Microbial host interactions

Foodborne Salmonella ecology in the avian gastrointestinal tract

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Abstract

Foodborne Salmonella continues to be a major cause of salmonellosis with Salmonella Enteritidis and S. Typhimurium considered to be responsible for most of the infections. Investigation of outbreaks and sporadic cases has indicated that food vehicles such as poultry and poultry by-products including raw and uncooked eggs are among the most common sources of Salmonella infections. The dissemination and infection of the avian intestinal tract remain somewhat unclear. In vitro incubation of Salmonella with mammalian tissue culture cells has shown that invasion into epithelial cells is complex and involves several genetic loci and host factors. Several genes are required for the intestinal phase of Salmonella invasion and are located on Salmonella pathogenicity island 1 (SPI 1). Salmonella pathogenesis in the gastrointestinal (GI) tract and the effects of environmental stimuli on gene expression influence bacterial colonization and invasion. Furthermore, significant parameters of Salmonella including growth physiology, nutrient availability, pH, and energy status are considered contributing factors in the GI tract ecology. Approaches for limiting Salmonella colonization have been primarily based on the microbial ecology of the intestinal tract. In vitro studies have shown that the toxic effects of short chain fatty acids (SCFA) to some Enterobacteriaceae, including Salmonella, have resulted in a reduction in population. In addition, it has been established that native intestinal microorganisms such as Lactobacilli provide protective mechanisms against Salmonella in the ceca. A clear understanding of the key factors involved in Salmonella colonization in the avian GI tract has the potential to lead to better approach for more effective control of this foodborne pathogen.

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1. Introduction

1.1. Salmonella nomenclature

Salmonella are gram-negative bacteria consisting of non-spor forming bacilli and are a member of the family Enterobacteriaceae. The nomenclature of Salmonella is quite complex and is based on both serotype and subspecies names. For example, Salmonella enterica subspecies enterica serotype Enteritidis is shortened to Salmonella serotype Enteritidis or Salmonella Enteritidis [1]. Salmonella can be further subdivided onto biotype and phase type with biotype being a biochemical variation between two microorganisms of the same serotype, whereas the phase type is based on the differences in susceptibilities of two microorganisms of the same serotypes to a lytic bacteriophage [2,3]. Salmonella are also classified by three distinct types of antigens including somatic O, flagella H, and capsular Vi antigens. Antigens have been used to isolate and identify more than 2500 serotypes of Salmonella [4]. There are two species of Salmonella, namely S. bongori and S. enterica. S. enterica is divided into six subspecies including enterica, salamae, arizonae, diarizonae, houtenae, and indica. The most common O-antigen serogroup within Salmonella is O:4,[5].
1.2. Epidemiology

Foodborne salmonellosis is responsible for over 600 deaths and 1.4 million illnesses in the US annually [6] and the costs for medical care and loss of productivity can range anywhere from $464 million to $2.3 billion [7]. In 1999, 22% of all culture-confirmed Salmonella infected individuals were hospitalized [8]. Salmonella have also been commonly associated with foods such as raw meat, poultry, eggs, and dairy products and cause a large fraction of the food-related deaths in the US annually [9]. In Europe, the number of human cases was reported to be greater than 100,000 in 1997 [10]. In the past few years, the incidence of salmonellosis has shown a significant decrease across Europe (73,000 cases in 2001) and in the US since 1996 [10,11]. Approximately 60% of human cases reported to the CDC (in 2001) were caused by four serotypes including S. Typhimurium, S. Enteritidis, S. Newport, and S. Heidelberg [11]. Salmonella cases associated with certain serotypes, however, increased in 1999 and were accompanied by decreases in Campylobacter jejuni, Shigella, and Escherichia coli O157:H7 [8]. In 2004, new incidences of S. Newport and S. javiana increased up to 41% and 167%, respectively, over the 1996–1998 baseline period [12].

