Possibilities of early life programming in broiler chickens via intestinal microbiota modulation

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ABSTRACT The strong selection in search for a higher growth rate in broilers has resulted in adverse effects such as metabolic disorders, low responsiveness of the immune system, and decreased resistance to pathogens. On the other hand, newly hatched chicks rely mostly on innate immune responses until their gut gets colonized with microbiota. In consequence, early access to active substances or bacteria (pre- and post-hatch) is particularly relevant here because in broilers much of the immune system development occurs early in life. Therefore, early stimulation of beneficial microflora is critical, as it affects, to a great extent, the entire life-span of an individual, and also because the nutritional manipulations of the gastrointestinal tract (**GIT**) microbiome to enhance productivity and health are rather limited by the resilience of the ecosystem once established in the chicken´s gut. Early life or developmental programming is based on the assumption that the development of diseases later in life can be modulated by perturbations or environmental exposures during critical pre- or early post-natal life. Substances such as plant derivatives, Na butyrate, pre- and probiotics, and β-glucans have been shown to induce beneficial microbiological and immunological changes within the GIT, and therefore are potential candidates to be used as tools to manipulate GIT functionality in the young chicken. Accordingly, substances as these might represent promising candidates to study intestinal microbiota/immune system modulation in broilers´ early stages of breeding. *In ovo*-delivered prebiotics and synbiotics have been shown to have no adverse effect on the development of the immune system in exposed chickens, while being able to affect lymphoid-organs' morphology in chickens. *In ovo* procedures have also been proposed as means of promoting a healthy microflora in embryonic guts and stimulating maturation of the cellular and humoral immune responses in central and peripheral immune organs, including those in the GIT. The purpose of this presentation is to discuss the potential usefulness of the instruments currently available to induce early life programming in broilers.

Key words: broiler, early life programming, immune system, intestinal microbiota

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INTRODUCTION

The monogastric (pigs and poultry) industry is at present leading meat production in the European Union (up 60% of the total value of production) with a 14.5% of the economic share in agricultural production (EUROSTAT, 2015.). Meanwhile, poultry producers are able to produce chicken meat at half price than in the 1950's, mainly due to a decline from 2.6 to 1.6 in the feed conversion ratios, which was the result in the first place of a quite successful genetic selection. However, this strong selection in broilers in search for a higher growth rate has at the same time inadvertently resulted in marked changes in the development of the digestive system of the birds, along with other adverse effects such as metabolic disorders, low responsiveness of the immune system, and decreased resistance to pathogens (Zuidhof et al., 2014). This extra load would benefit from an adequate management of the birds in the early development stages so that the functionality of the digestive and immune systems is as accomplished as possible as the birds grow. For example, feed restriction has been proposed as a means to overcome physiological problems such as ascites and sudden death syndrome associated with fast growth (Tottori et al., 1997; Butzen et al., 2013). Also, diluting the diet with 40% rice hull during 8 to 14 d of age might be a suitable method to improve feed efficiency, and to reduce carcass fat deposition in the production of meat-type ducks at 42 d of age, although the mechanisms involved have not been clarified (Guo et al., 2013). However, other ways of feed manipulation in the early stages of poultry production could be explored to enhance a healthier intestinal development in later stages. In this context, early life or developmental programming concept postulates that perturbations or environmental exposures during critical pre- or early post-natal life can have lasting

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impacts on the development of chronic diseases later in life (Waterland and Garza, 1999). Nutrition has been identified as one source of early exposure that might affect early development and later phenotype, and some investigations have reported the influence of gestational availability of different nutrients in mammalian models (Korotkova et al., 2005) and in birds (Cherian, 2011; Koppenol et al., 2015). The aim of this review is to explore the possibility that some potential tools/mechanisms linked to intestinal microbiota modulation in chickens´ early life might be used to induce durable beneficial changes in broiler production.

RELEVANCE, COMPOSITION, AND FUNCTIONS OF THE INTESTINAL MICROBIOTA

In the EU there is currently a need to seek for viable alternatives to antibiotic growth promoters (**AGP**) capable of increasing the defensive capacity of livestock while avoiding AGP use (EC Regulation 1831/2003; http://eur-lex.europa.eu/en/index.htm) and maintaining adequate production levels. One way to achieve this goal is the use of certain additives to favorably influence animal performance and welfare, particularly through changes in the composition of the IM, which exerts a direct influence on host´s health (Tuohy et al., 2005; Johnson et al., 2018). The intestinal microbiota is defined as the microbial community, including commensal, symbiotic, and pathogenic microorganisms, which usually colonize an area of human and animal organisms; it amounts around two times more plentiful than somatic and germinal cells of the host (Sender et al., 2016). With the information currently available, there is no doubt that the intestinal microbiota is directly or indirectly involved in all physiological and pathological processes that occur in the digestive tract of higher animals and man. The microbiota resident in the gastrointestinal tract (**GIT**) plays key roles in the normal nutritional, physiological, immunological, and protective functions of the host animals (Vispo and Karasov, 1997; Frick and Autenrieth, 2013), but also in the efficiency of nutrients uptake and utilization (Gabriel et al., 2006; Frick and Autenrieth, 2013). It is possible that the growth of beneficial bacteria, suppression of detrimental bacterial species, or both, is partially responsible for the improved productivity of diets supplemented with exogenous enzymes or antibiotics in broilers (Torok et al., 2008, 2011a, b). However, antibiotic administration not always results in better performance. Quite interestingly, Kumar et al. (2018) recently found that bird performance was improved with bacitracin dimethyl salicylate treatment only up to 7 d of age, whereas growth was better in the non-bacitracin supplemented group at the time of commercial processing. These discrepancies are due to a great extent to the fact that our knowledge of the gut microbiota composition, metabolic functions, and influence on animal health, welfare, and performance is far from complete. Information on intestinal microbiota composition and function is so far quite limited as many species have not yet been identified in vertebrates including poultry (Bäckhed et al., 2005; Wei et al., 2013). As indicated by Oakley et al. (2014), how changes in taxonomic composition relate to changes in metabolic functioning and morphological development of the intestine remains an important gap in our current knowledge of the chicken GIT microbiome. When available, such meta-omic data will provide important mechanistic insights into how the microbiome contributes to host development and nutrition.

