Gastrointestinal microbiomes of broilers and layer hens in alternative production systems

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ABSTRACT Alternative poultry production systems consisting of free-range or pasture flock raised poultry continues to increase in popularity. Based on the perceived benefits of poultry products generated from these alternative poultry production systems, they have commercial appeal to consumers. Several factors impact the health and well being of birds raised and maintained in these types of production systems. Exposure to foodborne pathogens and potential for colonization in the gastrointestinal tract has to be considered with these types of production systems. The gastrointestinal tract microbial composition and function of birds grown and maintained in alternative poultry operations may differ depending on diets, breed, and age of bird. Dietary variety and foraging behavior are potential influential factors on bird nutrition. The gastrointestinal tract microbiomes of birds raised under alternative poultry production systems are now being characterized with next-generation sequencing to identify individual microbial members and assess the impact of different factors on the diversity of microbial populations. In this review, the gastrointestinal tract microbiota contributions to free-range or pasture-raised broiler and egg layer production systems, subsequent applications, and potential future directions will be discussed.

Key words: alternative poultry systems, gastrointestinal tract, microbiomes, layer hens, broilers

INTRODUCTION

While there are a number of terms used to define the many different alternative poultry management systems (e.g., all-natural, free-range), the American Pastured Poultry Producers Association (APPPA, 2019) states that the 2 requirements for poultry to be considered pastured/pasture-raised are (1) continual access to pasture and (2) frequent movement/rotation of flocks onto fresh pasture (typically on a daily basis), which they consider different from free-range. In addition, the APPPA considers "free-range" a very broad USDA label only requiring access to the outdoors (APPPA, n.d.). For purposes of this review, free-range or pasture-raised poultry will be used interchangeability throughout the review to define birds raised and sustained on pasture with some mobility available to the birds along with the provision of a mobile shelter (Sossidou et al., 2011). More specifically, Elkhoraibi et al. (2017) has described these farms as any operation maintaining bird numbers below 3,000 layers or 20,000 broilers. In an online survey study on backyard flocks in the United States (US), Elkhoraibi et al. (2014) concluded that the majority of respondents owned less than 10 chickens for less than 5 yr, and rural owners generally possessed larger flocks and were more likely to derive income from their flocks as opposed to urban and suburban flock owners. Several factors have contributed to these alternative production systems becoming an economically viable component of commercial poultry production, including consumer preferences for specific animal production systems and overall marketing appeal (de Jonge and van Trijp, 2013; Pettersson et al., 2016; Bray and Ankeny, 2017). Among the specific drivers that have contributed to increasing popularity include the cage-free movement in the laying hen industry, the continued interest in locally produced food, and perceptions on poultry welfare and nutrition (Gifford and Bernard, 2011; Mench et al., 2011; Rainey et al., 2011; Elkhoraibi et al., 2014; O'Bryan et al., 2014; Pettersson et al., 2016; Phillips-Connolly and Connolly, 2017).

As expected from the highly varied profiles of free-range poultry flock owners, the corresponding management strategies and concerns are also relatively diverse. For live birds, several management topics have been identified in surveys of producers from 14 states by Elkhoraibi et al. (2017) as being primary concerns including access to sufficient feed at an acceptable cost, limiting exposure to predators, managing soil and vegetation, and deciphering food safety and egg product

regulations. Bird health issues were also considered a minor concern, but earlier surveys indicated a general lack of awareness of some poultry health conditions and the need for more information, mainly since there was a perceived limitation in available local veterinarians (Elkhoraibi et al., 2014, 2017). Readily available poultry processing facilities and lack of niche markets were also listed as concerns (Elkhoraibi et al., 2017). O'Bryan et al. (2014) attributed this lack of processing facilities to the historical displacement by large scale integrated poultry processing industries leaving minimal options for small-scale poultry farmers with annual sales ranging from $10,000 to $499,999 in rural, suburban, and peri-urban areas. The introduction of the mobile poultry processing unit that can be shared by multiple small flock owners has provided a viable alternative for converting birds into marketable poultry meat products (O'Bryan et al., 2014). However, environmental issues can be a potential issue with these types of processing facilities and access to sufficient water for processing may be problematic as well (O'Bryan et al., 2017; Micciche et al., 2018).

