuni*, were identified in the small and large intestines. These variations suggest that chicken gastrointestinal tract has evolved to support the colonization of *C. jejuni*. Moreover, N-linked glycosylation of surface proteins in *C. jejuni* enhances its fitness by protecting bacterial proteins from gut protease cleavage (Alemka et al. 2013).

Another critical component of innate immune system active in the poultry gut is antimicrobial peptides (AMP) present on the intestinal epithelial surface (Brisbin et al. 2008). Antimicrobial peptides have been described as a host defense that has coevolved with microbes (Zasloff 2002). Produced by all major eukaryotes, AMP provide immediate, effective, and non-specific defense against infections by bacterial, viral, or fungal organisms. Based on their secondary structure, these small molecules (15–50 amino acids) are classified into four major classes, namely, alpha helix, beta-sheet, and extended and loop peptides (Lai et al. 2009). Antimicrobial peptides act primarily by damaging the bacterial membrane (Shai 1999; Yang et al. 2001). Other mechanisms include suppression of protein, nucleic acid, or cell wall synthesis and inhibition of enzymatic activity (Brogden 2005). These attributes make them attractive candidates for the design of new antimicrobial agents. In poultry, beta-defensins are the well-characterized antimicrobial peptides that are produced by avian epithelial cells, macrophages, and heterophils (Jenssen et al. 2006; Derache et al. 2009). Brisbin et al. (2008) showed that infection with *Salmonella* resulted in an increase in the expression of beta-defensin genes in chickens. Ebers et al. (2009) profiled the expression of avian beta-defensin genes in chicken oviduct epithelial cells before and after infection with *S. Enteritidis*, where the pathogen was found to modulate the expression of select defensin genes. Moreover, the *pipB* mutant elicited significantly higher levels of avian beta-defensins 2 and 8, suggesting that the T3SS-2 effector protein PipB plays a role in dampening the beta-defensin-based innate immunity in birds during *Salmonella* invasion of chicken oviduct epithelial cells.

The cellular components of the avian immune system such as macrophages and heterophils also confer protection from enteric infection and are recruited to the site of infection to kill invading pathogens (Brisbin et al. 2008). Meade et al. (2009) studied the early host immune response to *Salmonella* and *Campylobacter jejuni* colonization in chickens. *Salmonella* infection induced significant changes in circulating heterophil and monocyte/macrophage populations, while *C. jejuni* infection increased monocyte/macrophage populations. Toll-like receptor 1 (TLR1) gene expression was decreased by *Salmonella*; however, beta-defensin genes (AvBD3, AvBD10, AvBD12) were significantly increased. In contrast, *Campylobacter* infection induced an increase in TLR21 expression but significantly reduced the expression of AMP genes (AvBD 3, AvBD 4, AvBD 8, AvBD 13, AvBD 14). Enteric pathogens have evolved to utilize some of the physiological changes in the gut to their advantage. For example, pathogen colonization of avian gut leads to mild inflammation with influx of macrophages and heterophils to the lamina propria and villus epithelium (Crhanova et al. 2011). *Salmonella* has developed mechanisms to survive in host cells such as macrophages which help in its systemic dissemination (Buchmeier and Heffron 1991; Cirillo et al. 1998; Malik-Kale et al. 2011; Swart and Hensel 2012). More recently, studies suggest that *S. Enteritidis* and *S. Typhimurium* can induce mild inflammation leading to influx of macrophages and heterophils to villus epithelium that aids in their systemic dissemination (Fasina et al. 2010; Van Immerseel et al. 2002). The major virulence factors participating in this pathogenesis and intracellular survival have been characterized (Ibarra and Steele-Mortimer 2009). Type III secretion systems, LPS, peptidoglycan, and flagellin that trigger inflammatory response through pathogen-associated molecular patterns and pro-inflammatory cytokines are some of the major virulence factors responsible for epithelial cell invasion and intracellular survival in macrophages (Ahmer and Gunn 2011; Zhou and Galán 2001; Abrahams and Hensel 2006). Taken together, these results suggest that the innate immune system of poultry responds differently to *Salmonella* and *Campylobacter* challenge and pathogens have evolved with strategies that facilitate their survival in the gut.

