

Application of microbial analyses to feeds and potential implications for poultry nutrition

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ABSTRACT Poultry nutrition and feed manufacturing are interrelated for a variety of reasons. Diet formulation is essential for optimizing bird growth and feed conversion, but compositional differences and the presence of certain feed additives can alter the gastrointestinal microbial composition and functionality. Not only does dietary composition and digestibility influence poultry performance, but specific physical characteristics such as feed particle size and thermal treatments can impact the avian gastrointestinal tract (**GIT**) microbiota. Poultry feeds also have a characteristic

microbial ecology consisting of pathogenic and non-pathogenic microorganisms. Some feed-borne pathogens such as *Salmonella* are well studied and linked with the colonization of birds consuming the feed. However, much less is known about the nonpathogenic feed microbiome and what impact that might have on the bird's GIT. This review discusses the potential interaction between poultry feed and the GIT microbiome, microbial ecology of feed, application of microbiome analyses to feed, and approaches for communicating these complex data sets to the poultry industry.

Key words: poultry, microbiome, microbial ecology, feed, nutrition

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INTRODUCTION

Nutrition is a critical component for achieving efficient poultry broiler production. A balance between maximum growth rate in birds and an efficient conversion of feed to weight gain represents the economic focus for optimal poultry meat production. This nutritional balance also holds for egg production, where maintenance of egg-laying hens for maximum egg production is a part of the overall dietary management strategy. The resulting nutritional demands emphasize optimal nutritional management programs with improved poultry genetics, housing management, and environmental advancements (Dittoe et al., 2020). Several factors influence nutritional management in poultry production. Indeed, specific nutrient requirements of either the broiler or laying hen impact feed formulation. For example, protein quality, essential amino acid availability, micronutrient supplementation, energy requirements are critical considerations for formulating diets. Other factors such as antinutritional factors from specific feed ingredients, loss of nutrients via formation of Maillard products

during thermal feed processing, and palatability must be considered as well. Crystalline essential amino acids such as lysine and methionine are often supplemented to meet specific protein synthesis requirements.

The importance of the microbial quality of feed and the interaction with the bird's gastrointestinal tract (**GIT**) also has become a factor in poultry nutrition. For example, the presence of harmful agents in poultry feeds such as mycotoxin-producing fungi has historically been a concern for bird health and well-being (Oguz, 2011). Not surprisingly, numerous antifungal strategies have been developed over the years to minimize these fungi and the subsequent production of mycotoxins. Likewise, the presence of foodborne *Salmonella* in feeds has been a food safety concern due to the potential of these pathogens to colonize the GIT of birds consuming these contaminated feeds (William 1981a; Maciorowski et al., 2004). As with antifungal treatments, several chemical and physical interventions have been proposed and implemented to decrease *Salmonella* in feed and potentially diminish colonization in the bird that consumes contaminated feed (Williams, 1981c; Wales et al., 2010; Ricke et al. 2019, 2020). While the emphasis on feed has been primarily focused on pathogenic agents, there has been an emerging realization that the microbiota in the feed may have some influence as well (Figure 1). The organisms that constitute the feed biota appear to be as

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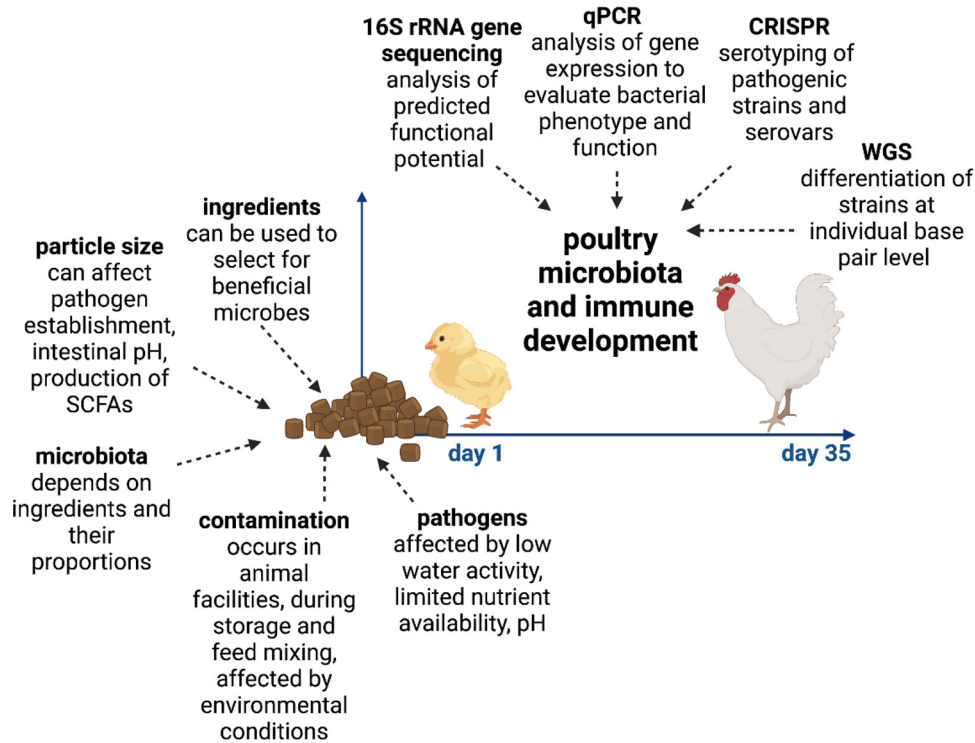