Several of the issues associated with alternative live production bird operations involve the gastrointestinal tract (**GIT**). Bird health and intestinal issues, such as exposure to parasites, present challenges due to the diverse sources for potential exposure including not only feed, water, and litter, but free-range bird consumption of snails, earthworms or various insects (Wuthijaree et al., 2019). Likewise, food safety is also a potentially critical issue as evidenced with the recent series of salmonellosis cases associated with backyard flocks (Behravesh et al., 2014; Hardy et al., 2019). As with exposure to multiple sources of parasites, free-range birds would likely encounter a wide range of vectors for foodborne pathogens such as *Salmonella* (Park et al., 2008, 2013). In addition to the usual risks associated with foodborne diseases, farms with mixed crop-livestock operations present additional challenges for the dissemination of zoonotic pathogens (Kijlstra et al., 2009; Salaheen et al., 2015). The other challenge is the development of economical feedstuffs that meet the specific requirements of pasture flock nutrition and also the acceptability of being considered "natural" feed ingredients. This has resulted in several efforts to examine alternative feed sources while taking into account the foraging aspect of pasture flock management as well the specialized needs of broilers vs. layers.

A common theme for all of these issues in live bird production is the GIT and its resident microbial population. Therefore, a comprehensive understanding of the chicken GIT microbial ecosystem under these production conditions is needed to determine the interaction between the bird host, GIT pathogens, and the indigenous GIT microbiota. The objective of this review is not to present a comprehensive overview of all the historical research on this topic, but rather to focus on the current status of GIT microbiota contributions to free-range broiler and egg layer production

systems, subsequent applications, and potential future directions.

THE PASTURE FLOCK BIRD GIT – GENERAL CONCEPTS

The avian GIT consists of several compartments beginning with the beak, followed by the esophagus, crop, proventriculus, gizzard, small intestine, ceca, and colon with each segment contributing to overall digestion in some fashion (Svihus, 2014). Once feed is consumed, the esophagus delivers feed materials to the crop, proventriculus, and gizzard, where digestion begins. The crop is believed to be involved in the storage of incoming feed, but passage rate and subsequent feed retention varies depending on the composition of the feed and frequency of meal feeding (Svihus, 2014). Fermentation also occurs in the crop by a predominant lactic acid population with primarily lactate and acetate as the end products (Rehman et al., 2007). The combination of the proventriculus and gizzard provides a "stomach-like" function with the proventriculus producing HCl and pepsinogen to start the digestion process (Svihus, 2011). The gizzard provides the grinding action to reduce the particle size to produce an outgoing uniform digesta that enters the small intestine. In the small intestine segments (duodenum, jejunum, and ileum) most of the digestion and subsequent nutrient absorption occurs (Svihus, 2014). The remainder of the digesta is passed onto the ceca, which is the primary site of microbial activity where a complex microbial population is harbored including strict anaerobes (Józefiak et al., 2004; Rehman et al., 2007). Extensive fermentation of carbohydrates occurs in the ceca with the generation of short-chained fatty acids (**SCFA**) and ammonia from the degradation of uric acid (Karasawa, 1989; Józefiak et al., 2004; Svihus et al., 2013). Finally, the digesta enters the colon, and eventually, the remaining digesta is excreted as waste materials.

Minimal comparisons have been made between conventional poultry GIT structure and function with birds raised on nonconventional, free-range environmental conditions. It is not clear whether substantial differences would occur, but the differences in diets and the ability of free-range birds to forage would suggest that there could be some impact on the GIT microbial activities and function. Likewise, the genetic line of bird may also have an impact as Lumpkins et al. (2010) noted differences in intestinal development between a modern multipurpose broiler strain, a high-yield strain, and a historic strain of bird. In a more recent study, Walugembe et al. (2015) compared Ross male broiler chicks with Hy-Line layer chicks fed dried distillers grains with solubles and wheat bran at different supplemented levels during different phases of their growth cycle (60 g/kg on days 1 to 12 and 80 g/kg on days 13 to 21). On day 21 they sacrificed the birds and used terminal restriction fragment polymorphism to compare