The interactions of host gut microbiota and innate immune response also trigger adaptive immune response activation (Palm et al. 2015; Lee and Mazmanian 2010). Studies in mice have revealed that a lack of adaptive immune system leads to alterations in the gut microbiota composition (Kato et al. 2014; Kawamoto et al. 2014). In the poultry gut, the B and T cells are found in organized lymphoid tissues (e.g., bursa of Fabricius, cecal tonsils, Peyer's patches) and some in lamina propria and epithelium (Brisbin et al. 2008; Bar-Shira et al. 2003). These cells primarily contribute to adaptive immunity through antibody-mediated and cell-mediated responses, respectively. Several researchers have investigated the effect of microbiome modulations on the adaptive immune response of chickens. Kim et al. (2010) investigated the effect of dietary plant-derived phytochemicals on the translational regulation of genes associated with immune modulation. Many of the genes contributing to metabolism, immunity, antigen presentation, and inflammatory response were modulated by the phytochemicals such as capsicum oleoresin and cinnamaldehyde. In another study, Du et al. (2016) tested the effects of thymol and carvacrol

on intestinal integrity and immune responses of broiler chickens challenged with *Clostridium perfringens*. Results revealed an increase in interleukin-1-beta and TLR2 mRNA expression. Moreover, the expression of secretory IgA was also upregulated in response to pathogen challenge. Interestingly, expression of claudin-1 and occludin mRNA (responsible for intestinal integrity) was downregulated. Dietary essential oil alleviated gut lesions and increased expression of occludin mRNA, suggesting that they modulate immune responses in *C. perfringens*-challenged broiler chickens. In addition to phytochemicals, probiotics have also been tested for their role as immune-modulators in chickens. Chickens receiving probiotics containing *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Streptococcus faecalis* showed increased systemic antibody reactive to tetanus toxoid and *C. perfringens* alpha toxin (Haghighi et al. 2006). In addition, intestinal IgG and serum IgG and IgM were also increased in chickens supplemented with the probiotics, suggesting the induction of natural antibodies for maintaining chicken health. Similar results have been reported with other groups of lactobacilli (Koenen et al. 2004; Brisbin et al. 2012). However, the exact mechanism(s) by which probiotic bacteria bring about aforementioned immune modulations is not clear and requires further research.

10.3.4 Pathogen Colonization

As discussed previously, the intestinal microbiota interacts with the gut mucosal immune system to maintain homeostasis, and disruption of this interaction leads to disease state. Perumbakkam et al. (2014) investigated the effect of Marek's disease virus on core gut microbiota of chickens. The virus targets lymphoid tissue such as the bursa of Fabricius, thymus, and spleen; however, limited literature exists on its interaction with gut-associated lymphoid tissue. The viral infection altered the core cecal microbiota early after infection (2–7 days) and in the late phase of infection (28–35 days) suggesting a correlation between viral infection and microbial composition of the intestinal tract. Necrotic enteritis caused by *C. perfringens* is another disease that affects poultry globally. Some of the factors that predispose the gastrointestinal environment for *C. perfringens* colonization include high levels of nonstarch polysaccharides, high protein fishmeal, and factors that induce epithelial cell damage (*Fusarium* mycotoxins, *Eimeria* infection). Recent studies suggest that the onset of necrotic enteritis is associated with changes in gut microbiota, including shifts in the alpha and beta diversity (Stanley et al. 2012, 2014). Butyrate-producing strains of *Ruminococcaceae* and *Lachnospiraceae* decrease with an increase in protein content of feed or *Eimeria* infection (Wu et al. 2014). Butyrate stabilizes intestinal integrity through its anti-inflammatory action (Eeckhaut et al. 2011) and activation of glucagon-like peptide 2(GLP-2). The GLP-2 hormone induces cytokine production that improves tight junction-based intestinal integrity (Hiramatsu et al. 2005). Pathogens such as *Campylobacter* and *Salmonella* which colonize chickens in high numbers tend to achieve this without perturbing the microbiome