Bacteria in the GIT of broilers are distributed in four main phyla (Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria) that account for >90% of all the sequences, among which *Clostridium, Ruminococcus, Lactobacillus,* and *Bacteroides* are the predominating genera. An important difference with other vertebrates is the relatively low diversity, probably due to the fast transit of food through the digestive system (4 to 5 h in a 29-d-old broiler chicken; Rougière and Carré, 2010; Wei et al., 2013). Though with limitations due to the bias generated by the polymerase chain reaction and by the depth of the sequencing involved, culture-independent analysis of the chicken cecal microbiota have estimated a bacterial population consisting of over 600 species from more than 100 genera, with a large proportion of these bacteria belonging to unclassified species or genera (Stanley et al., 2014). However, profiles of taxonomic composition differ greatly in reported studies because the microbiota in chickens varies according to diverse factors such as diet, location, and age (Oakley and Kogut 2016; Ranjitkar et al., 2016; Clavijo-López and Vives-Florez, 2018). Also, significant differences have been described in the taxonomic composition of the different organs and even sections within the GIT, which means that they could be considered separate ecosystems, and microorganisms perform independent functions in each of the organs (Van Der Wielen et al., 2002; Choi et al., 2014; Johnson et al., 2018). Thus, different species of *Lactobacillus* spp predominate in the crop, which are responsible for the fermentation of starch and lactate production; *Clostridiaceae* family and *Lactobacillaceae* predominate in the gizzard with less fermentation activity; the small intestinal microbiota is dominated by *Lactobacillus* spp, *Enterococcus* spp, and various *Clostridiaceae* (Han et al., 2016). Ileum was reported as a source of novel bacteria, especially butyrate producers. Since the function of the ileum is nutrient absorption, it is likely that a number of these unknown bacteria may influence nutrient availability and absorption rate and thus bird performance. The cecum is the organ with greatest taxonomic diversity and abundance most likely due to the longer time retention (12 to 20 h); the microbiota of the ceca (mainly *Clostridium, Ruminococcus, Eubacterium, Faecalibacterium,* and *Lactobacillus* species among a number of unknown and uncultured phylotypes)

is associated with the digestion of foodstuffs rich in cellulose, starch, and polysaccharides, which are resistant to bacterial digestion in the small intestine (Clench and Mathias, 1995; Stanley et al., 2014). Sergeant et al. (2014) found numerous polysaccharide and oligosaccharide-degrading enzymes with genetic evidence for the coordination of polysaccharide degradation with sugar transport and utilization as well as several fermentation pathways leading to the production of short-chain fatty acids (**SCFA**).

The main functions ascribed to the intestinal microbiota in broilers are: (i) nutrient exchange, (ii) modulation of the immune system, (iii) physiology of the digestive system, and (iv) pathogens exclusion (Oakley et al., 2014; Stanley et al., 2014). In the current review, the interest is focused mainly on the modulation of the immune system and the exclusion of pathogens. The activity of the microbiota is important in regulating the activation of both innate (e.g., abundance and composition of mucins) and acquired immune response. Although the precise mechanisms are not yet well elucidated, experiments with germ-free birds have shown that the intestinal microbiota has a dramatic effect on the repertoire of intestinal T cells and their expression of cytokines (Forder et al., 2007; Oakley et al., 2014). On the other hand, established intestinal microbiota is able to reduce the adhesion and colonization of pathogens in the intestine by competitive exclusion, which is regarded as the result of different mechanisms (physical occupation of space, competition for resources, and specific chemical substances as bacteriocins; Gabriel et al., 2006; Chaucheyras-Durand and Durand, 2010). The exclusion of pathogens is mainly performed in practice through the use of competitive exclusion products, which are anaerobic cultures of bacteria applied to poultry hatchlings to establish a protective enteric microbiota that excludes intestinal colonization by human food-borne pathogens. Although not without potential disadvantages (Wagner, 2006), competitive exclusion is regarded as one of the most effective approaches to prevent intestinal colonization by *Salmonella* in broiler chickens, and is in the basis of some probiotics aimed to improve birds´ health (see below).