Figure 1. Ingredients, particle size, pathogens, environmental contaminants are potential factors that affect the composition of microbiota associated with poultry feed. Feed microbiota may affect maturation of poultry's microbiota and the immune development. Techniques that may elucidate on their development are 16S rRNA analysis, qPCR analysis of gene expression, clustered regularly interspaced short palindromic repeats (CRISPR) serotyping of pathogenic strains, and whole genome sequencing (WGS) and analysis. Figure created with Biorender.com.

varied and diverse as the sources and ingredients used in mixing feeds for poultry and other animal species (Ricke, 2005, 2018a; Maciorowski et al., 2007). However, it is not clear what impacts this may have on the quality and storage shelf life of the feed before its utilization and how these organisms influence the development of the poultry GIT microbiota. This review will discuss the GIT and feed microbial ecology, feed processing, whole-genome sequencing (WGS), and the utilization of 16S rDNA-based microbiome sequencing for feed analyses. In addition, microbiome methods for characterizing feed microbial populations and communicating bioinformatic information to poultry nutritionists will be examined.

POULTRY GIT MICROBIOTA AND HOST RESPONSES

The GIT microbiota of food-producing animals, such as chickens, raised in large commercial scale housing environments (Concentrated Animal Feeding Operation; CAFO) can potentially differ when compared to the same age animals raised in the wild because of the influence of their immediate environment (Shi et al., 2019; Ricke and Rothrock, Jr., 2020). Differences may also hold for poultry raised in a free-range organic environment with limited housing and outdoor access vs. CAFO raised birds. Birds raised in these environmental settings not only are exposed to a wide range of microorganisms in their

respective environments but are likely to consume a more varied diet that includes exotic dietary sources such as forages and insects (Shi et al., 2019; Ricke and Rothrock, Jr., 2020). The GIT microbiome development in birds can be influenced by the bird's age as well. As birds mature, the relatedness among the respective GIT compartments, crop, small intestine, and ceca decreases, and diversity within the GIT microbial populations increases (Stanley et al., 2014; Rychlik, 2020). Consequently, distinct GIT populations can be associated with each GIT compartment in the bird. For example, strict anaerobes such as methanogens can be detected in the ceca of mature birds, indicating the occurrence of an extensive anaerobic fermentation (Saengkerdsud and Ricke, 2014). In older birds such as laying hens, distinct phases of GIT microbial population shifts can be distinguished as a function of age and phase of egg-laying (Videnska et al., 2014). Not surprisingly, differences in diets associated with various types of bird production may have a distinctive effect on the GIT microbiota (Shi et al., 2019; Feye et al., 2020a). Birds raised on pastures have access to a broader diversity of food types, such as insects and grass, in addition to formulated feed (Ricke and Rothrock, Jr., 2020). The grass may be ingested and play a significant role of fiber in the bird's diet, likely impacting the GIT microbiota and their functionality (Ricke and Rothrock Jr., 2020). Fiber-containing feeds benefit proper GIT function and birds' overall

health (Shi et al., 2019). More specifically, the cecal microbiota of layer hens has been shown to ferment various fibers such as alfalfa both in vitro and in vivo (Dunkley et al., 2007a,b; Ricke et al., 2013).