2. Salmonella in poultry

A variety of investigations of outbreaks and sporadic cases have indicated that food vehicles identified as the most common source of Salmonella infections are poultry and poultry by-products, including raw and uncooked eggs [13–19]. Salmonella cause asymptomatic intestinal infections in birds but acute outbreaks exhibiting clinical disease along with high levels of mortality occur in chicks younger than 2 weeks old [20–22]. Egg shells can be contaminated with Salmonella as a result of intestinal passage and the ability to penetrate into the avian egg [23]. Pullorum disease, for example, is caused by S. Pullorum and is spread from an infected parent bird via the egg to the chicken. While clinical signs are variable and non-specific, the outcome is an excessive number of dead-in-shell chicks and deaths shortly after hatching. Salmonella can be highly invasive in laying hens leading to systemic infections that can potentially be deposited in the internal contents of eggs by transovarian transmission following colonization of the intestinal tract [24–30]. S. Enteritidis, in particular, has shown a greater ability to colonize the vaginal epithelium of laying hens compared to other serotypes [31]. Birds that are asymptomatic carriers may facilitate the spread of disease infections among flocks [32,33]. S. Enteritidis contaminated eggs have proven to be extraordinarily difficult to detect internally and unless bacterial populations exceed log_{10} 9.0 per egg, no distinct changes in appearance or odor are usually observed [34]. S. Gallinarum is excreted in the feces of infected birds and can persist in feces for at least 1 month and longer in carcasses.

Even though Salmonella pathogenesis has been well characterized in the mammalian model, there is limited information on specific mechanisms in the avian species. Light and electron microscopic examinations of intestines taken from chickens experimentally infected with various Salmonella species demonstrated similar cellular responses to these organisms, including the influx of heterophils and macrophages to the luminal surface of the intestine [35,36]. Heterophils are considered to be the avian counterpart to mammalian neutrophils in their action as tissue phagocytes, and their importance to host defense against bacterial infections is well known [37]. The capabilities of the heterophils and avian macrophages to kill Salmonella have been demonstrated through bactericidal assays performed in vitro [38]. If Salmonella are not cleared by the immune system, colonization of the intestine occurs, and they are able to move through and colonize other cells by inducing them to take up the bacteria [39]. Studies have shown that at least Salmonella used to experimentally infect birds will migrate from the intestine to the liver, spleen, and ovaries [25,27,29,30,36,40,41]. This indicates that the pathogenesis of Salmonella in experimental avian model infections involves a sequential dissemination in the internal organs that is similar to what has been established in the mammalian model.

3. Microbial ecology in the avian GI tract

3.1. Avian GI tract and indigenous microflora

The lower gastrointestinal (GI) tract of most animal species including poultry is normally populated by large numbers of microorganisms [42], and through various competitive niches and virulence capabilities, some are able to survive. The capabilities of microorganisms associated with the mucosa of the GI tract to withstand the flow rates of food material is essential for the development of protective mechanisms such as surface mucus colonization [43], deep mucus, development of specialized insertion structures [44], and crypt association [45] by specific adhesions [46]. Changes in the passage rates that are representative of dilution rates can alter the limiting nutrients and therefore could ultimately affect microflora composition in the GI tract ecology.

Historically, the microbial composition of the GI tract of avian species has not been extensively defined compared to what is known about microorganisms in ruminants [47]. There is the perception that the role of microorganisms in chickens is not as important as is the case for ruminants [48]. However, extensive anaerobic metabolism including methanogenesis fermentation occurs in birds fed a variety of diets [49–52]. The ceca are the major fermentation sites in the GI tract of chickens and contain the largest number of bacteria [42,53–55]. Over 200 different bacteria have been isolated and characterized [56], and these bacteria are known to be influenced by various factors including diet, health, and age. However, Zhu et al. [57] indicated that only 10–60% of microorganisms in the ceca could be propagated using anaerobic culture techniques.

3.2. Methods for studying avian GI microbial ecology

Continuous culture (CC) techniques historically have provided in vitro models to study GI tract metabolism and fermentation of microorganisms [58–64]. A typical CC experiment involves a chemostat apparatus that simulates specific GI tract physical and chemical properties and consists of an afferent inlet which inputs substrates and buffer and an efferent outlet that facilitates outflow port of a homogenous mixture of microorganisms, fermentative metabolites and substrates [65]. The growth of bacteria in CC can provide a more accurate reflection and simulate conditions that are closely related to the natural GI ecosystem. Steady state can be attained and significant parameters can be quantified including rate of growth, manipulation of nutrient source, pH status and maintenance energy [58]. This however, depends on how constant the nutrient flows are in the particular GI tract systems. The flow rate (passage rate) may vary for different gut systems including cattle (approximately 80 h) [66], horse (48 h) [67], and chickens (2.5 h) [68]. Furthermore, flow rates depend on the feed composition and texture [69].