It is generally accepted that the establishment of an adequate microbiota is an effective barrier to colonization by opportunistic pathogens, provides metabolic substrates required by the animal (vitamins, SCFA, etc.), and is a stimulus for proper development of the immune system (Lan et al., 2005). Changes in the diet are among the main factors able to modify the balance and mutualistic relationship between gut microbiota and the host (Turnbaugh et al., 2009). In Animal Production, the relationship between intestinal microbiota composition and nutrients utilization is particularly relevant. For example, it has been reported that the presence of microorganisms modifies the use of dietary metabolizable energy in broilers (Lan et al., 2005). One of the main factors affecting growth in broilers in the first 1 to 2 wk is the digestibility of fat, which is affected by intestinal development, viscosity of the content, and quality of dietary fat (Preston et al., 2001). It is also known that the composition of the intestinal microbiota has a significant influence on the viscosity of the contents as an increase in digesta viscosity was more pronounced in conventional than in germ-free birds (Langhout et al., 2000). The intestinal microbiota has also been shown to affect the content of bile salts (Maisonnier et al., 2003; Ranjitkar et al., 2016), which are needed to digest and absorb fats. It would therefore seem possible to positively influence nutrients and energy utilization by inducing certain well designed changes in the composition of the intestinal microbiota modulating dietary composition.

USE OF ADDITIVES TO MODULATE THE INTESTINAL MICROBIOTA

Pre- and probiotics, active plant derivatives (phytobiotics), and other additives such as Na butyrate might become useful tools for improving health status, and as a consequence productive parameters, of farm animals through intestinal microbiota modulation (Gaggìa et al., 2010). In addition, although currently assayed for the control of *Salmonella* and *Campylobacter* in farms, due to their specificity bacteriophages may represent a novel tool for inducing well-focused durable changes in the intestinal microbiota composition in hatchlings (for a recent review see Clavijo-López and Vives-Florez, 2018). The concept of prebiotic (ingredients, usually carbohydrates, that are selectively degraded by beneficial microbial species thereby improving host´s health) has been formalized by the establishment of three scientific criteria that a food ingredient must satisfy to be considered as such: (i) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (ii) be a fermentable substrate by microorganisms belonging to the intestinal microbiota; and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing. A number of substances with potential (isomalto-oligosaccharides, lactosucrose, xylo-oligosaccharides) or confirmed (inulin, transgalacto-oligosaccharides, lactulose) prebiotic effect have been described (Candela et al., 2010). Prebiotics have been shown to modulate the microbiota towards beneficial bacteria, such as bifidobacteria and lactobacilli, enhancing the intestinal defense systems in poultry (immunomodulatory action, pathogen displacement, bacteriocin production, etc.; Gaggìa et al., 2010). Prebiotics in poultry have been found particularly effective in lowering intestinal pathogen counts (Biggs and Parsons, 2008). Thus, chicory fructans have resulted in *Campylobacter*, *Salmonella,* and *Clostridium perfringens* decreases (Kleessen et al., 2003; Yusrizal and Chen, 2003); yeast cell wall containing MOS reduced intestinal *Salmonella* spp concentrations in broiler

chicks (Spring et al., 2000); isomaltooligosaccharide showed a significant reduction in the level of inoculated *S. enterica serovar typhimurium* in the ceca of young broiler chickens (Thitaram et al., 2005). Overall, authors generally agreed that a symbiotic product (mix of pre- and probiotics) displayed a greater effect than individual preparations (Gaggìa et al., 2010). However, in all those reports, prebiotics were used over the whole experimental period. No indication is found to date in the literature on the effects that the inclusion of the prebiotic only during the first days might have on the intestinal microbiota in later stages of bird´s life span. Anyway, it is known that at hatch the innate and adaptive immune systems are immature and functional maturation occurs mainly over the first 2 wk of life in broilers (Schokker et al., 2009). Therefore, as immune system maturation and intestinal microbiota composition are closely connected (see below), it would not seem unlikely that well-selected prebiotics, probably better if combined with probiotics, might be effective tools in early programming in birds.