These changes in the GIT microbiome are reflected in the host as well. It is relatively well established that nutritional supplements and feed additives have a noticeable impact on the presence of pathogens in the GIT and the GIT immune system response (Hume, 2011; Clavijo and Flórez, 2018; Swaggerty et al., 2019). These changes are likely reflected in the GIT microbiota's nonpathogenic members (Stanley et al., 2014; Rychlik, 2020). Malmuthauge et al. (2015) observed a relationship between the microbiome and the expression of genes controlling the mucosal lining and nonspecific defense mechanisms in neonatal cattle. Regional distinctions in the microbiome were correlated with local differences in the innate immune gene transcription. Comparable conclusions have been made on the broiler's microbiome and the expression of avian cytokine RNA transcripts by Oakley and Kogut (2016). In their study, a negative correlation between the phylum of Firmicutes and the proinflammatory cytokine genes was detected. In contrast, a positive correlation was revealed with the phylum Proteobacteria and the proinflammatory cytokines. In addition, Arsenault and Kogut (2015) have concluded that several immune-signaling pathways can be activated at the asymptomatic *Salmonella* infection site. The host and pathogen interactions lead to numerous immune and biochemical changes at the infection site that potentially can decrease nutrient uptake by the host and further bacterial establishment in the GIT (Oakley and Kogut, 2016). Even when diets may be relatively similar, variances between GIT microbiota may still be detected. For instance, de Greeff et al. (2010) observed differences in expression of 49 genes, including those associated with immune response, while comparing jejunal gene expression in layer hens that consumed either conventional or organic feed of comparable composition.

POULTRY GIT MICROBIOTA AND IMPACT OF FEED

High-quality nutritional diets are a fundamental component of poultry protein production (Dittoe et al., 2020). It follows that microbiota in the GIT poultry likely will interact with the diet being consumed by the bird. Commensal bacteria play an essential role in animals' fitness, while pathogen establishment can lead to deleterious outcomes (Yadav and Jha, 2019). When fluctuations in ingredients or quantity of nutrients in animal diets occur, selection for particular resident GIT microbiota can vary (Yadav and Jha, 2019). Indeed, some feed additives such as prebiotics and organic acids are well known for their selective impact on the poultry GIT microbiota (Dittoe et al., 2018; Ricke, 2003, 2015, 2018b). For example, prebiotics serve as substrates for specific GIT microbiota, such as lactic acid bacteria and

Bifidobacteria. Selection for these particular microorganisms can have favorable impacts on bird health and performance and limit the establishment of foodborne pathogens. More recently, there are indications that a wide range of cereal grains and other carbohydrate sources can elicit prebiotic-like impacts on the poultry GIT (Zhuang et al., 2017; Ricke, 2018b).

Other characteristics associated with feed can also impact the GIT microbial ecology. One of the most significant factors that define feed utilization in these animals is the particle size of the feed (Kiarie and Mills, 2019). Feed particle size has been demonstrated to influence the occurrence of enteric pathogens in the GIT. Research indicates that coarse feed particle size decreases pH in the stomach content compared to fine particle size, which is linked to biochemical and microbial properties (Kiarie and Mills, 2019). Mikkelsen et al. (2004) demonstrated that coarse size particle feed increased anaerobic bacteria numbers and the concentration of organic acids in swine. Further *in vitro* studies with the pig's stomach content fed coarse particle size feed led to an increased reduction of *Salmonella* Typhimurium DT12 and higher concentrations of undissociated lactic acid (Mikkelsen et al., 2004). In addition, beneficial bacterial counts, such as *Lactobacilli*, were amplified at the lower gastric pH, and pathogenic bacterial growth, such as enterotoxigenic *Escherichia coli*, was inhibited. Lower pH also supported a higher proportion of short-chain fatty acids in undissociated form, which, in turn further enhanced antimicrobial potency (Kiarie and Mills, 2019). Subsequently, the gastric environment generated by the coarse feed created an additional obstacle against the spread of fecal/feed-oral pathogens (Kiarie and Mills, 2019). Larger particle sizes have been shown to increase the flow of starch in the GIT, increasing short-chain fatty acids production and inhibition of coliforms and *Salmonella* (Mikkelsen et al., 2004). Huang et al. (2006) used the *Salmonella* Typhimurium DT12 model developed by Mikkelsen et al. (2004) to assess whether the physical characteristics of feed manipulated *Salmonella* colonization in broiler GIT. The results were similar to those observed in pigs; less *Salmonella* reduction was associated with higher pH in the gizzard of birds fed fine feed particles. Finely ground feed particles have also been shown to stimulate the proliferation of *Clostridium perfringens* which can contribute to necrotic enteritis infection in birds (Branton et al., 1987).