significantly. Thibodeau et al. (2015) observed that the alpha diversity was conserved, while beta diversity was moderately affected during high cecal colonization by *C. jejuni*. Similar effects were observed by Videnska et al. (2013) with *S. Enteritidis* colonization in young chickens. The efficacy of trans-cinnamaldehyde and caprylic acid in reducing *S. Enteritidis* colonization in 24- and 40-week-old layer chickens and corresponding shifts in the microbiome was investigated (Upadhyaya et al. 2015a). Results revealed that the phytochemicals (caprylic acid 0.7, 1%; trans-cinnamaldehyde 1, 1.5%) were effective in reducing *S. Enteritidis* in the cecum, on the eggshell and in the yolk. There was no change in the alpha diversity, beta diversity, and major bacterial phylotypes (*Firmicutes*, *Bacteroides*, and *Proteobacteria*) across treatments and time (days 0, 1, 7, 10, 20, 30, 60). Overall, these results suggest that pathogen colonization in chickens usually occurs without major shifts in the microbiota.

10.4 Applications of Transcriptomics and Proteomics to Study Poultry Gut Microbiota Function

After characterizing the gut microbiota, it is critical to delineate the underlying mechanisms by which they impact gut physiology and health of the host. Technological advancements in RNAseq have enabled us to study genes that are actively expressed in a complex bacterial community such as the gut microbiota. This facilitates gaining insight into microbial function, interactions with the host, and changes that occur during disease state. Several researchers investigated the transcriptomic profile of gut microbiota, enteric pathogens, and poultry host to develop an understanding of various host-pathogen interactions. Taveirne et al. (2013) used RNAseq to study the complete *C. jejuni* transcriptome during colonization in chickens. A total of 272 genes that are differentially expressed during chicken colonization were identified. Some of the *C. jejuni* genes that were increased in abundance include genes coding for transport (pstSAC, ChuABCD), stress response (katA, cgb), and energy metabolism (dsbAB, sulphite oxidase, cytochrome c family). In addition to differential gene expression, several noncoding RNAs were also identified that are likely induced due to stress or nutrient limitation and potentially contribute to chicken colonization.

Li et al. (2010) investigated the cecal response of two genetic lines of chickens with different susceptibility to *C. jejuni* colonization. The more resistant line A birds responded by an upregulation of lymphocyte activation and increased expression of oligomerization domain (NOD)-like receptor. Also known as NALP1, these proteins function as sensors for detecting microbial components in the host cell similar to the role of toll-like receptors. In another study, Matulova et al. (2012) studied the changes in spleen transcriptome after infection with *S. Enteritidis* to identify potential markers of infection. A total of 40 genes were upregulated. Genes coding for avidin, immune responsive gene (IRG1), fatty acid binding protein (EXFABP), chemokine ah221, and trappin-6-like protein (TRAP6) were some of the upregulated genes.

Complementary DNA sorting revealed that the abovementioned genes were preferentially expressed in the macrophages. Furthermore, some of the abovementioned genes (EXFABP, IRG1, TRAP6, AH221) were also induced in the cecum of infected birds on day 1 post challenge. Ciraci et al. (2010) studied the genome-wide transcriptome profile of chicken macrophages exposed to *S. Typhimurium*-798 derived endotoxins. Pathway analysis showed that 10% of total differentially expressed genes were involved in inflammatory response. Endotoxin exposure significantly affected the mRNA expression of IL1B, IL6, IL8, and TLR15 in chicken macrophages (HD11). Overall, these studies provide insights into the expression of key genes during host-pathogen-microbiota interplay in the gut.