Probiotics are defined as microbial supplements able to beneficially affect the host by improving its intestinal balance (Gibson and Roberfroid, 2007). Probiotics could thus be a possible strategy to control pathogen shedding and maintain a healthy indigenous gut microbiota. A variety of well-characterized probiotic strains (such as *Lactobacillus* spp, *B. cereus* var. toyoi, *Bacillus subtilis*, *Bifidobacterium* spp, *Enterococcus* spp, etc.) have been selected for modulation of the avian gut microbiota and protection against a variety of pathogens. The application of probiotics in poultry is associated with the concept of competitive exclusion, which is considered as their main mechanism of action (Wagner, 2006; Gaggìa et al., 2010). According to the literature, a mixture of *B. subtilis* plus *Clostridium butyricum* in the feed increased bird performance (Chen et al., 2013), but *C. butyricum* administered alone caused the same effect (Zhao et al., 2013). *C. butyricum* decreased cecal *E. coli*, *Salmonella* spp and *Clostridium perfringens* from d 14 to 42, and increased cecal *Lactobacillus* spp and *Bifidobacterium* spp counts, and promoted growth performance and immune function (Yang et al., 2012). *C. butyricum* is presently authorized for use in the EU, and its safety for a major poultry species (chickens for fattening) has been previously established (EFSA, 2011). The information reported on other species such as *Enterococcus* spp is much more limited. Zhao et al. (2013) found no interaction between *C. butyricum* and *E. faecium* on growth performance, lipid metabolism, and cecal microbiota of broilers. Also, dietary addition of microencapsulated *E. fecalis* CG1.0007 enhanced growth performance of broilers, and increased the numbers of lactobacilli and bifidobacteria (Han et al., 2013). However, although the supplementation with probiotics during early life is of great importance to the host because bacteria can modulate the expression of genes in intestinal epithelial cells, thus creating a favorable habitat for themselves, the available body of literature offers a variety of conflicting results on the efficacy of probiotics for increasing growth performance in broilers (Gaggìa et al., 2010). Anyway, probiotics have been used in competitive exclusion models in poultry since Nurmi and Rantala (1973) showed that feeding recently hatched chicks with a suspension of the intestinal contents of adult chickens protected them against *Salmonella* spp. Probiotics reported to reduce the levels of adhesion of pathogens include bacteria of the genera *Bifidobacterium* spp and *Lactobacillus* spp, preferably if they are obtained from chickens (Servin and Coconnier, 2003; Collado et al., 2005). The mechanism of action is based in the production of substances such as hydrogen peroxide (H_2O_2), bacteriocins, and organic acids including SCFA (Stern et al., 2006). Although factors such as the composition of the probiotic bacteria in the mix, origin of the bacterial strain, etc. are important, in the present context, it is relevant to underline that: (i) the composition of the probiotic may be beneficial for one breed of chicken but not for others (which indicates that the genetic differences are relevant), and (ii) the time point at which the probiotic is administered affects its effectiveness. Thus, Nakphaichit et al. (2011) found that the administration of *Lactobacillus reuteri* to broiler chickens only during the first week of the life cycle had a positive effect on the intestinal microbiota composition (diversity, abundance, and reduction of pathogens) for up to 6 wk. This is indicative that early treatment with properly selected probiotics may be able to induce durable effects in the intestinal microbiota composition and/or immune system functionality.

Phytobiotics are primary or secondary components of plants with putative or described positive effects on the growth and health of animals. They are commonly classified into four groups: (i) herbs (products from flowering, non-woody, and non-persistent plants); (2) botanicals (whole plants or processed parts); (3) essential oils (hydro-distilled extracts of volatile plant compounds); and (4) oleoresins (extracts based on non-aqueous solvents). The benefits ascribed to this quite heterogeneous group of substances are improved intestinal health, improved digestion, modification of digestive secretions and improved histological structure of the intestine (Diaz-Sanchez et al., 2015). However, extracts or complex mixes of substances have been used in most reported trials, which results in uncertainty to ascribe an effect to a given chemical compound. The effectiveness of phytochemicals remains controversial as they can differ depending on the method of extraction, geographical origin, plant genotype, and storage time (Clavijo-López and Vives-Florez, 2018). Anyway, the effectiveness of some chemically defined molecules such as garlic derivatives for modulating the composition of the intestinal microbiota in vitro and in vivo has been demonstrated. Thus, previous work carried out in vitro with sulphinates such as PTS/PTS-O has shown a significant antimicrobial effect, specifically against some Gram-bacteria.

PTS (propyl propane thiosulfinate) and **PTS-O** (propyl propane thiosulfonate) are naturally occurring organosulfurate compounds obtained by decomposition of initial compounds present in garlic such as allicin and alliin. PTS/PTS-O was highly effective *in vitro* at doses ≥50 ppm as bactericidal agents against pathogenic or potentially entero-pathogenic species (*S. typhimurium, E. coli,* enterobacteriaceae, coliforms; Ruiz et al., 2010). In vivo tests with growing broiler chickens have shown very significant effects of PTS/PTS-O on the counts of pathogenic and potentially pathogenic bacteria, intestinal histological structure, and feed conversion index (Peinado et al., 2012, 2013a, b). In addition, Kim et al. (2013) reported that treatment of chicken spleen cells with PTSO/PTS increased their proliferation and treatment of *Eimeria acervulina* sporozoites with PTSO/PTS decreased cell viability. Even more, chickens given a PTSO/PTS-supplemented diet and infected with *E. acervulina* had greater body weight gain, reduced faecal oocyst excretion, and increased profilin antibody responses compared with chickens fed a non-supplemented diet. Differential gene expression by microarray hybridization of chickens given a PTSO/PTS-supplemented diet identified the altered transcripts as belonging mainly to the function identified was "Inflammatory Response," and "Cardiovascular System Development and Function." No information can as yet be found on the potential effects of feeding broilers with PTS/PTSO-supplemented diets for limited initial periods of time, but it is not unlikely that the effect(s) induced persist in later stages.