However, the impact of feed on the poultry GIT may be more than just a few specific feed additives or physical forms. It is conceivable that the general microbial composition of poultry feed could be a critical factor for the development and growth of food-producing animals and their GIT microbiome. Certainly, the presence of antimicrobial feed additives in animal feed plays a crucial role in structuring animals' commensal intestinal microbiota, host gene expression, and immunity (Torok et al., 2011). However, the question remains whether the microorganisms associated with that feed also directly or indirectly impact the development of the

GIT microbiome. There is indirect evidence that feed microbial composition may affect GIT microbial composition. In an *in vitro* broiler cecal culture model where various cultivars of rice bran were supplemented in a commercial basal diet, the level of *S. Typhimurium*, microbiome profile, and the metabolites were evaluated over a 24 h period post-adaptation (Rubinelli et al., 2017). There were differences in rice cultivar bran reduction of *Salmonella*, with some being much more inhibitory. When microbiome populations were compared for particular rice bran, there were detectable differences in cecal populations associated with cecal contents incubated with only basal feed versus the combination of rice bran and basal feed. This finding indicated that even for subtle feed compositional differences, distinct microbiome population taxa could be detected (Rubinelli et al., 2017).

While diet can play a discriminatory role in the local GIT population, it is not clear whether naturally occurring bacteria in the feed can colonize the GIT of the bird consuming that feed. Feed-borne pathogens such as *Salmonella* can be introduced to the poultry GIT when contaminated feed is consumed and subsequently colonize the GIT, particularly young birds. Likewise, numerous studies have been published on the colonization of the poultry GIT via artificially inoculated feed with *Salmonella* and successful administration of probiotics and competitive exclusion cultures. Less is known on whether indigenous feed microbiota directly becomes established in the poultry GIT. Olson et al. (2020) have noted that there are similarities between the feed microbiota and neonatal intestinal microbiota of chicks, which predominantly consist of Enterobacteriaceae and Firmicutes phyla. It has been suggested that feed microorganisms could be a contributor to GIT microbial development in young chicks (Diaz Carrasco et al., 2019). The impact these feed bacteria might have on the composition and maturation of GIT microbiota in neonatal chicks is not established. To develop an understanding of this relationship requires a more in-depth characterization of the feed microbial populations, their ecology, and how this could relate to the poultry GIT.

FEED MICROBIAL ECOLOGY

The ecology of microorganisms in animals and poultry feeds is complex. There are a variety of reasons for this. The components that comprise a complete feed originate from a wide range of sources, including different cereal grains and various amendments such as crystalline amino acid supplements, micronutrients, and other specialized ingredients. These can potentially contribute to microorganisms in the feed. Cereal grains may carry microorganisms from the soil where they were grown, during storage before shipping, and those encountered during transportation to the feed mill. In addition, if protein sources such as meat and bone meal or other rendered animal protein sources are introduced, these can make specific contributions to the microbial composition

of the feed. Further microbial contamination likely occurs during the feed mixing and blending processes where microorganisms residing on the surfaces of the feed mill equipment come in contact with the feed as individual ingredients are added and mixed into the feed. Other sources such as aerosols and dust present during feed mill operations are likely contributors. In addition, given the low water activity and other environmental conditions, some selectivity potentially occurs as feeds remain in storage before delivery to poultry and animal facilities. Finally, microbial cross-contamination can also happen at the animal facilities if the feed comes in contact with microbial carrying vectors such as rodents or insects. These vectors have been well documented as carriers for specific pathogens such as *Salmonella* and can lead to *Salmonella* colonization in poultry flocks (Park et al., 2008). It will not be surprising if these vectors are also sources of nonpathogenic organisms that contaminate feeds and contribute to the microbial composition of the feed.

Most of the research in feed microbial ecology has been focused on the presence and control of foodborne pathogens and toxigenic fungi (Ricke, 2005, 2018a; Oguz, 2011; Ge et al., 2013). Bacteriophages also have been detected in dry feeds and silage, but their impact on overall feed bacterial ecology remains essentially unknown (Maciorowski et al., 2001a; Vongkamjan et al., 2012). Much of the bacterial pathogen effort has centered on *Salmonella*, but other pathogens such as spore-forming *Clostridium perfringens* and *C. botulinum* have been associated with feeds and silage (Xylouri et al., 1997; Wojdat et al., 2006; Ricke, 2018a). Silage also has been identified as a source for *Listeria* spp. (Ryser et al., 1997). A wide range of factors impacts the frequency and occurrence of bacterial pathogens in feed. Some of these are better characterized than others. Certainly, the low water activity, limited nutrient availability, and other variable environmental conditions are likely to contribute to the frequency and level of pathogens detected in feeds (Ricke, 2018a). The presence of feed amendments such as organic acids, aldehydes, and other chemicals designed to decrease the pathogen populations in feeds can impact levels of detectable pathogens and also can influence the accuracy of detection methods for specific pathogens such as *Salmonella* (Carrique-Mas et al., 2007; Wales et al., 2010). Likewise, thermal treatments such as pelleting can decrease pathogens. However, spore formers can survive higher temperatures, and there is the risk of recontamination of the pelleted feed by non-spore formers such as *Salmonella* as it cools off after pelleting (Ricke, 2005, 2018a; Jones, 2011).

