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OVERVIEW OF PREBIOTICS AND PROBIOTICS: FOCUS ON PERFORMANCE, GUT HEALTH AND IMMUNITY – A REVIEW

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Abstract

Due to the threat and emergence of bacterial resistance against antibiotics, the use of in-feed antibiotics at therapeutic and subtherapeutic levels has been limited. Complete withdrawal of antibiotics as growth promoters (AGP) has led to poor gut health signs in chickens that include conditions like wet litter, intestinal bacteria overgrowth, poor growth performance, malabsorption and various diseases. Two of the most common alternatives to AGP are prebiotics and probiotics. Both prebiotics and probiotics have become the potential feed additives that improve the gut health, immune system and microbiota by various mechanisms of action, and enhance growth performance of chickens. The review discusses the modes of action like antibacterial, competitive exclusion (CE), and immunomodulatory properties of prebiotics and probiotics, particularly in poultry. *In ovo* feeding of prebiotics and probiotics with promising effect on growth performance and reduction of pathogens like *Salmonella* is also discussed in this review. However, it is necessary to conduct more research with prebiotics and probiotics as well as other feed additives to understand the detailed mechanisms of action and identify better alternatives for poultry production and health.

Key words: prebiotics, probiotics, poultry, antibiotics alternatives

Prebiotics

Prebiotics are non-digestible feed ingredients that beneficially affect the host by selectively stimulating the activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). Prebiotics influence intestinal bacteria and immunity of chickens (Bozkurt et al., 2014; Kim et al., 2011). Prebiotics should have the characteristics such as: 1) being not absorbed in the upper gastrointestinal tract (GIT), 2) being resistant to acidic pH, 3) stimulating the growth of beneficial bacteria, 4) modulate host defense system (Patterson and Burkholder, 2003). The predominant prebiotics tried in chickens include types of oligosaccharides like fructooligosaccharides (FOS), inulin, mannanoligosaccharides (MOS) and xylooligosaccharides.

FOS are linear polymers of β -(2-1)-linked fructosyl units, terminated by one glucose residue and are not digested in the upper gut of avian species (Roberfroid et al.,

2010). Inulin is the longer chain version of FOS. MOS are mannose-based oligomers linked together by β -1,4 glycosidic bonds, found in cell wall of *Saccharomyces* yeast (Pourabedin et al., 2015). Xylooligosaccharides are oligomers consisting of xylose units linked through β -(1-4) linkages (Aachary et al., 2008). Other potential oligosaccharides used in chickens are galactooligosaccharides (GOS) (Jung et al., 2008) and lactose (Hajati and Rezaei, 2010). Several commercial prebiotics are prepared from yeast cells including cell walls and fermentation products (Ding et al., 2014; Santin et al., 2001). Other compounds that show prebiotics-like effects include *Saccharomyces cerevisiae* fermentation products or yeast culture (Roto et al., 2015).

Mechanism of action of prebiotics

Major prebiotics mechanisms of action include modulation of gut microbiota by selectively regulating beneficial groups of bacteria by providing food for them (Hajati et al., 2010) and by reducing undesired intestinal colonization of pathogenic bacteria, thus improving the integrity of gut mucosa (Iji et al., 1998). Prebiotics are not digested or absorbed in the upper GIT and instead provide food source for host beneficial bacteria such as *Lactobacillus* (LAB) and *Bifidobacteria* in the lower GIT. This eventually excludes the attachment of pathogens including *Salmonella* and promotes microbiota in the gut. Some sugars are able to block the binding of pathogens to the mucosa. For example, MOS is able to bind to mannose-specific lectin of gram-negative pathogens that express Type-1 fimbriae such as *E. coli* resulting in their excretion from the intestine (Thomas et al., 2004). MOS are commonly derived from yeast and the outer cell of yeast. MOS are found to modulate the immune system and eliminate pathogens from intestinal tract (Fernandez et al., 2002). GOS have been shown to increase certain beneficial bacteria such as LAB, *Bifidobacteria* or their fermentation products (Macfarlane et al., 2008). Production of short chain fatty acids (SCFA), mainly butyrate, propionate and acetate as a part of fermentation process, is one of the main mechanisms of prebiotics (Pourabedin et al., 2015). SCFA lower the pH of gut lumen and provide energy to epithelial cells. This modulates the inflammation and regulates the metabolic functions (Pourabedin et al., 2015).

Prebiotics in chickens (effects on growth performance, immune response, microbiota, intestinal morphology and pathogenic bacteria)

Growth performance is the general and direct indicator in poultry as it involves feed utilization and overall effectiveness of poultry production (Ajuwon, 2015). Some of the major prebiotics that have shown beneficial effects in performance and gut health are given in Table 1. Replacement of antibiotics as growth promoters (AGP) with prebiotics or probiotics to observe the effect mainly in growth is the major reason for the researches.