Amongst the intestinal SCFA, which have been found to function as major energy sources for colonic cells, butyrate seems to be the main one as far as energy metabolism and immune function are concerned (Gourbeyre et al., 2011). Its practical interest has increased particularly in protected form such as Na butyrate (Zhang et al., 2011). Butyrate has been proposed to have a homeostatic effect, improving the interactions among gut, resident bacteria, and the immune system. In particular, butyrate has been shown to be effective in increasing host defense peptides (**HDP**) synthesis both in vitro (Sunkara et al., 2011) and in vivo (Sunkara et al., 2014). In poultry, butyrate might also improve GIT mucosa integrity (Peng et al., 2009), enhance resistance to pathogenic bacteria such as *Salmonella* spp and *Clostridium* spp (Fernández-Rubio et al., 2009; Namkung et al., 2011), enhance bird´s innate immune response and disease resistance (Sunkara et al., 2011), and induce an anti-inflammatory response (Guilloteau et al., 2010). Butyric acid has been reported to increase the villi length in small intestine, stimulate the pancreatic exocrine thus increasing the secretions of digestive enzymes such as amylase and lipase, have a bactericidal effect, both direct (toxicity by reduction of the cytoplasmic pH) and indirect (pH lowering in the medium), and increased gene expression of antimicrobial HDP in chicken macrophage cells, monocytes, bone marrow cells, and jejunal and caecal explants (Ahsan et al., 2016). Moquet et al. (2016) summarized the results originating from recent poultry studies and proposed putative mechanisms for the beneficial effects of butyrate on production and health: lower pro-inflammatory response to nutritional, environmental, and immune challenges, associated with improved digestibility and absorption of dietary nutrients; modulating effects of butyrate on gut microbiota; gut endocrine regulation. It is important to indicate that most of the mechanisms are likely to be affected by the delivery site of butyrate within the GIT (for example bacteriostatic properties require an acidic environment), but commercially available butyrate derivatives offer poorly documented release kinetics. Therefore, due to its cell growth and immune system stimulating effects, Na butyrate could be another candidate to early programming in birds.

INTESTINAL MICROBIOTA, GROWTH, AND THE IMMUNE SYSTEM

The genotype—microbiota interaction was first described in mice (Turnbaugh et al., 2006), where genetically obese animals showed a composition of the intestinal microbiota different from lean mice with respect to the relative abundance of Bacteroides and Firmicutes. In pigs, Guo et al. (2008) found that storage of fat may affect the proportion of Bacteroidetes division in the gut of obese and lean animals. In chickens, the impact of genotypic variation on early life microbial colonization in relation to the functional development of the gut is largely unknown, as is unknown the effect of host genetic background on microbial colonization and microbiota composition. However, recent data suggest that the genetic background influences colonization of gut microbiota after hatch in combination with the functional development of intestinal mucosal tissue, including the programming of the immune system, and that genetically different chicken lines have different coping mechanisms in early life to cope with the outside world (Schokker et al., 2016).

No doubt that the study in more detail of the possible relationship between intestinal microbiota composition and productive and physiological parameters is currently very relevant for the Animal Production sector. Torok et al. (2008) showed that the use of operational taxonomic units of the intestinal microbiome for T-RFLP analysis may represent a useful tool to relate changes in the microbial population with productive parameters in broiler chickens. Multivariate statistical methods were then used after the T-RFLP analysis to establish the relationship between the composition of the intestinal microflora and certain production parameters (energy use of feed, digestibility, etc.). Torok et al. (2008) used an indirect model based on the use of enzymes in the diet to modify the composition of the microbiota. However, the relationship between changes in the composition of the

intestinal microbiota and production parameters can be studied more specifically by using additives that have a more direct effect on the intestinal microbiota composition. Substances such as antibiotics, bioactive compounds, prebiotics, etc. would be suitable for this purpose. By using T-RFLP analysis in combination with multivariate statistical techniques, we have recently (Ruiz et al., 2015) found that Clostridiaceae 1, Lachnospiraceae, Ruminococcaceae, and Micrococcaceae are among those families most likely implicated in defining the microbiota composition of growing broiler chickens, and also those more closely related with differences in productive parameters. As for the genera contributing most to dissimilarity, these were *Clostridium* sensu stricto spp (family Clostridiaceae 1), *Streptomyces* spp (family Streptomycetaceae), *Clostridium XlVa* spp (family Lachnospiraceae), *Blautia* spp (family Lachnospiraceae), *Arthrobacter* spp (family Micrococcaceae), *Acetivibrio* spp (family Ruminococcaceae), and *Ruminococcus* spp (family Ruminococcaceae). All these genera belong to phyla Actinobacteria and Firmicutes, which, together with Proteobacteria, were those most represented in the cecal bacterial contents (Lu et al., 2003). Furthermore, significant positive correlations have been determined for the relative amounts of bacteroides (bacteroides/total bacteria) in the ileal contents with faecal NDF, ADF, hemicellulose, and cellulose digestibility. Also, the relative amounts of *Escherichia–Shigella* (*Escherichia–Shigella*/total bacteria) in the crop contents correlated negatively with weight gain and fecal fat digestibility of broilers, and total bacteria in ileal or caecal contents of growing chickens correlated negatively with ileal N digestibility (Rubio et al., 2015). These correlations had a relationship with the biological activity of the different bacterial groups. Very much in line with these observations, Johnson et al. (2018) recently identified many taxa positively correlated with performance, while negatively associated potential pathogens were also identified in the absence of clinical disease, indicating that subclinical dynamics occur that impact performance. Moreover, Oakley and Kogut (2016) reported that the transcription of proinflammatory cytokines was generally negatively correlated with the relative abundance of various members of the phylum Firmicutes and positively correlated with Proteobacteria. Correlations of the microbiome with specific cytokine mRNA transcription highlight the importance of the GI microbiome for bird health and productivity, as inflammation is usually linked to lower performance values in productive animals and birds (Gessner et al., 2017). However, at present we can only give information on the statistical relationships between variables, but it cannot be concluded from it that a given bacterial group(s) is (are) responsible for differences in any given functional effect.