Experimentally, CC techniques have been used to simulate the GI tract microenvironment of humans [61,62] and various animal species including chickens [63,64], pigs [70], and ruminants [58]. Studies which model the human colon microecology [61] demonstrated antagonism of indigenous microflora against enteropathogens from...
crude human fecal cultures in anaerobic culture systems. In these studies, it was observed that five human fecal microorganisms provided levels of antagonism that mimic the crude fecal flora in GI tract in the presence of carbon sources such as lactose, sucrose, and starch that were fermentative only by antagonistic bacteria. The enteropathogen (S. Typhimurium) was suppressed to a \(10^4\) CFU/mL by 5–7 days post-challenge [61]. Parameters such as competition for growth-limiting amino acids and microfloral density are known to contribute to the superior competitiveness of the normal microflora to outcompete and eliminate pathogens [62,71,72]. Using CC techniques, even small variations in media pH (6.17–7.35) were shown to influence S. Typhimurium physiology by altering the bacterial cell parameters [73]. While ammonia release into media was favored by low pH, the increases in pH resulted in higher cell protein concentrations, glucose disappearance, and bacterial ATP yields.

4. Salmonella physiology in the avian GI tract

4.1. Salmonella growth physiology

Under nutrient limiting conditions, bacteria reach stationary phase of growth rapidly [74]. When this occurs, bacterial replication ceases and cell density begins to decrease. Historically, research has shown that transition into a survival mode during stationary phase is a more physiologically controlled event by bacteria than previously thought [74,75]. Cessation of growth can be caused by many environmental factors, including acid pH, osmotic stress, heat shock, and redox potential [76–80].

Considerable attention has been given to nutrient starvation with a primary focus on carbon, nitrogen, or phosphorous sources because these are identifiable with already highly characterized genetic changes that occur in bacteria including S. Typhimurium [81]. Ševčık et al. [82] demonstrated that under anaerobic conditions when electron acceptors are scarce, the stationary phase of S. Typhimurium growth may be reached not only by nutrient deprivation but due to a limited availability of electron acceptors such as oxygen. When Salmonella became exposed to such conditions upon infection of a susceptible host, they multiplied rapidly and reached a density of \(10^8\) CFU/g cecal content [82].

The utility of animal cell culture has become a popular model for studying adhesion and penetration through epithelial cells by Salmonella [83,84]. It has been shown that an adhesion-invasion-deficient mutant of Salmonella is largely controlled by genetics and multiple chromosomal loci [85,86]. Invasion is genetically mediated [87] by several invasion genes found on a 40 kb Salmonella pathogenicity island 1 (SPI 1) located between \(fha\) and \(mutS\) chromosomal genes at centromere 63 on the S. Typhimurium chromosomes [88]. Rodriguez et al. [89] observed that S. Typhimurium incubated in the presence of high osmolarity and low oxygen for 8 h exhibited reduced \(hilA\) expression during the exponential growth phase. However, at stationary phase (3 h post-inoculation), there was an apparent increase in \(hilA\) expression most probably due to less optimal growth conditions such as limited nutrients, low oxygen tension, and other stresses created after the first 2 h in the medium. Cell-association and invasion of S. Typhimurium into cultured epithelial cells may also be influenced by short chain fatty acids (SCFA) as a function of both SCFA concentration and pH of the media [90–92].