Animal feed harbors a wide array of microorganisms, many of which are probably nonpathogenic. Identifying pathogens such as *Salmonella* within this highly diverse and complex animal feed microbial consortia is difficult due to bacteria's random and inconsistent occurrence throughout the feed matrix (Maciorowski et al., 2004; Ricke, 2018a). Several challenges such as achieving representative sampling of large volumes of feed, infrequent

occurrence, and the wide range of serovars make routine *Salmonella* monitoring difficult. Characterizing and monitoring the distribution of indicator nonpathogenic organisms, which are analogous in physiology with associated pathogens, throughout the feed processing steps may pose a potential means for predicting the presence of such pathogens (Ricke, 2018a). In contrast to pathogens, indicator organisms commonly occur and are found ubiquitously throughout the feed and feed-associated matrices in high enough numbers that are readily detectable by most molecular and cultural methods (Ricke, 2018a). However, little is known about the taxonomy of nonpathogenic bacteria associated with commercial feeds. In one of the few studies that has been reported, Olson et al. (2021) described the microbiota associated with a select group of poultry feeds. Using a 16S rDNA sequencing approach, Olson et al. (2021) detected 24 bacterial taxa identified with 4 discrete phyla among the 11 morphologically distinct aerobic plate count (APC) colonies from poultry feed isolates. The four phyla included the most abundant phyla of Firmicutes, followed by Proteobacteria, and one taxon in each phylum of Epsilonbacteraeota and Actinobacteria. Low levels of *Campylobacter* genus were noted within 89 of 11 bacterial feed isolates, indicating *Campylobacter* occurrence in animal feed and leading to the suggestion that this could be a route of exposure to poultry.

Identification of nonpathogenic indicator microorganisms associated with animal feed and manufacturing would be useful for quantitating the effect of feed additives on these candidate organisms. Isolating bacteria from nonselective media that support a diverse, viable microbial population, followed by 16S rRNA gene analysis, can offer a much more rapid means to screen animal feeds and precisely identify unique strains, symbiotic relationships, and bacterial niches. Taxonomic description of the isolates from aerobic plate count media (APC) colonies described in Olson et al. (2021) reflects the bacterial taxa that generally have been considered feed microbiota from microbial culture-based earlier studies. For example, Loken et al. (1968) described *Micrococcus* and *Bacillus* as the main genera associated with rendered protein additives and *Salmonella* and *E. coli* as potential pathogens. Cox et al. (1983) isolated *Enterobacter agglomerans*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* as commercial poultry feed representatives of *Enterobacteriaceae* and *Salmonella*.

DETECTION AND IDENTIFICATION OF FEED PATHOGENIC MICROORGANISMS

Most microbial detection and identification methods for feed microorganisms have focused on pathogens. *Salmonella* has received most of the research attention, with considerable early work on adapting culture methods such as enrichment and selective plating to detect and enumerate *Salmonella* from feed sources (Williams, 1981b; Maciorowski et al., 2006; Feye et al., 2021). More rapid molecular and immuno-based methods have

emerged to complement the traditional culture-based methodologies (Maciorowski et al., 2005, 2006). Application of polymerase chain reaction (PCR) assays for the detection and quantitation of *Salmonella* in feeds have provided much more rapid results and delineation of specific serovars (Maciorowski et al. 2005). More recently, the application of quantitative PCR (qPCR) has allowed for the molecular quantitation of *Salmonella* in feeds and, depending on the genes, targeted assessment of physiological and virulence status. For example, Park et al. (2011) measured the expression levels of *Salmonella* virulence regulatory gene *hilA* qPCR to not only detect viable *Salmonella* in heat-treated feeds but to demonstrate increased levels of expression after exposure to higher temperatures (Park et al., 2011). The authors concluded that increased *hilA* expression could not only be used as a quantitative assessment of viable bacterial cells, but increased expression could be an indicator of the increased capability of *Salmonella* to colonize susceptible young chicks. Andino et al. (2014) compared 15 strains of 11 *Salmonella* serovars using qPCR measurement of the survival and virulence genes. They concluded that in the presence of dry feed sampled over time, the *cfa* gene (cyclopropane fatty acid synthesis) was upregulated by most strains and that survivability in feeds varied among strains. Given these differences in *Salmonella* serovars and strains, it may be essential to apply methods capable of differentiating these differences in *Salmonella* isolated from feeds to better determine potential risk as a function of strain and serovar. Indeed, conventional serotyping can accomplish this, but molecular-based serotyping may represent an improvement. For example, Shariat et al. (2021) applied clustered regularly interspaced short palindromic repeats (CRISPR)-typing to serotype *Salmonella* isolates from samples collected from over 100 U.S. animal feed mills. They reported that serovars Infantis and Tennessee were the most commonly isolated *Salmonella*. As more becomes known about *Salmonella* CRISPR, this approach may have utility for routine screening of feed samples.