microbial population composition in the ceca and gas chromatography to quantitate cecal SCFA. Acetate was the SCFA produced in the most significant quantity followed by propionate and butyrate, which is consistent with previous reports (Rehman et al., 2007). Production of SCFA, acetate, and propionate, was higher in broiler chicks vs. layer chicks and the individual SCFA were not different when the fiber level was changed, except for a decrease in butyrate when birds were shifted to the higher level of fiber. Cecal microbial populations differed both as a function of diet and genetic line of bird and metagenomic analyses revealed relative abundance differences of specific microbial group differences such as *Helicobacter pullorum* (nearly 52% in low fiber-fed layers), *Megamonas hypermegale* (12.7% in broilers fed high fiber), *Bacteroides* (17.5% in low fiber-fed broilers), *Faecalibacterium* (11.3% in low fiber-fed broilers, 10.8% in high fiber-fed layers), and *Escherichia coli* (12.4% in high fiber-fed broilers). Interestingly, *Campylobacter jejuni* and *C. coli* were detected in high fiber-fed broilers vs. only *C. jejuni* in low fiber-fed broilers, but not in layer chicks fed either low or high fiber diets. This may suggest that the appearance of *Campylobacter* is somewhat dependent on the overall microbial consortia present, which is consistent with what is known about *Campylobacter* ecology in the poultry GIT (Indikova et al., 2015).

Before overall conclusions can be drawn on whether GIT differences may occur in birds grown and maintained under alternative poultry production conditions vs. conventionally raised birds, more studies with a greater number of birds will need to be conducted. This is in part due to the fact that the choice of bird genotype appears to be essential for alternative poultry broiler and egg-laying production. Consequently, slower-growing birds are favored to meet the animal welfare and environmental conditions associated with outdoor access and free-range pasture due to their behavior patterns being more suited for actively foraging compared to conventional, fast-growing breeds (Sossidou et al., 2011). In addition, interest in utilizing local bird genotypes continues to increase for niche meat and poultry meat markets (Rizzi et al., 2013; Mosca et al., 2019). The dietary choices for birds raised in alternative poultry production systems is also a factor in the types of feeds utilized, as well as the pasture forages available for grazing (Singh and Cowieson, 2013; Sossidou et al., 2015; Bodie et al., 2019). Age of bird is known to be a factor in conventional bird GIT development, and this is likely also the case for the slower-growing breeds. In short, it is not known how the GIT microbial composition and functionality of these types of birds and the production conditions they encounter differs from conventional breeds. The introduction of next-generation sequencing (**NGS**) and microbiome characterization has allowed a more comprehensive examination of the GIT microbiota in these birds raised in alternative poultry production operations.

FREE-RANGE LAYING HEN GIT MICROBIAL ECOLOGY

The emergence of nonconventional egg production systems has occurred as a function of the movement towards cage-free systems and the rise of organic and speciality egg markets (Michella and Slaugh, 2000; Patterson et al., 2001; Windhorst, 2005; Anderson, 2009; Rossi, 2011; Sumner et al., 2011). With the introduction of aviaries, cage-free, and free-range management operations, several issues have been identified that need to be considered for further development of these types of systems. Among these issues, food safety, animal welfare, and economics have all been identified as focal points for further research (Anderson, 2009; Holt et al., 2011; Mench et al., 2011; Sumner et al., 2011). In conventional egg production, *Salmonella*, particularly the serovar *S.* Enteritidis, has been the primary foodborne pathogen concern for a number of years although other pathogens such as *Listeria monocytogenes* have been detected in some floor housed layer flocks (Chemaly et al., 2008; Howard et al., 2012; Ricke, 2017). When considering alternative laying hen housing, Holt et al. (2011) suggested that factors such as housing environment, stress exposure, and breed difference should be considered. In a survey of Californian commercial poultry pasture layer flocks, Dailey et al. (2017) concluded, based on *Salmonella* Pullorum positive whole blood agglutination tests, that primary indicators for a likely positive test included flock size and mobile coops with raised flooring consisting of either chicken wire or wood slats. Jones et al. (2011) reported that the season of the year could influence environmental and egg microbial levels in free-range layer hen operations. In an overview of research on different egg-laying housing systems, De Reu et al. (2008) concluded that aerobic bacterial contamination of eggshells was greater for nest eggs in non-cage operations vs. nest eggs produced by hens in furnished cage or conventional cage houses. They also identified microbial contamination from environmental sources, such as dust in aviaries, as contributing to microbial contamination of nonconventional produced eggs (De Reu et al., 2008). Egg handling may also be a factor as Whiley et al. (2016) reported that washed free-range eggs held at a higher storage temperature led to increased penetration by *S.* Typhimurium when inoculated on the eggs.