Supplementation of MOS and FOS in broilers is found to be associated with improved body weight gain (BWG), feed conversion ratio (FCR) and carcass weight (Baurhoo et al., 2007; Sims et al., 2004; Xu et al., 2003). Improving broiler performance by dietary beta-glucans and MOS has been found to be associated with the improvement of innate immune function (Bozkurt et al., 2012). Also, production of SCFA is the reason behind better growth performance as this increases the partition

of nutrients into other tissues of body (Lu et al., 2012; Ajuwon, 2015). The improvement of growth performance in chickens by prebiotics is affected by many factors. Prebiotics may increase SCFAs which are directly absorbed in the hind gut and used as an energy source in tissues (Chapman et al., 1994). Performance, egg cholesterol and gut microflora were improved by addition of inulin in laying hens diet (Shang et al., 2010). Improvement in egg shell and bone quality that increased the overall mineral metabolism due to inulin or oligofructose was also observed (Swiatkiewicz and Arczewska-Wlosek, 2012).

Prebiotics like MOS, FOS and inulin were found to modulate the immune responses in the gut-associated lymphoid tissue (GALT) of chickens like cecal tonsil, enhanced antibody titers of plasma IgM and IgG, cecum IgA levels, mucin mRNA expression and also enhanced intestinal immune functions (Janardhana et al., 2009 b; Huang et al., 2015). Prebiotic treated group (both MOS and FOS) had similar performance to an AGP treated group with better GALT immunity in chickens (Janardhana et al., 2009). Prebiotic-mediated immunological changes may in part be due to direct interaction between prebiotics and gut immune cells as well as due to an indirect action of prebiotics via preferential colonization of beneficial microbes and microbial products that interact with immune cells (Janardhana et al., 2009 a). In a study by Huang et al. (2015), dietary inulin supplemented at 5–10 g/kg had better effects on a starter phase (0–21 d) in both feed intake (FI) and intestinal IL-6, IgA, CD8, CD4 lymphocytes, and did not have any effect on d 42 broiler chicks.

Length of time for adaptation and the exposure of GIT microbes to the supplemented FOS plays major role in producing positive effect due to FOS. When FOS was added for a longer duration, it produced better results with villi height and crypt depth of intestine (Hanning et al., 2012). It is presumed that increased villi height is associated with the increased absorption of feed due to increased surface area transporting more feed nutrients (Amat et al., 1996). Feeding MOS and lignin in poultry has resulted in low pH, high production of SCFA like butyric acids and healthy gut, particularly increased villi height (Baurhoo et al., 2007). A study with MOS showed improved intestinal development as well as a healthy microbial community in broilers (Baurhoo et al., 2009).

Prebiotics beneficially interact with animal's physiology by selectively stimulating favorable microbiota in the intestinal system (Macfarlane et al., 2008). Abundance of LAB and *Bifidobacteria* in chicken gut have been associated with the prebiotics supplementation, mainly MOS, FOS and inulin type fructans (Geier et al., 2009; Kim et al., 2011; Baurhoo et al., 2007) (Table 1). Microbial flora such as LAB and *Bifidobacterium* sps. support the defense system of animal against invading pathogens by stimulating GIT immune response (Mead, 2000). According to Seifert and Watzl (2007), prebiotics such as inulin and oligofructans can modulate immune system directly. However, it is not clear if prebiotics directly affect the pathogen or host in a microbiota-independent manner. Oligosaccharides like beta-glucans stimulate the performance by enhancing phagocytosis and proliferating monocytes and macrophages (Novak and Vetvicka, 2008). Prebiotics compete for the sugar receptors thus preventing adhesion of pathogens like *Salmonella* and *E. coli* (Iji and Tivey, 1998). MOS have receptor properties for fimbriae of *E. coli* and *Salmonella* that

leads to elimination of such pathogens with the flow of digesta instead binding mucosal receptor (Fernandez et al., 2002).

Table 1. Role of prebiotics supplementation in growth performance, immune modulation and pathogen reduction (research from 2002 to 2015)