It is becoming clear that tracking *Salmonella* and other pathogens can be enhanced as more becomes known at the genomic level. The technology for bacteria's whole genome sequencing (WGS) continues to improve and become more economically accessible. This availability has encouraged the advancement of surveillance and quality control in the food safety industry on a national and global level. Whole genome sequencing offers methods for differentiating bacterial strains and geographical variations within a strain (Laing et al., 2017). It is anticipated that food and feed safety will become more dependent on a complementary alliance between federal agencies, local health and veterinary departments, and food and feed manufacturing industries. Whole genome sequencing is becoming more of an investigative technique and offers a culture-independent approach with only one colony for practical construction of DNA library using an Illumina platform (Kährström, 2014). Based on WGS, the U.S. FDA Veterinary

Laboratory Investigation and Response Network has linked animal clinical isolates to one or more raw pet food *Salmonella* spp., *L. monocytogenes*, and *E. coli* isolates (Jones et al., 2019).

The main goal of source attribution is to separate animal disease over several presumed sources of infection, including animal feed, environment, and the presence or absence of additives. Data from WGS provides the network for analog comparison and is beneficial in food-borne disease surveillance, inspection and monitoring, outbreak detection and containment, and food technology developments (FAO, 2016). This technology further revolutionized the application of microbiology in the food industry by offering benefits such as pathogen detection, microbial profiling in various food environments, genotype-phenotype correlation, and the use of starters in food manufacturing (FAO, 2016).

Whole genome sequencing allows for the characterization of bacterial strains that can be differentiated at the individual base-pair level on the genomic chromosome leading to single nucleotide polymorphisms (SNPs) profiles (Ricke et al., 2018). In addition to genomic resolution at the SNP level, WGS may be a necessary means for identifying specific pathogenic bacterial traits in feeds, such as their respective antibiotic resistance genes and virulence factors. Additionally, WGS has been used extensively to characterize and identify pathogens such as *Salmonella*, as described in detail previously (Ricke et al., 2018), and will not be discussed in the current review. Further advancements may eventually lead to more direct connections between the pathogens present in the feed, their physiological status, and the potential for colonization in animals and birds consuming those feeds. Integrating numerous levels of -omic techniques into a single data set that can represent a distinct environmental variation that could provide a highly detailed understanding of how bacteria survive and interact in poultry feeds and what impact that has on the bird.

MICROBIOME IDENTIFICATION OF NONPATHOGEN FEED MICROORGANISMS

Most of the research on the ecology and identification of feed microorganisms has been conducted via conventional culture methods (Ricke, 2005, 2018a). However, unlike specific pathogens, identifying particular non-pathogens in feed microbial communities is more of a hurdle because of the inherent selectivity of most culture media. Consequently, it had been challenging to capture a more comprehensive and complete survey of the various microbial taxa potentially present on a particular feed. The microbial taxonomy based on individual members of microbial communities can now be specified with the general advancements in sequencing methodologies and the development of a 16S rDNA microbiome sequencing approach (Ricke et al., 2017). In addition to identifying individual taxa, diversity comparisons among microbial populations became possible. Feed

microbial analyses offer the opportunity to assess the impact of feed treatments such as antimicrobials on indigenous microbial populations on feeds and the effect on the GIT microbial populations of the animals consuming that feed. Only limited microbiome analyses of feeds have been done thus far. For example, Solanki et al. (2019) compared the bacterial microbiomes of wheat grains stored over time with the insect fumigant phosphine added. They demonstrated that the wheat grain microbiomes were more diverse immediately after harvest and 3 months later.

However, limitations for applying microbiome analyses of feeds remain to be resolved. For example, biological compounds, such as proteins and lipids, that are present in feed matrices may not entirely be separated during the DNA extraction procedure and can affect the integrity of DNA or inhibit the following PCR process (Maciorowski et al., 2001b, 2002; Piskata et al., 2017, 2019). These inhibitors can restrict PCR efficiency by reducing or blocking DNA polymerase activity. DNA extraction can be highly subjective by the method used for extraction and the starting sample medium (Feye et al., 2020b). Because feed is exposed to various treatments during the manufacturing process that drastically affects the quality of DNA, it becomes necessary to optimize DNA isolation procedures for each type of commercial animal feed. The optimal DNA extraction technique depends on bacterial quantities, chemical composition, and physical properties. Different methods have been found to vary in purity and DNA yield, impacting the molecular results (Maciorowski et al., 2001b; Kennedy et al., 2014). Kennedy et al. (2014) found 16S rRNA gene sequencing variation when comparing different DNA extraction kits. There is no particular DNA extraction kit available for animal feed due to its variability in composition (Maciorowski et al., 2001b, 2002, 2005). Therefore, an optimized DNA extraction technique associated with feed is necessary for comparable downstream sequencing analyses.