Dietary variety and access to pasture forage may also play a role in the GIT microbial composition of free-range layer hens. Al-Ajeeli et al. (2018) noted that when egg production and quality attributes of cage vs. free-range birds were compared for birds fed the same diet, the production results for free-range birds were more variable. Some of the variations in performance values associated with free-range layers may be due to the ability of the hens to be mobile and potentially consume available forage. The impact of forage on laying hen nutrition and GIT microbial activity is difficult to define due to the lack of quantitative precision for

assessing the level of range impact and the potential for behavioral differences among birds within a flock. For example, Larsen et al. (2017) using Radio Frequency Identification to track individual laying hens in free-range flocks concluded that most hens accessed the range every day both in close proximity to their sheds as well as long distances from the sheds, but the daily duration and frequency varied extensively among individual birds. In summarizing free-range laying hen pasture consumption studies, Singh and Cowieson (2013) concluded that pasture forages were consumed by hens and could be detected in crop contents, potentially contributing to the nutrient requirements of the bird. However, the nutritional contribution may be influenced by foraging activity as Golden et al. (2012) observed that increased nutritional demands of foraging activity led to a reduction in nutrient partitioning for egg production. This may also factor in the level of hen manure on the open range and the subsequent environmental impact during the runoff occurring from rainfall (Xin et al., 2011).

The impact of forage intake in free-range laying hens is also potentially influenced by the type of forage and corresponding nutrient content. Indeed, there is potential for fiber utilization by the GIT microbiota of the layer hen (Ricke et al., 2013). Layer chick cecal and intestinal microbiota composition responds to the presence of fiber, and the cecal populations appear to be able to ferment different fiber sources included in the diet (Escarcha et al., 2012; Walugembe et al., 2015; Zheng et al., 2019). Dunkley et al. (2007a), using an in vitro cecal culture screening method, demonstrated that a wide array of fiber sources could support adult laying hen cecal microorganisms and influence their fermentation pathways. Results from adult hens fed high fiber molt diets also suggest that the cecal microbiota are capable of explicitly responding to the inclusion of fiber and generating SCFA (Hume et al., 2003; Dunkley et al., 2007b; Callaway et al., 2009). Redig (1989) noted that many avian species were capable of modifying their GIT and that as fiber intake increased, gizzard and cecal size increased accordingly. Redig (1989) concluded that this, in turn, allowed for more energy extraction from cellulose via SCFA production.

How important fiber and forages are to free-range layers remains to be determined. Based on avian crop content studies and vegetation cover changes, it appears that free-range laying hens do consume a certain quantity of pasture forage (Lorenz et al., 2013). However, Singh and Cowieson (2013) pointed out that there was minimal information on bird selectivity or preference for particular types of forages. More recently, De Koning et al. (2019) examined saltbush plants (*Atriplex nummularia* and *A. amnicola*) as a source of shelter, shade, and potential forage source for free-range layers. When comparing groups with or without saltbush over 11 wk, they observed that the hens ate 5% of their dry matter intake as saltbush forage with no impact on egg production. In a follow-up experiment, they (De Koning et al., 2019) incorporated saltbush at levels up to 20% in diets fed to layer hens for 28 D and detected no influence on egg production, hen live weight, or feed intake while excreta increased proportionally with increased dietary levels of saltbush. More studies need to be done on long term foraging pattern adaptation of free-range layer hens and the impact on their GIT compartmental functionality and microbial compositional development over time, including changes in crop storage capacity, gizzard size and grinding activity, and cecal level of fermentation.