4.2. Genetics of Salmonella

The central regulator of stationary phase is expressed by \(rpoS\) [80,93] which is responsible for the induction of a specific subset of bacterial genes expressed during stress. \(RpoS\) is known to be positively regulated by a starvation specific molecule ppGpp [78] accumulated as a part of the stringent response. In addition, induction of sigma factor can alter the efficiency of metabolism including reduction in cellular concentration of cAMP [94] and UDP-glucose. \(RpoS\) is an alternative sigma factor (\(\sigma^d\)) which has been demonstrated to be essential for stationary phase stress response in Salmonella and E. coli and is an important gene regulator in S. Typhimurium [81,95,96]. \(rpoS\) encodes an RNA polymerase sigma factor (\(\sigma^d\) or \(\sigma^d\)) that is known to regulate at least 60 genes in response to environmental signals including various stress conditions, nutrient limitation, osmotic challenge, acid shock, heat shock, oxidative damage, redox potential, and growth in stationary phase [97–102].

S. Typhimurium is an intracellular pathogen, residing in the macrophages upon infection and can be exposed to a wide range of antimicrobial effectors including the phagocyte NAD(P)H oxidase (Phox). An initial oxidative bactericidal phase associated with the production of superoxide anion and hydrogen peroxide is followed by bacteriostatic phase where nitric oxide is produced [103]. The combination of nutrient limitation and stress conditions in the intracellular environment is probably a stimulus for rpoS induction [104]. Starvation also increases the intracellular levels of ppGpp which in turn enhances the level of \(RpoS\) [105]. This is evident because ppGpp-deficient strains fail to synthesize \(RpoS\) as cells enter into stationary phase in a rich medium and under starvation [105]. The major effects of ppGpp induction are not exerted on \(rpoS\) mRNA abundance or on protein turnover but instead influence translational efficiency [106].

4.3. Acid tolerance response of Salmonella

Salmonella elicit several strategies to avoid or repair damages that are caused by exposure to acid stress. In Salmonella, \(RpoS\) is also integrally involved in the development of several low-pH inducible acid defense systems, collectively referred to as acid tolerance response (ATR), that expand the range of pH tolerance [107–111]. There are two major ATR that have been identified and are based on the particular growth phase in which they become induced. The first type, the log-phase ATR system, operates during the exponential growth phase of cells undergoing a rapid transition to low pH [108]. Over 50 acid shock proteins (ASP) are produced during this response [112]. The second type of ATR system is known as a stationary phase ATR and is induced by exposing cells to low pH during stationary phase [111]. In contrast to the log-phase type system, it is induced by the onset of the stationary phase regardless of the pH of the growth. Bearson et al. [113] reported that both ATR systems in S. Typhimurium are able to protect against the two types of acid stress including organic (weak acids) and inorganic acids (low pH). \(RpoS\) and Fur are believed to protect against organic acids, whereas PhoP along with \(RpoS\) protect against inorganic acid stress. Bearson et al. [113] demonstrated that \(rpoS\) in S. Typhimurium encodes for a shock protein (ASP) and its expression is induced 4-fold by transition from normal to acid conditions (pH less than 4.5). The importance of this induction has been demonstrated for S. Typhimurium to initiate and sustain induction of the ATR [110]. It is a complex adaptive response that induces both an \(\sigma^d\)-independent transient ATR which is maximally induced by 20 min of pH 4.4 acid shock but progressively lost during longer adaptation [114] and a \(\sigma^d\)-dependent sustained ATR which can be seen during a longer period of acid environment of 60–90 min [110].

4.4. Response to short chain fatty acids (SCFA)

SCFA are end-products of microbial fermentation in the GI tract of humans and animals and include acetate, propionate, butyrate,
valerate, isovalerate, and isobutyrate [53,115–117]. Previous studies with four species of birds indicated that metabolizable energy obtained from total SCFA production was equivalent to 5–15% of the daily requirement for maintenance [53,118,119]. However, young chickens do not contain a wide diversity of anaerobic bacteria as a dominant fraction of the microflora [120], and low concentrations of acetate (below 70 μmol g⁻¹), propionate (below 8 μmol g⁻¹), and butyrate (below 24 μmol g⁻¹) are expected during the first week of life [54,120,121]. In the first 15 days after hatching, the concentrations of SCFA in the young chick’s ceca vary which may explain their low protective efficiency against pathogen colonization [122]. The production of SCFA reaches concentrations which are considered optimal for pathogen exclusion (acetate at 70 μmol g⁻¹; propionate 8 μmol g⁻¹; and butyrate 24 μmol g⁻¹) and stabilizes after chickens reach 15 days of age [120]. Increases in acetate, propionate, and butyrate in ceca have been assumed to lead to a decrease in the viable population of Enterobacteriaceae in ceca of chickens [123]. However, pre-exposure of Salmonella to high levels of various SCFA (100 mM) at neutral pH may enhance survival by increasing acid resistance and stimulating virulence response [124–127].