Other factors such as a high degree of sequence homology in closely related bacterial species may lead to difficulties in identification (Devanga Ragupathi et al., 2017; Peker et al., 2019). For example, targeted 16S rRNA gene sequencing does not discriminate between *Shigella* species and *E. coli* due to 99% sequence homology (Devanga Ragupathi et al., 2017; Peker et al., 2019). In this case, Devanga Ragupathi et al. (2017) suggest using WGS to distinguish the 2 types of bacteria. Furthermore, Rychlik (2020) noted that the abundance of the Actinobacteria phylum might be underestimated due to a limited number of 16S rRNA gene copies, potentially resulting in a high variation of 2 to 10% of the phylum detected in a healthy chicken GIT microbiota.

Feye et al. (2020b) discussed in detail several critical principles that must be assumed with microbiome sequencing prior to data execution, and these will be described briefly in the current review. As these authors emphasized microbiome data is not quantitative, and the microbial populations must be chosen with a statistically based foundation. The use of a *Q*-value rather than

a *P*-value must be implemented in pairwise and individual compositional effects. Feye et al., (2020b) concluded that statistical analyses such as *t* tests and analysis of variance (ANOVA) are less appropriate as the assumptions correlated with microbiome sequencing, and the statistical methods can be in contrast to each other. Although statistical power remains a debatable topic in microbiome sequencing, numerous R-based approaches do exist that can enhance the power of the analysis and the applicability of the results (Feye et al., 2020b). Without appropriate statistical power and compositional analyses, the data are not informative and could be misconstrued. Another limitation to microbiome sequencing is that it incorporates all the bacterial DNA present within the sample (Feye et al., 2020b). It should be noted that the presence of bacteria alone does not indicate how those populations functionally contribute to microbial ecosystems such as poultry feeds. It will still be important to combine microbiome sequencing with the phenotypic characterization and microbiological plate enumerations to relate microbiome-based identification with culturable and not-culturable feed microbial populations. As more poultry feed microbiome data are collected and analyzed, these criteria and precautions will be highly relevant when comparing different feed sources and ingredient microbial populations. This will also be important when studies are conducted that attempt to connect the feed microbial composition with the GIT microbial population of the birds consuming the feed.

COMMUNICATING MICROBIOME ANALYTICS TO FEED AND NUTRITION AUDIENCES

It is beginning to become apparent that poultry nutrition, feed manufacturing, and the GIT microbiome are interrelated and can influence overall poultry production and health. As the understanding of the interactions between poultry nutrition, health, the GIT microbiome, and bird productivity has grown, the two spheres of food and GIT microbial ecology have begun to become more interconnected in the field. The introduction of microbiome analyses and bioinformatic interpretation has accelerated the need to further integrate these seemingly separate research pursuits. As nutritionists become more concerned with concepts such as GIT health, it is essential to keep in mind that they approach these topics from a different starting point and set of expectations than researchers focused on characterizing the GIT microbiome, exclusively. Therefore, it is crucial to present a newly derived complex data set, including the microbiome, metabolome, histology, and host gene transcription abundance interpretations in a way that is both intelligible and actionable, but more importantly, that delivers economically meaningful performance applications. While the microbiome data and its associated bioinformatic analytics offer potentially new, ground-breaking information, translating this seemingly

complex data into formats that can communicate the potential impact is critical for widespread practical application. And those impacts are only going to be pursued by industry if there is a corresponding economic benefit. After all, commercial animal production is a financial endeavor, and every advancement, whether nutritional, immunological, or behavioral, needs to be economically justifiable as well if widespread implementation is to occur.

Introducing microbiome analyses directly to poultry feeds adds a new dimension to potential applications of this type of information for poultry nutrition interpretations and production operations. While minimal data has been generated thus far, the opportunities and rationale for conducting additional microbiome studies directly on poultry feeds are becoming an apparent, if not obvious, application. The connection between feed microbiota and microbiota establishment in the young bird's GIT has not been made. However, some properties of poultry feed, such as particle size, do appear to influence GIT microbial composition, so it would not be surprising if other feed characteristics also influence the GIT microbial ecology. As more research is conducted, the assessment of poultry feed microbiota on the development and maturation of the GIT microbial populations will undoubtedly become more definitive.