Age of bird must also be considered as a potentially influential factor. Van den brand et al. (2004) reported more significant fluctuations in egg characteristics from free-range hens vs. those housed in cages and concluded that environmental conditions influenced these responses. If bird age can alter egg production, it is conceivable that the GIT microbiota of free-range layer hens also responds to age as is seen with other commercial birds (Stanley et al., 2014; Oakley et al., 2014a). Cui et al. (2017) compared intestinal and cecal microbiota of 8 and 30-week old cage fed or free-range laying hens. They used denatured gradient gel electrophoresis (**DGGE**) for overall microbial diversity comparisons as well as isolation of individual DGGE bands for taxonomic identification of specific bacteria. They reported considerable differences in intestinal diversity between caged and free-range birds and between young vs. older hens with the small intestinal microbial populations more impacted than the cecal populations. The free-range birds exhibited more distinguishable bacterial sequences at both 8 wk and 30 wk than their cage fed counterparts. They noted that the abundance of *Coprococcus*, *Clostridium*, *Butyricimonas*, *Paraprevotella*, and *Acinetobacter* was greater in free-range hens. Based on these results, it appears that both age and type of housing environment can influence the development and compositional makeup of the laying hen GIT microbial communities. It would be of interest to examine more incremental age differences between 8 and 30 wk and include samples from laying hens both younger than 8 wk as well as older than 30 wk similar to the study reported by Videnska et al. (2014) for commercially raised layer hens. This would help to identify the timing of transitions in the GIT microbial populations as well as compare with other factors such as forage availability and grazing behavior. However, the application of more advanced taxonomic characterization based on NGS is needed to achieve a higher resolution of the individual members of the laying hen GIT microbial populations.

FREE-RANGE BROILER GIT MICROBIAL ECOLOGY AND FOODBORNE PATHOGENS

Free-range broiler production faces several of the same issues as free-range layer hen operations including threats from predation, bird health, and environmental

exposure, but there are distinctions as well (Sossidou et al., 2011, 2015). In addition to differences in production outcomes of eggs vs. poultry meat, other differences such as breed, diet, and length of time on pasture are likely different as well. While free-range broilers would be expected to have shorter pasture time than laying hens, the introduction of slower growing breeds would still mean longer growing cycles than conventional broiler production systems that use fast-growing breeds. Fast-growing broiler breeds can be market-ready in as little as 7 wk, whereas slow-growing breeds might require up to 12 wk before entering retail markets (Fanatico et al., 2009). Sossidou et al. (2015) has suggested that slow-growing poultry breeds are preferred for free-range operations because they are more adaptable to the environmental conditions associated with extended pasture occupation due to their growth rate, skeletal development, behavior characteristics, and a well-developed immune system. Also, Sossidou et al. (2011, 2015) pointed out that there may be animal welfare benefits and lower nutritional requirements for slow-growing breeds on pastures vs. fast-growing breeds under these same conditions. In addition, Fanatico et al. (2007) demonstrated that meat quality differences existed between slow-growing and fast-growing bird genotypes raised on alternative production conditions.

Food safety concerns also have to be considered with free-range broiler production and most aspects involving free-range broilers have recently been summarized by Shi et al. (2019) and will not be discussed in detail in the current review. The primary foodborne pathogens associated with poultry, namely *Campylobacter* and *Salmonella* have been identified in free-range broiler flocks, processing operations, and retail products (Bailey and Crosby, 2005; McCrea et al., 2006; Hanning et al., 2010; Melendez et al., 2010; Trimble et al., 2013a,b; Tangkham et al., 2016; Li et al., 2017). *Listeria* spp. have also been detected in pasture flocks, but it remains unclear how prevalent and under what environmental circumstances their presence would be favored in alternative poultry operations, although age of flocks and time of year have been identified as leading to the likelihood of increased detection of *Listeria* spp. in the feces and soil (Chemaly et al., 2008; MililIo et al., 2012; Rothrock et al., 2016, 2017, 2019; Locatelli et al., 2017; Golden et al., 2019). The routes for foodborne pathogen colonization in the GIT and systemic invasion would presumably be similar to conventional poultry (Shi et al., 2019). Certainly, this would be dependent on host factors such as intermittent dietary intake that is potentially experienced during free-range grazing. For example, the temporary removal of diet and a subsequent emptying of the GIT contents in commercial broilers have been shown to predispose market-ready birds to relatively rapid pathogen GIT colonization (Ramirez et al., 1997). However, research needs to be conducted with specific foodborne pathogen challenge studies under free-range conditions to determine whether any differences occur. Although retail preva-