SCFA can inhibit Salmonella growth when present in the dissociated form. Van der Wielen et al. [123] demonstrated in a batch fed commensal exclusion (CE) culture that acetate, propionate, and lactate inhibited Salmonella growth at pH 5.8, but failed to do so at neutral pH. At pH 5.8, the total undissociated SCFA were significantly higher compared to the dissociated form at neutral pH [128]. At a lower pH (5.8), it is thought that SCFA promote bacteriostatic action by increasing the concentration of undissociated acids. Undissociated acids enhance permeability of the cell membrane [129,130] and cause bacteria to lose energy generating capacity in the form of ATP, thus compromising replication [130]. While [129,130] and cause bacteria to lose energy generating capacity in the form of ATP, thus compromising replication [130]. Therefore, a role in the survivability of acid sensitive pathogens exposed to a reduced pH by induction of ATR which is associated with virulence. SCFA promotes bacteriostatic action by increasing the concentration of undissociated acids. Undissociated acids enhance permeability of the cell membrane [129,130] and cause bacteria to lose energy generating capacity in the form of ATP, thus compromising replication [130].

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5. Potential for Salmonella control in avian GI tract

5.1. Probiotics

Probiotics are generally referred to as any live microbial feed supplements that benefit the host animals by largely improving intestinal microbial balance [132,133]. Intestinal microorganisms that are recognized as possessing probiotic properties include but are not limited to Lactobacillus and Bifidobacteria spp. They exhibit identifiable beneficial effects for the respective host via promotion of gut maturation and integrity, antagonism against pathogens (Salmonella) and immune modulation [134–136]. The effects of probiotics in poultry also include maintaining normal intestinal microflora by CE, increasing metabolism, decreasing enzymatic activity and ammonia production, as well as an increase in feed intake and the neutralization of digestive enterotoxins [137,138]. Therefore, the overall goal of probiotics intervention is to promote the general growth of healthy microorganisms that are competitive with or antagonistic to enteropathogens [133].

The application of such probiotics has been referred to as the Nurmi concept of CE. Nurmi and Rantalä [122] were the first to utilize CE as a viable pathogen-reduction strategy. They demonstrated that Salmonella colonization in juvenile chickens was reduced by the administration of a preparation of gut bacteria originally isolated from healthy adult chickens. Currently, CE approaches essentially involve pathogen-reduction strategy via introduction of a (non-pathogenic) bacterial culture to the intestinal tract of food animals resulting in reduced colonization or decreased populations of pathogenic bacteria in the GI tract [133,139–141]. Over the past three decades, CE cultures have been extensively studied in several laboratories [64,70,139,142–144] with a primary focus on limiting Salmonella colonization in the GI tract of chickens. There have been several efforts designed to understand and reduce their microbial complexity, improve their resistance and identify successful colonization after introduction of the CE culture [47,70,142,143]. CE cultures in which the bacterial composition is unknown are termed undefined, while those of known bacterial composition are referred to as defined CE cultures [70]. An established and mature GI microbial population theoretically occupies all available environmental niches nutritionally, metabolically and physically, making an animal more resistant to colonization by opportunistic pathogen infections [133].