A major challenge while using DNA-based approaches for characterizing microbiota functionality is that the data predict potential functions based on the presence of certain genes. In addition, questions such as expression levels of genes and source of DNA (live/dead cells) require additional experimentation. However, such limitations can be addressed using proteomics as a tool for characterizing microbiota function. High-throughput metaproteomic analysis provides a clear fingerprint of the metabolic state of a microbial community such as in the gut (Verberkmoes et al. 2008) and is a useful resource for providing meaningful data on host-microbiota interactions and microbiota function. Since the identified proteins can be assigned to taxa as well as functions, it is very useful to study different functional properties of a microbial community, including any posttranslational modifications. Tang et al. (2014) conducted a metaproteomic analysis of fecal samples to study the adaptation process of chicken gut microbiota. Metaproteomic analysis identified 3673 proteins of which 380 proteins belonged to *Lactobacillus* spp., 155 to *Clostridium* spp., and 66 to *Streptococcus* spp. The most frequently identified proteins were chaperon proteins (GroEL, DnaK), dehydrogenases, elongation factor proteins, heat shock chaperones, and pyruvate kinases. In addition to studying the metaproteome, researchers have also investigated the proteome of a poultry enteric pathogen as well as the host response. Upadhyay et al. (2017a) investigated the effect of trans-cinnamaldehyde (essential oil obtained from cinnamon bark) on the proteome of *C. jejuni*. Results revealed that trans-cinnamaldehyde downregulated the expression of several proteins (AspA, FrdA, AhpC, PstS, CeuE, HemC) critical for aero tolerance, acid tolerance, stress response, and colonization in chickens. Follow-up investigation revealed that the phytochemical was able to significantly reduce *C. jejuni* colonization in broiler chickens (Upadhyay et al. 2017b). In another investigation, Upadhyaya et al. (2017) studied the changes in *C. jejuni* proteome in response to subinhibitory concentrations of eugenol (essential oil obtained from cloves). Interestingly, the group of proteins downregulated by eugenol (PorA, CadF, CheA, CheV, CheY, LuxS, TatA, TatB, MotA, MotB) primarily contribute to bacterial adhesion, locomotion, and cell-to-cell communication, and they were different from those observed with trans-cinnamaldehyde, suggesting that the two essential oils work via different mechanisms/pathways. O'Reilly et al. (2017) investigated changes in chicken intestinal proteome in response to microbial challenge and age of birds and observed significant changes in the small intestinal proteome sampled from 12 to 22 days of age in chickens. Proteins such as actin and

actin-associated proteins increased over time. Villin-1, an actin-associated anti-apoptotic protein, was reduced in abundance in birds challenged with *C. jejuni* and coccidial oocysts, indicating that many of the changes in cytoskeletal protein abundance in the challenged birds were because of an increased rate of apoptosis. Several heat shock proteins also decreased over time, especially in challenged birds. Some of the challenges that still need further research include (1) high complexity of the miscrobial community, (2) low coverage of the complete proteome by existing technology, and (3) high sequence similarity between many proteins, especially those that perform similar functions (Haange and Jehmlich 2016). One potential approach that is being employed is the use of gnotobiotic animal models that have a relatively simple microbiota consisting of only well-characterized species. This may facilitate greater protein coverage during analysis and enable better interpretations (Woting et al. 2014).

10.5 Applications of Metabolomics to Study Host-Microbiota Interactions

The metabolome is the final transcript of the genome that consists of all low molecular weight compounds (metabolites; less than 1500 Da) in a cell, tissue, or organism. The metabolites produced by the microbiota are key signaling compounds that, along with proteins, form the biochemical basis of cross talk with other microorganisms and hosts. Metabolomics is a powerful scientific approach that can be used to study such metabolite-based chemical cross talk. More recently, metabolomics is being extensively employed to study gut physiology in health and disease (Holmes et al. 2011), identify biomarkers for rapid diagnosis of a physiological state (Dunn and Ellis 2005), and characterize microbial metabolism (Vaidyanathan et al. 2006).

10.5.1 Analytical Tools to Study Microbial Metabolomics

Metabolomic methodologies fall into two broad categories: untargeted metabolomics, a comprehensive analysis of all measurable compounds in a sample and targeted metabolomics, and the measurement of defined classes of well-characterized and annotated metabolites (Roberts et al. 2012). With the rapid development of a plethora of analytical platforms, we can effectively detect and quantify metabolites and characterize relevant metabolic pathways. Some of the popular analytical platforms include liquid and gas chromatography (LC, GC), high-pressure and ultra-pressure liquid chromatography (HPLC, UPLC), Fourier transform infrared spectroscopy (FTIR) coupled with mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (NMR) (Zheng et al. 2011; Vernocchi et al. 2012, 2016). A brief description of these methods is presented in this section. Additional details are described elsewhere (Roberts et al. 2012; Vernocchi et al. 2016).