lence levels can vary, Sossidou et al. (2015) concluded that the risk of zoonotic disease was not strictly linked to housing conditions but noted that there is exposure to a broader and more variable range of potential vectors in free-range/pasture-raised broilers.

Since antibiotics are generally excluded from birds in free-range production, the occurrence of antibiotic resistance in microorganism isolated from birds produced under these conditions would presumably be less frequent. Most of the antibiotic resistance profiling has been conducted on foodborne pathogen isolates from free-range birds or retail meat products. Rothrock et al. (2016) conducted a 6 farm survey of 15 all-natural antibiotic-free broiler flocks using the Centers for Disease Control National Antimicrobial Resistance Monitoring System for Enteric Bacteria (**NARMS**). Of the foodborne pathogens isolates screened via the NARMS panel, antibiotic resistance was most prevalent in *Listeria* and *Salmonella* and least prevalent in *Campylobacter* with *Salmonella* antibiotic resistance being farm-specific, but not with the *E. coli* isolates used as indicator organisms. They reported that several isolates of *Listeria*, *Salmonella*, and *Campylobacter* exhibited multidrug resistance to 3 or more antibiotics. Siemon et al. (2007) and Melendez et al. (2010) also detected multidrug resistance in some of their *Salmonella* isolates from free-range broiler farms as did Nguyen et al. (2016) for *Campylobacter* isolated from small scale and backyard layer flocks in Kenya.

How much risk this is to consumers that purchase free-range poultry meat retail products is not definitive. Kamboh et al. (2018) compared *Enterobacteriaceae* liver isolates of individual birds removed and slaughtered from both commercial broiler flocks and backyard flocks in Pakistan. They screened the isolates for antibiotic resistance with a battery of antibiotics using an agar disk diffusion method. Overall, the multidrug-resistant *E. coli*, *Klebsiella*, and *Salmonella* isolates from commercial bird livers yielded a higher level of resistance for 9 antibiotics than their counterparts from backyard flocks. However, there were exceptions as more backyard flock *Klebsiella* and *E. coli* liver isolates were resistant to chloramphenicol and oxytetracycline than the commercial isolates, while all three pathogens from backyard flocks were more resistant to oxytetracycline. A more comprehensive analysis of antibiotic resistance in free-range birds will require a metagenomic assessment of the GIT microbiome to assess whether nonpathogenic GIT microorganisms harbor antibiotic resistance genes and how extensive they are in the overall GIT microbial population.

MODULATION OF THE FREE-RANGE BROILER GIT MICROBIOME

Diet, poultry breed, bird age, and housing conditions can all exhibit potential influence on the development of the broiler GIT microbiological consortia (Kers et al.,

2018; Shi et al., 2019). Low nutrient diets are typically promoted to support slower growth of pasture flock birds, and intake restricted cereal-based diets are known to lead to increased leguminous pasture intake and improvement in bird performance (Fanatico et al., 2007; Ponte et al., 2008). These types of dietary differences between conventionally raised poultry and pasture flock birds may also be a distinguishing influence on the respective GIT microbiota. Lourenco et al. (2019a) fed pasture raised broilers either soybean-based or soy-free diets and the birds were grown over a 12-week period. Microbiome composition was examined during this time from fecal samples collected during the trial, and cecal and whole carcass rinsate samples were also taken when birds were slaughtered and processed. At day of processing, after 12 wk, the fecal and carcass samples yielded more richness in diversity of the soy-free diet fed birds than the soybean fed birds. The beta diversity analyses revealed that the 2 diets led to distinct microbial populations, particularly for the 12 wk fecal samples and the carcass rinses.