Van der Wielen et al. [120] reported that in adult chickens the microbial population becomes more complex, stable, and better able to resist enteropathogens than their younger counterparts. In the 1990s, there was considerable progress achieved in developing cultures maintained in CC which were shown to effectively control Salmonella colonization when administered to chickens [139,140,145–147]. The inhibitory mechanism against Salmonella colonization has been associated with a reduction in cecal pH, increase in cecal lactic acid and SCFA, competition for attachment sites, competition for growth-limiting nutrients, production of antimicrobial compounds, immunomodulation, and synergistic and antagonistic interaction [61,71,72,128,142,148,149]. It has been stated that CE should be used as a prophylactic treatment rather than a therapeutic agent [150] and should originate from the intestinal content of the animal of interest. For instance, a CE culture for use in chickens must be derived from healthy chickens, likewise for pigs [70]. Administration of a bacterial community to newly hatched chickens can lead to an early colonization of adherent bacteria on the intestinal mucosal surface forming a mat of microorganism occupying environmental niches [151–153]. In the food animal industry, the use of probiotics and CE can be administered as a synbiotic by combining them with external dietary ingredients that will favor the specific growth and establishment of the probiotic bacteria [154,155]. Roller et al. [156] established that while an inulin-enriched oligofructose dietary supplement increased the production of intereleukin-10 in Peyer’s patches and secretory immunoglobulin (sIgA) in the cecum of rats, the probiotic mixture (L. rhamnosus and B. lactis) affected the immune functions only modestly. The combined application of both supplements resulted in enhanced production of sIgA in ileum and decreased oxidative activity of blood neutrophils. They concluded that simultaneous administration of probiotics and selected dietary supplements may have different effects than when applied separately.

5.2. Prebiotics

Prebiotics can be defined as non-digestible carbohydrate fractions fed in diets that are beneficial to the host by stimulating the growth of one or more bacteria in the GI tract [157,158]. Prebiotics (dietary fibers) are predominantly a constituent of plant cell walls and also consist of non-starch polysaccharides (NSPs) along with
non-carbohydrate compounds including lignin, protein, fatty acid, and wax [159]. Upon ingestion, dietary fiber may influence the GI tract by altering its microbial activities, rate of passage, metabolites, and digestive efficacy [159,160]. Certain dietary fractions including polysaccharides have been identified for their potential to be utilized as prebiotics [161,162], possibly by reducing pH and increasing VFA concentrations [121,148,163]. Beneficial species of Lactobacilli and Bifidobacteria that are considered to be inhibitory towards pathogens are known to be supported by some of these compounds [157].

Some of the more extensively studied prebiotic sources are fructooligosaccharide (FOS), oligofructose and inulin [154,155]. FOS are naturally occurring oligosaccharides that originate from plants such as onions, wheat, barley, and rye and consist of one to three fructose residues attached to a sucrose molecule. When fed to animals, FOS have been shown to impact bacterial populations by promoting the growth of Lactobacilli [164] and Bifidobacterium spp. [165]. Bailey et al. [166] demonstrated reduced susceptibility of broiler chickens to Salmonella invasion after inclusion of FOS in their diets which was explained by a probable shift in gut microorganisms. The efficiency of FOS in the same study was enhanced by a combination with a protective CE culture which resulted in a 3-fold reduction of S. Typhimurium chicken colonization compared to chickens given CE alone. Lactobacillus paracasei administered in combination with FOS resulted in a significant increase of Lactobacillus spp., Bifidobacterium spp., total anaerobes, and total aerobes, as well as a decrease in Clostridium and Enterobacteriaceae observed in piglets [167]. In a series of in vitro studies, Donalson et al. [168,169] demonstrated that a combination of FOS, alfalfa and grain, incubated with cecal inoculum exhibited a significant reduction in Salmonella population, while increasing propionate, butyrate, other SCFA, and lactate. However, in vivo work with laying hens was less conclusive indicating some adaptation of the cecal microflora was required [170]. In at least half of the trials, the S. Enteritidis colonization of ovary and liver of hens fed FOS (0.375% and 0.750%, w/w) containing diets were reduced compared to hens subjected to complete removal of feed. Significant decreases in cecal S. Enteritidis counts were also observed in only half of the trials. However, no substantial differences in Salmonella colonization of hens’ organs were observed due to FOS. Although the addition of FOS to cereal or high-fiber diets did not improve the production of the total cecal VFA, hens fed high fiber with or without FOS yielded greater cecal lactic acid concentrations than hens subjected to complete removal of feed [170].