Timmerman et al. (2006) underlined the importance of way and timing in the administration as main factors affecting the productive efficacy of probiotic preparations. Administration via the feed, compared to administration in the drinking water, resulted in a greater increase of average daily gain. Moreover, the supplementation of probiotics during early life is of great importance to the host because bacteria can modulate the expression of genes in intestinal epithelial cells, thus creating a favorable habitat for themselves. It is known that broiler chickens have at birth very few immunoglobulin-producing cells in the intestine, but their number increases in response to microbial colonization of the GIT, possibly due to the mitogenic activity of bacterial lipopolysaccharide on B lymphocytes. Therefore, the period after hatching is crucial in immune system development because the chicken is immediately exposed to environmental antigens. B cell population of gut-associated lymphoid tissue (**GALT**) starts at 4 d after hatching, and increases further during the first 2 wk of age (Bar-Shira et al., 2003). This may directly affect the relationship between the rate of colonization of normal flora and the development of infection with *Salmonella* spp (Nurmi et al., 1992). The establishment of an adequate intestinal microbiota has proved essential for the production of antibodies and for the stimulation of early maturation of the cellular components of the intestinal immune system. Therefore, the early handling of the intestinal microbiota can be effective in improving the intestinal immune response of treated birds, especially in relation to local activation of the secretion of IgA (Revolledo et al., 2006). The adaptation to the post-hatching period and the increased stressors, deriving from practices used in modern broiler production, e.g., feed changes or imbalances, transportation, processing at the hatchery and high stocking densities, may weaken immune functions and thus predispose broilers to colonization of the GIT by bacterial pathogens, posing a threat to birds health and food safety. Among pathogens, *Salmonella* spp has been the most studied because of its ability to infect chickens and hens increasing the risk of human contamination through the food chain, but in the last years, application studies have been extended to other bacteria such as *Campylobacter jejuni* and *Clostridium perfringens*, which could be both considered an emerging and increasing threat for poultry and human health (Gaggìa et al., 2010).

EARLY LIFE PROGRAMMING IN BROILERS

The concept of early life programming is also valid in humans, where evidence has been provided that initial appropriate newborn colonization is necessary to stimulate innate and adaptive immunity development and for the prevention of infant intestinal inflammatory and immune mediated diseases (allergy and autoimmunity) in later life. Also, oligosaccharides in human milk given preferentially during the first few postnatal months stimulate health-promoting (probiotic-like) organisms, such as *Bifidobacteria infantis*, and evoke gene transcriptions in this microorganism, which

promotes anti-inflammation. Adequate colonization must occur in the immediate post-partum period to prevent inadequate colonization and its short-term and longer term clinical consequences (necrotizing enterocolitis, allergy, asthma). Alternatively, probiotics can be used as a surrogate colonizer during the period of inadequate colonization to prevent the expression of these diseases (Weng and Walker, 2013). In productive animals other than birds, recent studies suggest that it would indeed be possible to promote different microbial populations establishing in the rumen of the young animal by manipulating the feeding management early in life that persisted in later life (Yáñez-Ruiz et al., 2015), and many of the changes in the development of the progeny in pigs take place in embryonic life (Foxcroft et al., 2009).

In broilers, as already mentioned, the strong selection in search for a higher growth rate has resulted in adverse effects such as metabolic disorders, low responsiveness of the immune system, and decreased resistance to pathogens (Tottori et al., 1997; Cheema et al., 2003). Also, the nutritional manipulations of the GIT microbiome to enhance productivity and health are rather limited by the resilience of the ecosystem once established in the chicken´s gut, as explained above for the competitive exclusion mechanism. However, it is important to bear in mind that amongst the animal production systems, poultry are somewhat unusual in that the young have a markedly reduced parental influence on the development of microbiota post-oviposition. As a consequence, newly hatched chicks are exposed to a diverse range of bacteria from environmental sources and hence varying colonization of the chicken GIT may be a consequence of the high diversity in non-avian bacterial sources (human handlers, bedding material, feed, and transport boxes) during the first hours and days of life (Stanley et al., 2013). In this context, and as broiler chickens reach slaughter weight at a physiologically younger age, investigating the consequences of early life programming has important practical implications for improving bird health, welfare, and productivity in broiler production (Cherian, 2011). Also, the short life-span of broiler production practices makes it easier and faster to study the effects on the whole productive cycle of early life interventions.