10.5.1.1 Gas Chromatography Mass Spectrometry (GC-MS)

Gas chromatography mass spectrometry (GC-MS) is the gold standard in metabolomics (Harrigan and Goodacre 2012) that is used to study heat stable and volatile compounds such as alcohols, esters, aldehydes, ketones, and fatty acids. The metabolites are separated by GC followed by detection by electron-impact (EI) mass spectrometer. The samples are prepared by liquid/solid phase extraction or by headspace-solid phase microextraction based on specific requirements (Dettmer et al. 2007; Pawliszyn 1997). The metabolites are stabilized by a two-stage derivatization process (Roessner et al. 2000). Metabolite quantification is conducted by external calibration or response ratio (peak area of test metabolite/peak area of internal standard). Identification of metabolites is conducted by matching retention time and mass spectrum of the sample peak with a pure compound previously analyzed under identical conditions (Fiehn et al. 2000) or against a commercial database.

10.5.1.2 Liquid Chromatography Mass Spectrometry (LC-MS)

Liquid chromatography mass spectrometry is an analytical technique with a wide range of applications in biotechnology, food, pharmaceutical, and cosmetic industry. The LC-based metabolite separation is followed by electrospray ionization (ESI) or atmospheric pressure chemical ionization (Bakhtiar et al. 2004). The combination of LC with MS permits analysis of polar, nonpolar, and neutral compounds. Unlike GC-MS, the LC-MS technique does not require sample volatility or sample derivatization, thereby facilitating accessibility to much greater mass ranges than permitted by GC-MS. Metabolite quantification is obtained by external calibration/response ratio. Moreover, ESI does not provide direct metabolite identification due to lack of molecular ion fragmentation and mass spectral libraries. With the development of HPLC and UPLC, the analysis time has been shortened along with higher resolution and sensitivity (Smirnov et al. 2016).

10.5.1.3 Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectroscopy studies the vibrational properties of compounds based on the presence of functional groups in their structure (Berthomieu and Hienerwadel 2009). These functional group-specific IR signatures are often used to identify the metabolites in a sample. Although comparatively insensitive as compared to GC/LC-MS, this technique allows high-throughput screening and classification of biological samples (Ellis and Goodacre 2001). It has been used to detect spoilage in meat (Ellis and Goodacre 2001), milk (Nicolaou and Goodacre 2008), and strawberries (Dong et al. 2013) and to detect the bovine mastitis marker (Schabauer et al. 2014) and characterization of food spoilage fungi (Shapaval et al. 2013).

10.5.1.4 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance refers to the phenomenon in which nuclei in the presence of a magnetic field absorb energy leading to a high-energy state followed by release of the energy at a specific resonance frequency. This energy is quantified using a spectrometer. NMR spectroscopy is used to study the structure of molecules, intermolecular interactions, composition of biological mixtures and metabolites. Some of the advantages of using NMR spectroscopy include (a) simpler sample preparation, (b) identification of compounds with identical masses and low ionization state, and (c) nondestructive nature (Lenz et al. 2004; Smolinska et al. 2012). Moreover, site-specific NMR imaging offers strategies for metabolic investigations in live animals (Markley et al. 2017). Some of the disadvantages of NMR spectroscopy include low sensitivity and restricted annotation capability (Jansson et al. 2009). Le Roy et al. (2016) used NMR spectroscopy to characterize chicken tissues (liver, kidney, spleen, egg yolk, plasma, colon, cecum, fecal water, pectoral muscle, brain) followed by metabolite identification. Approximately 80 metabolites were identified to develop the first chicken metabolome atlas. Only eight metabolites were found to be common across all tissue samples. In another study, Quirk et al. (1989) studied the metabolites present in chicken small intestinal cells using NMR spectroscopy. High concentrations of serine ethanolamine phosphodiester (SEP), creatine, aurine, and acidic amino acids were found in all segments of the intestinal tract. Taurine (~8 mM), choline (0.5 mM), and betaine (~0.5 mM) were evenly distributed throughout the segments. These works constitute development of a database for future NMR-based metabolomic investigations in relation to poultry production and health.