Reference	Type of prebiotics	Major outcomes
Fernandez et al. (2002)	MOS	Reduced <i>Salmonella</i> infection
Baurhoo et al. (2007)	MOS and lignin	Increased <i>Lactobacillus</i> (LAB) and <i>Bifidobacteria</i> , decreased <i>E. coli</i> , low intestinal pH, increased villi height
Baurhoo et al. (2009)	MOS	Increased intestinal microbes community and development of intestinal morphology
Xu et al. (2003)	FOS	Improved body weight gain, feed conversion and carcass weight, increased LAB and <i>Bifidobacteria</i>
Sims et al. (2004)	MOS	Improved body weight gain
Macfarlane et al. (2008)	GOS	Increased growth of LAB, <i>Bifidobacteria</i> , and/or their fermentation products
Zhao et al. (2013)	Fructan, FOS	Increased cecal LAB and <i>Bifidobacteria</i> , decreased <i>E. coli</i> and <i>C. perfringens</i>
Janardhana et al. (2009)	FOS, MOS	Increased immunity in GALT, increased IgG and IgM
Huang et al. (2015)	Inulin	Increased mucin mRNA expression of jejunum, increased cecum IgA level, increased intestinal immune function at d 21 but did not affect at d 42
Kim et al. (2011)	FOS and MOS	Increased LAB and <i>Bifidobacteria</i>
Geier et al. (2009)	FOS, MOS and inulin	Increased LAB and <i>Bifidobacteria</i>
Hanning (2012)	FOS	Improved villi height and crypt depth
Cao et al. (2005)	FOS + tea polyphenols	Reduced mortality in 28–42 d old broilers, FOS selectively promoted favorable microbes and inhibited microflora metabolites except volatile fatty acids in the cecum

Studies have showed an increase in *Bifidobacteria* and LAB count and decrease in *Salmonella*, *E. coli* and *Clostridium perfringens* numbers in broilers fed MOS, FOS, fructan and lignin supplemented diets (Baurhoo et al., 2007; Macfarlane et al., 2008; Zhao et al., 2013; Cao et al., 2005; Fernandez et al., 2002; Spring et al., 2000) (Table 1). The population of *Clostridium* and *E. coli* decreased with 0.25% FOS and 0.05% MOS supplementation whereas LAB diversity increased in ileum by these two prebiotics (Kim et al., 2011). MOS have been reported to promote LAB growth contributing to overall microbial diversity in the contents of chicken cecum (Pourabedin et al., 2014). Feeding lignin or MOS increased cecal population of LAB and *Bifidobacteria* whereas reduced *E. coli* in cecum of broilers (Baurhoo et al., 2007). The reason behind this might be the competitive exclusion (CE) where LAB and *Bifidobacteria* competed against *E. coli*. On the other hand, bacteriocin produced by LAB and organic acids produced by *Bifidobacteria* might suppress the colonization

of pathogenic bacteria. The increase in intestinal microbial diversity is believed to have positive effects on gut and overall host health (Janczyk et al., 2009).

Due to the low pH created by SCFAs, pathogens like *Salmonella* and *Campylobacter* are reduced from the gut. Fermentation products such as SCFA increased after prebiotic supplementation as a result of oligosaccharide fermentation by resident microbiota (Macfarlane et al., 2008). SCFA such as acetate, propionate, butyrate etc. modify the bacterial ecosystem by lowering the pH that becomes intolerant to pathogens. Due to low pH of the cecum, prebiotics have been shown to inhibit pathogens growth and stimulate the growth of beneficial bacteria like *Bifidobacterium* and LAB, and the process is the most effective in cecum (Cummings et al., 2001). The overall integrity of gut is also improved due to the production of SCFA (Alloui Mohamed et al., 2013). Stimulation of immune system includes increase in antibodies like secretory IgA and activation of phagocytic cells (Macfarlane et al., 2008). Thus, production of SCFA and reduction of gut pH are key mechanisms of prebiotics in order to limit pathogen colonization and maintain optimal growth performance and health in poultry.

Probiotics

Probiotics are either mono or mixed culture of live microorganisms which beneficially affect the host animal by improving its intestinal microbial balance (Fuller, 1989). According to FAO/WHO, probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host. The characteristics of good probiotics are: 1) they should be a strain capable of exerting beneficial effects on the host animal, 2) they should be non-pathogenic and non-toxic, 3) they should be present as viable cells, 4) they should be capable of surviving and metabolizing in the gut environment and 5) they should be stable and capable of remaining viable for periods under storage and field conditions (Fuller, 1989). Probiotics are also called 'direct fed microbials'. Commonly used probiotics in animals are: LAB (*L. bulgaricus*, *L. plantarum*, *L. acidophilus*, *L. helveticus*, *L. lactis*, *L. salivarius*, *L. casei*, *Bacillus subtilis*), *Enterococcus* (*E. faecalis*, *E. faecium*), *Bifidobacterium* spp., *Streptococcus*, *Enterococcus*, *Lactococcus*, *E. coli* and fungi and yeast (*Aspergillus oryzae*, *Saccharomyces cerevisiae*) (Huang et al., 2004). LAB and *Bifidobacterium* species have been used most extensively in humans as well. *Bacillus*, *Enterococcus*, and *Saccharomyces* yeast have been the most commonly used organisms in livestock (Ferreira et al., 2011). Multiple strains may be more beneficial than single strain as they act on different sites and provide different modes of action that create synergistic effects (Klose et al., 2006; Timmerman et al., 2004; Sanders and Huis in't Veld, 1999).