Specific feed additives have been examined in free-range broilers in an attempt to modulate the GIT microbial populations in ways that benefit the broiler host (Shi et al., 2019). There are a wide array of different types of chemical compounds, organic acids, botanicals, and several biological additives, bacteriophage, probiotics, and prebiotics, that either have potential or have been examined directly for the use as a feed amendment for free-range poultry (Shi et al., 2019). Botanicals, in particular, may be attractive since free-range birds are already grazing on forage and their GIT microbial populations should be adapted to more efficient fiber utilization. Islam et al. (2019) evaluated organic low-bush blueberry (*Vaccinium angustifolium*) pomace (**LBBP**) as a feed additive for free-range broilers. Birds were fed 2 levels of LBBP (1 and 2%) for the first 21 D, and ileal samples were collected incrementally over the 64 D trial for microbial culture-dependent enumeration along with DNA sequencing and analysis for taxonomic identification and microbial community diversity comparisons. Based on enumeration, the ileal *Lactobacillus* populations appeared to be higher in the birds fed diets containing LBBP than those fed the control diet from days 21 to 42, with day 42 populations significantly higher in birds supplemented LBBP than those fed the control. However, the ileal *Lactobacillus* populations were significantly lower in birds fed diets supplemented with LBBP than those fed the control at day 64. The primary phyla identified by sequencing were *Firmicutes*, *Proteobacteria*, *Bacteridetes*, and *Tenericutes*, and as the trial progressed the authors noted some displacement of *Firmicutes* by *Bacteroidetes* in the LBBP fed birds. At the genera level, *Lactobacillus* was the predominant inhabitant identified in ileal samples overall, and at day 21 the 2% LBBP birds had the highest proportion of *Lactobacillus* compared to the other treatments. After day 29, *Lactobacillus* decreased in the control

fed birds and eventually were replaced by other genera at day 64 for all treatments. In general, ileal microbial diversity increased as birds matured, and the authors concluded that age was a primary influential factor as distinct groupings between LBBP treatments and control were detected on day 29. These results support the prominence of the genera *Lactobacillus* in the small intestine, but it would be of interest to delineate individual species in the various sections of the small intestine to determine if different *Lactobacillus* species present in the different sections as reported by Adhikari and Kwon (2017) respond in a similar manner or differ depending on their individual metabolic characteristics.

Prebiotics, which are characterized as complex carbohydrates that cannot be used by the bird but can be hydrolyzed and fermented by the bird's GIT microbial population, have received some attention as candidates for free-range broiler feeds (Ricke, 2018; Shi et al., 2019). Several performance trials have been reported and summarized in previous reviews (Ricke, 2015, 2018; Shi et al., 2019). While certain oligosaccharides are believed to target specific GIT bacteria such as lactic acid bacteria and bifidobacteria, GIT microbiome sequencing has been done on free-range broilers to determine if other GIT bacteria are also impacted by prebiotics (Ricke, 2015; Shi et al., 2019). Park et al. (2016) compared 2 commercial yeast cell-wall prebiotics fed to Naked Neck broilers over an 8 wk period and collected cecal contents at the end of the trial for sequencing the respective microbial populations. They concluded that both prebiotics exhibited a minimal influence on phyla, but the genus *Faecalibacterium* was increased by one of the prebiotics, and all 3 treatments (2 prebiotics and control) were distinguishable from each based on the unweighted UniFrac Beta diversity principal coordinated analyses. Park et al. (2017) compared broilers fed 1 of 4 treatments of either control diet, the prebiotics fructooligosaccharide or galactooligosaccharide, or plum fiber. The plum fiber and fructooligosaccharide birds appeared to increase the diversity of their cecal populations as the birds matured from 2 to 6 wk, but overall age was the more influential factor rather than dietary treatment for development of diverse cecal microbial populations. Shi et al. (2019) suggested that in future studies, diversity changes need to be analyzed from much younger birds when the prebiotic is initially added to the diet to determine if diversity change varies in younger birds. An additional point of interest would be to compare cecal populations before and after the additive has been introduced to detect any temporal shifts in either or both the microbial composition and changes in the metabolomic profiles.