Early life or developmental programming is based on the assumption that the development of chronic diseases later in life can be modulated by perturbations or environmental exposures during critical pre- or early post-natal life (Waterland and Garza, 1999). Although not clearly established, mechanisms associated with early life programming have been attributed to functional and structural changes in genes, cells, tissues, and even whole organs, and nutrition has been identified as one source of early exposure that might affect early development and later phenotype (Korotkova et al., 2005). Although not much information exists specifically on this issue in birds, literature data allow to conclude that this is a potentially useful procedure to control microbiota development in broilers, which would open new ways to improve birds´ health, welfare, and productivity. Thus, Yin et al. (2010) found that an initial exposure to different bacterial communities could lead to the development of distinct microbiota and gene expression in the gut, and concluded that it is possible to manipulate the gut microbiota by feeding a proper bacterial composition at an early age. Indirect evidence that early life programming is possible in broilers also comes from studies with antibiotic administration during the initial stages of the bird´s life. Thus, Schokker et al. (2017) recently reported that a short term (24 h) oral perturbation with an antibiotic during the first day of life of chickens affects microbial colonization and especially intestinal immune development over a period of at least 2 wk. Furthermore, they found that the observed changes at the gene expression level most probably lead to alterations at the cellular immune level, i.e., changes in the number of macrophage-like cells. The authors indicate that their data support the assumption that early life colonization of the gut by microbiota is an important driver of immune development and/or immune programming, as has been found for other species such as pigs and mice. They also suggest that although modulation of the microbiota via antibiotics (Amoxycilin) had a negative impact on immune development (reduction in the number of macrophage-like cells), it may also be possible to modulate the early life colonization of "beneficial" microbiota by the application of innovative dietary-based or management procedures.

Early access to substances or bacteria (pre- and post-hatch) is particularly relevant here because in broilers, which are selected for rapid early growth, much of the immune system development occurs early in life. In this respect, experiments have been conducted in models related with the early stages of birds´ life such as breeder hens, *in ovo* techniques or hatchlings. Research on breeder hens has conventionally been focused on studying the nutrients and allowances that maximize production and hatchability of eggs from broiler breeders so as to achieve not only egg production but also proper egg size, chick quality, and feather structure. Several factors could affect the chick quality and some complex interactions must be considered, including hen physiological status, hen nutrition, diet formulation, farm and hatchery management, as well as transportation and brooding efficiency. However, actual studies on the effect of nutrients other than protein and energy in breeders on chick quality are sparse, with the exception of the role of vitamins and some minerals (Chang et al., 2016). Thus, there are indications that maternal dietary energy, protein, and concentrations of Se, Zn, and Mn influence immune function and livability (Hocking, 2007). Although no information is found as yet in the literature, it is not unlikely that those effects of layers dietary composition on the immune function of broiler hatchlings have an effect as well on the amount and composition of the chickens IM. In an interesting recent report,

Hynd et al. (2016) explored the impacts of the intake and composition of broiler breeder hen rations on organ development, growth, and immune status of their progeny. They found that developmental programming in chickens appears to be influenced not only by feed restriction in the breeder hens, but also by the composition of the diet, which supports the notion that specific nutrients in hens feed can act as programming agents to influence chicken health. Interestingly, only females showed responses to maternal nutrition in terms of heterophil: lymphocyte ratio and response to an immune challenge. Although the mechanisms underlying embryonic programming are yet to be elucidated, the prevailing paradigm is that the nutritional environment influences methylation reactions and microRNA expression, which in turn alter chromatin structure, gene expression and protein production with consequent phenotype changes.

Among the very few substances already assayed in early life programming experiments in young broilers are fatty acids, β-glucans, probiotics, and high-density amino acid diets. Early access of chickens to n-3 PUFA led to increased retention of n-3 PUFA in cell membranes, reduction in plasma non-esterified fatty acids, alteration in the expression of pro-inflammatory cyclooxygenase-2 protein, reduced production of pro-inflammatory eicosanoids, suppression of cell-mediated immunity, and alteration in the expression of several genes associated with lipid metabolism. The effects of an early exposure persisted up to 14 to 35 d after birth, which means 36% to 47% of posthatch life (Cherian, 2011). However, Koppenol et al. (2015) recently reported that maternal supplementation with n-3 PUFAs played a minor role in perinatal programming of the immune response of broiler chickens. β-glucans are also known to influence immune function in broilers. Huff et al. (2006) found that supplementation of broiler diets with β-1,3/1,6-glucan may be valuable for decreasing production losses due to *E. coli* respiratory disease. Rodríguez-Lecompte et al. (2012) showed that birds supplemented with combined probiotics (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Streptococcus faecium*, and *Saccharomyces cerevisiae*) and organic acids (sorbic and citric acid) for 7 d showed similar responses in TLR-2, IL-12p35, and IFN-γ compared with those supplemented for 14 d, which indicates that shorter periods of supplementation might be enough to elicit beneficial responses. Even more, Baldwin et al. (2018) recently showed that broiler chicks inoculated immediately post hatch with three species of *Lactobacillus* (namely *L. ingluviei, L. agilis,* and *L. reuteri*) reached significantly higher weight by 28 d of age, and the effects of at-hatch administration of the *Lactobacillus* mix on modifying microbiota development and structure remained persistent. There was a tendency of promotion of beneficial and reduction in pathogenic taxa in the probiotic administered group. As for high-density amino acids diets, Keerqin et al. (2017) recently found that supplementing 10% extra digestible amino acids over the recommended level proved to have long-lasting benefits to birds subjected to multiple stress conditions, namely post-hatch fasting and necrotic enteritis. This was likely because the high amino acid diet appeared to fulfill the increased demand for essential amino acids in the presence of inflammation or immune stress, and promote intestinal *Lactobacillus* spp population in the lower small intestine.