5.3. Dietary strategies to limit Salmonella in the avian GI tract

Adding specialized prebiotics may be economically limiting depending on the cost of the original sources of the compounds used, so recent research has focused on examining dietary regimens that elicit similar properties. This has been studied in some detail for certain egg-laying hen management practices in the poultry industry. In particular, molting diets for layer hens have been a focal point for development of these types of diets. Natural molt of hens is associated with the temporary interruption of egg production. Instead, hens utilize their energy in staying warm and growing new feathers [171]. Historically, the shortening of the natural molt and rejuvenation of hen flocks in poultry industry were achieved by withdrawal of feeds [172]. Feed deprivation was a procedure employed to achieve a rapid and economical new egg-laying cycle [173,174] and could last anywhere from 4 to 14 days [172,175]. However, this method, although possessing several management advantages, has become less popular due to a variety of animal and food safety issues [28,29,176–178]. It was suggested that the avian microbial ecology is altered during dietary stresses such as feed removal which in turn can lead to higher vulnerability of the host to pathogen infection and colonization [28,40,127,179]. Changes in dietary composition of the GI tract of poultry during feed withdrawal clearly have negative consequences on microbial population.

It has been proposed that dietary fiber can be utilized preferentially by Lactobacillus and Bifidobacteria species which leads to the production of lactic acid and SCFA, resulting in the maintenance of normal microbial populations, low pH and also prevents the establishment of Salmonella in the GI tract [180–182]. Studies have shown that feed deprivation can alter the hen’s immune system and physiological status [183–189]. Laying hens also become more susceptible to pathogen infection including Salmonella spp. with molted hens shedding significantly more S. Enteritidis in their feces [26,27,190], and higher levels of S. Enteritidis invasion in their internal organs including liver, spleen, and ovaries [25,27,40]. These findings suggested that complete removal of feed promotes pathogen invasion in molted hens.

Diets that regulate the passage rate by slowing it down could be advantageous since this mechanism may prolong fermentation which in turn increases metabolites needed to maintain GI tract integrity. The altering of passage rate (flow rates) represents changing the amount of digesta that passes along the GI tract in a given time [191]. Passage rate may vary in different segments of the GI tract and is dependent on the feed composition and texture [192,193]. Adequate feed retention time is essential especially in the ceca in order to encourage microbial degradation for longer periods of time [194] leading to the production of important metabolites, which subsequently maintain the integrity and an optimal range of microbial diversity.

Several high-fiber dietary approaches have been utilized as alternative molting diets to expedite an additional laying cycle for hens. This includes insoluble plant fiber such as grape pomace [195], cotton meal [196], wheat middling [197], and alfalfa [30,41,179,185,186,198–203]. Studies by Tsukahara and Ushida [204] demonstrated that feeding a plant protein-based diet to chicks generated a higher concentration of SCFA than a diet based on animal proteins and implied that the difference in SCFA concentration was due to a higher concentration of the dietary fiber component in the plant diet. In addition, it has been reported that certain microorganisms that are indigenous to the GI tract of poultry have the potential to hydrolyze dietary fiber into oligosaccharides and other low molecular weight carbohydrates which leads to production of SCFA [48,49,127,205,206].

Alfalfa is one of the more extensively studied high-fiber dietary sources in poultry. It has been widely used as animal feed and as a high-fiber feed source [207–209], as it is relatively high in protein exhibiting one of the slowest rates of passage (more than 24 h) through the avian system and components such as saponins can influence digestion and consumption of feed [210,211]. Alfalfa is well balanced in amino acids and rich in vitamins, and contributes to the desirable yellow color to carcasses and egg yolks when fed to chickens as a dietary supplement [208,212]. In addition, alfalfa may have advantages associated with the fermentation properties by cecal microflora that are capable of limiting in vitro growth of S. Typhimurium and has been shown to limit in vivo S. Enteritidis colonization in laying hens [30,41,170,202,203]. An in vitro study examined the fermentation of alfalfa and layer feed incubated with chicken cecal content in rumen fluid using nitrocompounds and indicated that both feed materials influenced SCFA production with acetate being the predominant component [50]. It was observed that incubation with the methane inhibitors nitroethanol and 2-nitropropanol produced significantly higher propionate than nitroethane, while layer feed produced more butyrate than alfalfa. The addition of nitropropanol to layer feed incubated with cecal...
contents was suggested to promote gram-positive, saccharolytic SCFA-producing bacteria especially *Clostridium* spp. which is a predominant group in the ceca of chickens [57,213,214]. High-fiber feed substrates (soybean meal, soybean hull, beet pulp, wheat middlings, ground sorghum, cottonseed meal, alfalfa, and different ratios of alfalfa and commercial layer ration) have also been observed to influence microbial diversity and stimulate SCFA production when incubated with chicken cecal inocula *in vitro* [206]. While isobutyrate and isovalerate were barely detectable, acetate production was pronounced, followed by propionate and butyrate.