While diet modulation may contribute to GIT microbial diversity poultry breed, bird age, and housing conditions can also exhibit considerable influence on the development of the broiler GIT microbial communities. Lourenco et al. (2019b) when following free-range broilers fed either soybean or soy-free diets found that the

number of cecal operational taxonomic units increased at 12 wk from the previous fecal samples in the soy-free birds, but remained relatively constant in the soybean fed birds. Age can impact diversity in free-range birds, even when the diet does not. For example, Ocejo et al. (2019) compared a fast-growing poultry broiler breed raised under conventional production conditions over 42 D with a slow-growing poultry meat breed raised in a free-range operation for 84 D. They fed the conventionally raised broilers a standard two-phase grain diet of starter ration followed by grower-finisher ration with feed supplements containing either whey powder, calcium butyrate, or both. The free-range broilers were fed a corn diet combination supplemented with combinations of soy and wheat and allowed access to pasture forage. Both groups of birds were sacrificed at different incremental times during their respective grow out periods, and cecal samples were collected for microbiome analyses. When microbiome populations were characterized for the respective production systems, no dietary influences were detected, but age-related differences occurred in both systems. The phyla *Proteobacteria* and *Firmicutes* were the major taxonomic groups in the young birds with *Proteobacteria* decreasing proportionally as the birds aged. At the end of the growout period for each of the 2 breeds, they shared 30 common genera while 35 unique genera were detected in free-range birds compared to only 7 exclusive genera in conventional birds. Overall, the authors concluded that while age impacted cecal microbial community development in the end, the free-range breed generated a richer, more complex cecal microbial population than the conventionally raised birds. The lack of impact of butyrate supplementation on cecal microbial communities has been observed with other organic acids fed to conventional broilers and some organic acids such as propionate are known to be absorbed in the upper GIT and never reach the ceca (Hume et al., 1993; Oakley et al., 2014b). It would be of interest to follow the development of the other compartments of the GIT in these respective groups of birds to determine how these factors influence their respective microbial populations and whether they are in conjunction with the shifts occurring in the ceca.

CONCLUSIONS

Poultry production systems involving free-range production of broilers and layer hens continue to be of public interest as attractive alternative commercial poultry products. However, practical operational farm management challenges remain. In addition to the threat from predators, food safety, and general bird health issues, economic concerns related to acquiring sufficient feedstocks that are considered acceptable to alternative production systems is an ongoing issue. Establishing reliable feed supplies requires a certain level of nutritional standardization to be able to evaluate non-traditional feed sources. However, this becomes complicated because of variable factors such as breed differences, the impact of the environment, and different dietary management approaches. Part of the difficulty lies in acquiring accurate estimates of bird foraging activity and the corresponding contribution that fiber consumption makes to dietary requirements of the bird. In addition, more in-depth nutritional assessment of alternative cereal grains is needed. Likewise, feed additives such as prebiotics and botanicals have potential utility for alternative poultry production, but more evaluation is needed to optimize dietary administration and assess the ability to limit foodborne pathogen establishment.

Microbiome sequencing and characterization of GIT microorganisms of birds raised in free range environments has contributed to a better understanding of some of the differences associated with breed and nutritional responses associated with alternative poultry production. While diet and environment can be influential factors, it appears that bird maturity and rate of growth are critical contributors to changes in GIT microbial diversity. As slow growing breeds become increasingly popular, more microbiome characterization studies will need to be conducted on these breeds to delineate potential differences further and determine the contribution of the GIT microorganisms to the overall nutrition and well being of the bird host. In addition, most of the microbiome studies on free range birds have focused on the cecal microbial populations, but more characterization of the other GIT compartments of the bird are needed to assess the contributions of these microorganisms. As more microbiome information becomes known and microbial metabolic network analysis is applied, it should become possible to elucidate in detail the contributions of individual members of the GIT microbial consortia and ascertain levels of GIT functions such as fiber degradation and contribution of forage grazing to the overall nutrition of the free range bird.

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