Newly hatched chicks rely mostly on innate immune responses until their gut gets colonized with microbiota, which stimulates GALT maturation, and attaining mature immune functions (including competitive exclusion) by GALT in neonate chickens has been associated with the seeding of the GIT with primary microflora. Therefore, early stimulation of beneficial microflora is critical, as it affects, to a great extent, the entire lifespan of an individual (Ballou et al., 2016). Developmental programming has in this context enormous potential application in the poultry industry, perhaps more so than in any other for various reasons: (i) broiler chickens now spend almost 40% of their lives *in ovo* due to the faster growth to market weight; (ii) the environment *in ovo* has a profound effect on the phenotypic development of the resulting chicken (Ho et al., 2011); (iii) avian embryos are readily accessible, can be cultured *ex-ovo*, the embryonic environment can be manipulated readily by *in ovo* injection of prospective agents into the yolk sac, albumen or chorioallantoic membrane, and the developmental stages are rapidly traversed; and (iv) the continued presence of potential programming "agents" within the egg throughout the entire embryonic development phase (Hynd et al., 2016).

In ovo experiments have also been conducted with pre-and probiotics. Thus, Madej et al. (2015) observed that *in ovo*-delivered prebiotics and synbiotics had no adverse effect on the development of the immune system in exposed chickens, and were able to affect lymphoid-organs' morphology in chickens. Authors speculated that embryonic development of the immune system was probably affected/modulated by the *in ovo* presence of prebiotics (inulin or transgalacto-oligosaccharides), while *in ovo* delivered probiotics (*Lactobacillus lactis* strains) may inhibit colonization of the GIT by wild strains. Given that in commercial hatcheries, hatchlings are born in sterile environments and with no contact with the dam, which restrains chicks from having their guts colonized with commensal microbiota, *in ovo* procedures have also been proposed as a means of promoting a healthy microflora in embryonic guts and stimulating maturation of the cellular and humoral immune responses in central and peripheral immune organs, including those in the GIT (Slawinska et al., 2016). Thus, Madej and Bednarczyk (2016) found that *in ovo* delivery of prebiotics and synbiotics gave also place to an increased proportion of adaptive immune cells within the cecal tonsils, predominantly helper T cells (CD4+), and cytotoxic T cells (CD8α+), together with a release

of the young B cells from primary lymphoid organs (bursa of Fabricius) and increased colonization of the secondary lymphoid organs (cecal tonsils). The authors conclude that synbiotics delivered *in ovo* stimulate the development of mucosal and systemic (spleen) humoral immunity in chickens. In addition, *in ovo*-delivered prebiotics and synbiotics have been shown to affect gene expression modulation in gut and immune-related tissues of adult broiler chickens, and the effects of early modulation of chicken embryos depend on the bioactive substance used and the tissue analyzed, which indicates different modes of action. For example, galactooligosaccharides proved to be a potent stimulator of the host–microbiome interaction by triggering a strong down-regulation of immune-related genes and pathways in cecal tonsils because prebiotics delivered *in ovo* were able to infiltrate the chorioallantoic membrane in the egg and stimulate the growth of indigenous microbiota, which resulted in an enhanced tolerance of the local immune system. The major benefit of such an evolutionary adaptation is that the healthy microbiome, which is tolerated by the host, prevents pathogen colonization in the GIT (Slawinska et al., 2016). It seems therefore that *in ovo* technology is a potentially effective procedure to facilitate early life programming of the immune system and the microbiota colonizing in broilers.

Finally, it is worth saying a few words on turkey production, as an increasing amount of information has been appearing recently in the literature in this poultry species. The most predominant intestinal genera found in both chickens and turkeys after analyzing species sequence data sets were *Clostridium, Ruminococcus, Lactobacillus*, and *Bacteroides*, but with different distribution between the two. Chickens and turkeys were also shown to have distinct intestinal microbiomes, sharing only 16% similarity at the species-equivalent level (Wei et al., 2013). As with chickens, field and controlled battery studies in turkeys (Bahl and Sorgente, 2002, Huff et al., 2002) have suggested that β-1,3/1,6-glucan may be useful as an alternative to AGP. Competitive exclusion procedures have also been assayed in turkeys. Fresh cecal turkey contents were superior to the commercial competitive exclusion products (of chicken origin), which were superior to the commercial *L. acidophilus* culture in preventing colonization of turkeys with *Salmonella* spp (Hofacre et al., 2000). In addition, some preliminary *in ovo* assays in turkeys have rendered promising albeit controversial results. Thus, *in ovo* feeding of turkey embryos consistently sped up the digestive and nutrient uptake capacity of the digestive tract around the pre-hatch period, but the effects on body weights at hatch appear to be somewhat inconsistent (Foye et al., 2005; De Oliveira, 2007).

In conclusion, a number of instruments (plant derivatives, Na butyrate, pre- and probiotics, β-glucans) and procedures (*in ovo* technology) are currently available to induce early life programming in broilers. Early life programming has potential to circumvent some of the problems (metabolic disorders, low responsiveness of the immune system, and decreased resistance to pathogens) that nowadays broiler production has to face due mainly to the strong selection imposed to fulfill the needs of an increasingly demanding market.

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