Developing these microbiome data sets and delineating the intricate relationships between nutrition, performance, and feed composition will be challenging. The solution will require several approaches to accomplish widespread adoption and utilization of this data type. A critical initial requirement will be to develop data interpretation tools that readily can be incorporated into poultry feed formulation and nutritional recommendations. However, the more significant challenge will be to provide interpretations that offer practical applications for the feed industry and poultry nutritionists.

A comprehensive meta-analysis assessment of key microbial variables in feeds that align with the GIT microbiome and ultimately with bird performance would contribute to the identification of the data critical for practical implementation. This knowledge also would direct future research studies to provide specific data sets for supplementing missing information required to generate more robust modeling of the interactions between feeds, the GIT, and poultry production. Once some of the data can be modeled into more practical applications, educational training strategies need to be launched that ensure appropriate utilization of this information. Indeed, the production of educational materials that offer definitions and examples of microbiome applications would merit short courses and workshops catered explicitly to poultry nutritionists and production managers. Delivery of this information by academic and technical advisors accompanied by the appropriate laboratory and computer exercises would be an efficient means to accelerate a more general understanding among different audiences. However, an essential key to such practices is to provide discussions through round table interactions and other types of

open-ended sessions to encourage questions and input on how microbiome and bioinformatics data can be used for specific situations in real-world settings. Such interactions will require a dynamic relationship between academic instructors and industry personnel to identify optimal communication approaches for encouraging the implementation of these new tools.

CONCLUSIONS AND FUTURE DIRECTIONS

Poultry production has evolved into an integrated industry that produces broilers with a rapid and proficient metabolism for transforming feed into the meat (Dittoe et al., 2020). Nutritional management and diet construction are essential components of an optimal broiler grow-out production cycle. This is also true for optimizing egg production in layer hens. Regardless of the type of poultry production system, quality of feed is a crucial consideration for a variety of reasons. Chemical and physical characteristics such as heat damage and feed particle size can influence the microbial activities in the poultry GIT and potentially bird health and performance. Likewise, pathogenic microorganisms such as *Salmonella* and mycotoxin-producing fungi in the feed can have harmful effects on the bird when consuming these contaminated feeds. Much less clear is the potential influence of the nonpathogenic microbiota on the development and functionality of the poultry GIT microbiota. There is limited data to suggest that some members of the feed microbial community are similar to those that inhabit the poultry GIT. Still, a direct demonstration of colonization of feed microbiota in the GIT has not been established. The emergence and application of 16S rDNA sequencing technologies should help develop a better understanding of the poultry feed microbiome and its interaction with the poultry GIT.

While molecular techniques have a relatively long history of targeting pathogens such as *Salmonella* in feeds, microbiome analysis of nonpathogenic microbial populations is currently underutilized. An opportunities for expanding the application of microbiome analyses to feed microbial ecology is a likely research target that may have practical implications. However, challenges remain for routine application. As observed with PCR methods, extraction of DNA from complex organic matrices such as feeds can be difficult. In addition, the tremendous volumes of feed that are processed and mixed at the feed mill can be an obstacle to collecting representative samples. Despite these difficulties, the possibility of using microbiome analyses on poultry feeds offers some potential practical applications. For example, identifying nonpathogenic indicator microorganisms to parallel the much less frequent pathogen microbial populations would be of practical utility for evaluating process control and intervention measures during feed manufacturing. Less obvious but perhaps more intriguing would be the characterization of the potential impact of the indigenous feed microbiota on the evolution of the poultry GIT, particularly in young birds.

The opportunities to use microbiome analyses and bioinformatics for poultry nutrition and feed manufacturing would appear to offer new insight into poultry production's microbial ecology. However, the generation of these datasets represents the assessment of relatively complex computations to effectively interpret the biological relevance of the data outcomes. Consequently, analyses and interpretations need input from a practical standpoint focusing on applications of interest to the poultry and feed industry. The communication must be easy to understand and provide practical merit because the microbiome data interpretation has to be presented in a manner that can be understood by a lay audience and used for realistic recommendations. Efforts to develop targeted educational forums such as workshops, webinars, and other media packages for presenting these research findings and interpreting and utilizing the data will be critical. Media events that encourage open discussion, such as round table sessions where questions and practical issues can be offered and discussed, would provide some of the input needed to expand the use of this type of information to a broader audience.

DISCLOSURES

Joshua Jendza was employed by BASF Corporation during the execution of the current project. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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