In order to derive maximum benefit from fermentable high-fiber prebiotic sources, physical modification may also be necessary to derive uniform particle size. Coarsely ground mash over whole grain wheat has been demonstrated to be effective on the physiologic function on GI tract of broiler birds. It has been shown that an increase in feed structure caused an increase in gizzard size [215–218]. A reduction in gizzard pH and an increase in small intestinal pH were observed with an increase of the grain particle size [217,218]. The particle size of feed structure is also known to influence *Salmonella* numbers [193]. It has been demonstrated that pigs fed a coarse non-pelleted diet significantly exhibited an increased number of anaerobic bacteria, increased concentration of organic acids, and reduced pH in the stomach compared to fine pelleted diets [193]. Furthermore, changes in these parameters as well as a significant higher concentration of undissociated lactic acid were presumably influential in reducing *Salmonella* population in the gut of pigs.

Numerous studies have been carried out to evaluate the effects of feed structure on performance of poultry [215,218–221]. In previous reports [215,219–221], the addition of whole grains to feed instead of pelleted compound feed was also shown to increase feed conversion and growth of broilers. Furthermore, whole wheat feeding significantly increased gizzard weight, increased retention time, and reduced pH in gizzard contents compared to pelleted fed birds [219,222] which in turn decreased the *Salmonella* population. In addition, uniform particle size was shown to contribute to the development and integrity of the GI tract which subsequently enhanced gut motility and backflow mechanisms in poultry including reverse peristalsis from the cloaca to the ceca [223]. Alfalfa when fed in a crumble form appears to support microflora that are accompanied by increased production of SCFA in a pattern similar to a grain-based diet [179]. While feed removal resulted in decreased fermentation capacity [30], the negative effect was neutralized by hens fed alfalfa crumbles as acetate, propionate and butyrate were observed to be the most pronounced SCFA in feces and ceca [179].

6. Conclusions

The microbial diversity of the GI ecology plays an essential role in the food animal industry and human medicine. A thorough understanding of microbial interactions can be a valid tool to prevent the environmental conditions that accompany management practices suspected in proliferating foodborne pathogens. Foodborne pathogens such as *Salmonella* possess the capability to survive in external environments during transmission from one host to the next [224]. The determination of microbial genetics and physiology associated with these mechanisms could have great potential for better control of pathogen colonization.

There have been attempts to use feed ingredients that are conducive to the growth of beneficial GI tract bacteria as well as the introduction of a bacterial population that favors optimal health and nutrition in animals to promote normal microbial growth in GI tract ecology [137]. A modulation of bacterial community in the GI tract through the use of probiotics and prebiotics remains an active research area and has shown great potential in reducing enteropathogens as well as enhancing the beneficial effects of normal microflora including *Lactobacilli* and *Bifidobacteria*. Historically, in *vitro* models, including CC-based studies, have been extensively utilized to study the ruminant GI tract ecology. More effort is needed to evaluate significant parameters of enteropathogens including steady state, nutrient status, pH status, energy requirements, and direct comparisons of metabolic and genetic responses. A better understanding of these indicators could assist in designing more novel approaches to minimize the spread of *Salmonella* in the food animal industry and decrease the consequences to human health.

Acknowledgments

This review was supported with funds from USDA-NRI Grants #2001-02675 and #2002-02614, US Poultry and Egg Association Grant 485, Hatch Grant H8311 administered by Texas Agricultural Experimental Station, and an USDA Food Safety Consortium Grant, University of Arkansas.

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