10.5.2 Microbial Metabolites

Several metabolites are produced by the gut microbiota that facilitates host-microbiota cross talk. Short-chain fatty acids are produced as a result of fermentation of indigestible polysaccharides, fiber, and proteins (Arora and Sharma 2011). Bacterial groups such as *Lactobacillus*, *Actinobacteria* (*Bifidobacterium* spp.), and *Clostridium* clusters IV and XIVa play a major role in SCFA synthesis and metabolism (Nicholson et al. 2012). Acetate is an important SCFA that facilitates development of colonic epithelium through its trophic effect. It is also absorbed by tissues participating in cholesterol synthesis (Scheppach et al. 1991). Propionate, on the other hand, inhibits cholesterol synthesis (Scheppach 1994), and the ratio of acetate to propionate is used as an index for determining the risk of cardiovascular disease in humans (Wong et al. 2006). Butyrate represents the major energy source for distal gut and nourishes the colonic mucosa (Walton et al. 2013). The production of SCFA in poultry gut has been studied to elucidate their association with health and productivity. Chang et al. (2016) studied the beneficial effect of *Bidens pilosa* (flowering plant in aster family; commonly known as beggartick or Spanish

needle) on body weight gain, FCR, gut microbiota composition, and susceptibility to coccidiosis in chickens. Results revealed that administration of *Bidens pilosa* significantly elevated body weight gain and reduced feed conversion ratio. Metagenomic analysis revealed an increase in probiotic genera such as *Alistipes*, *Bacteroides*, *Lactobacillus*, and *Ruminococcus*. *Bacteroides* and *Ruminococcus* have been previously reported to be involved in polysaccharide degradation and production of propionate in chicken gut (Sergeant et al. 2014). Vitamins are another group of critical micronutrients that play an essential role in biochemical reactions/pathways in the majority of animal hosts. *Bacteroides* and *Lactobacillus* were reported to produce essential vitamins such as vitamins K and B12 and folic acid (Luo et al. 2003). These results suggest that the beneficial effects observed with *Bidens pilosa* could be partially due to production of useful metabolites such as SCFA and vitamins.

In addition to SCFA and vitamins, other microbiota-transformed compounds include bile salts and polyphenols (Vernocchi et al. 2016). The metabolism of bile salts is primarily associated with bacterial genera such as *Bacteroides*, *Clostridium*, *Lactobacillus*, *Bifidobacterium*, and *Enterobacter* (Ridlon et al. 2006). In poultry, bile salts contribute to lipid digestibility and weight gain, and dietary interventions that modulate bile salts levels in the gut also affect digestibility and poultry health. Maisonnier et al. (2003) studied the effect of infeed supplementation of guar gum on lipid digestibility, intestinal bile salts levels, and health in broiler chickens. Results revealed that guar gum reduced bile salts levels, thereby leading to altered lipid digestibility and reduced weight gain in birds. Polyphenols are plant nutraceuticals that are considered as bioactive components in the diet (Manach et al. 2004). They have high structural diversity; however, most of them occur as glycosylated derivatives and require intestinal transformation through host enzymes or gut microbiota-mediated metabolism (Marin et al. 2015). Major groups of polyphenols include tannins, flavonoids, chlorogenic acids, and coumarins. Several investigations have studied the interaction of polyphenols with the intestinal microbiota in mice (Duda-Chodak et al. 2015; Ozdal et al. 2016) and humans (Van Duynhoven et al. 2011). In addition, the role of several polyphenols as effective antimicrobials for controlling foodborne pathogens in chickens has been investigated (Upadhyaya et al. 2015b, c; Kollanoor-Johny et al. 2012). However, studies investigating the metabolism of polyphenols including their absorption kinetics in chickens require further research.

10.6 Conclusion

Scientific advancements, especially in genomics, transcriptomics, and metabolomics, have contributed to rapidly accumulating knowledge in gut health. Several studies have elucidated the connection between microbial metagenome, metatranscriptome, proteome, and metabolome in relation to poultry health, productivity, and safety. As new research delineates the complete gut metabolome in various

physiological states (health and disease), a better understanding of host response to various environmental cues, microbiota, and chemicals would possibly emerge. The role of microbial metabolites in epigenetic activation/repression of gene expression through posttranslational and posttranscriptional modifications is a relatively less explored field that holds promise for new discoveries in gut health and productivity in poultry production.

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