Mechanism of action of probiotics

The most common mechanism of probiotics to work is competitive exclusion (CE), which was originated on the finding that the newly hatched chicken could be protected against *Salmonella* colonization of the gut by providing it with a suspension of gut content prepared from healthy adult chickens (Nurmi and Rantala, 1973). By competing for the common niche in the gut, probiotics exclude the sites

for pathogen replication (Wu et al., 2008). CE refers to the physical blocking of opportunistic pathogen colonization and altering the environmental niches within the intestinal tract like intestinal villus and crypts leading to better immune system (Duggan et al., 2002). It involves the addition of a non pathogenic culture either single or multiple strains in order to reduce the pathogenic bacteria in the GI tract (Fuller, 1989). CE due to probiotics includes competition for physical attachment sites, enhancement of host immune system, and production of antimicrobial compounds like SCFAs and bacteriocins or colicins from metabolic reactions (Callaway et al., 2008; Stahl et al., 2004).

Enhancement of the epithelial barrier, increased adhesion to intestinal mucosa, production of antimicrobial substances and modulation of immune system are other mechanisms of action by probiotics (Bermudez-Brito et al., 2012). A front line of defense against the adverse effect of pathogens is provided by probiotics showing their antimicrobial effect. For example, lactic acid producing probiotics show antimicrobial effects by reducing the pH of the gut (Fayol-Messaoudi et al., 2005; Corr et al., 2007). On the other hand, some strains of LAB that are used as probiotics inhibit the virulence factor expression of pathogens like in *Shigella* and *Yersinia* and directly reduce their invasiveness (Carey et al., 2008; Lavermicocca et al., 2008). It has been shown that lactic acid producing bacteria produce lactic acid, which is used by anaerobic butyrate producing bacteria for producing large amount of butyric acids, and this is called cross feeding (Duncan et al., 2004). A study showed that cross feeding mechanism, particularly due to butyric acid was able to promote growth performance (Qaisrani et al., 2015).

Mechanisms of action of probiotics to modulate immune system mostly depend on the strains of bacteria or microorganisms used (Huang et al., 2004), probiotic preparation method, routes of administration and environment where birds are raised (Ajuwon, 2015). Through the interaction of host and the probiotic cultures, enhancement of both natural and specific antibodies, interferon or cytokines as well as activation or suppression of T-cells that eventually leads to the cytokine expression have been observed in many studies (Haghighi et al., 2008; Castellazzi et al., 2007; Haghighi et al., 2005).

Probiotics in chickens (effect on growth performance, immune response, microbiota, intestinal morphology and pathogenic bacteria)

The major effects observed in poultry due to probiotics including yeast cultures supplementation are in growth performance, meat quality, immune response, intestinal morphology, and intestinal microbiota (Table 2) (Gao et al., 2008; Samanya and Yamauchi, 2002; Bai et al., 2013). In poultry, probiotics feeding has been shown to maintain normal flora mainly by CE (Kizerwetter-Swida and Binek, 2009), improve feed consumption/digestion and gut health (Awad et al., 2009), and stimulate the immune system (Brisbin et al., 2008). Probiotics may potentially stimulate growth through increased SCFA production in poultry and through selective regulation of insulin signaling in different tissues (Ichikawa et al., 2002). Short chain fatty acids like acetate, propionate and butyrate are used as energy source in tissues. Particularly in chickens, butyrate has shown beneficial effects by selectively partitioning the nu-

trients away from liver and adipose tissues towards muscles through upregulation of insulin receptors (Matis et al., 2015). Another mechanism by which probiotics may stimulate growth is by regulating the immune system. When immune system is regulated, it suppresses the negative effects of chronic immune activation. When immune system is activated, there is diversion of nutrients from production process towards immune response (Gabler et al., 2008). On the other hand, there is direct effect on epithelial barrier, thus producing better growth (Awad et al., 2010).

Table 2. Role of probiotics supplementation in growth performance, immune modulation and pathogen reduction (research from 2003 to 2015)

Reference	Type of probiotics	Major outcomes
Vicente et al. (2008)	<i>Lactobacillus</i>	Increased lactic acid producing bacteria, decreased gut lesions score in broilers due to <i>Eimeria</i> and <i>Salmonella</i>
Lee et al. (2010)	<i>Bacillus</i> (direct fed microbials)	Improved gut morphology and immunity against <i>Eimeria</i>
Yörük et al. (2004)	Humate and probiotic	Increased egg production, decreased mortality
Pelicano et al. (2003)	<i>Bacillus subtilis</i> ; <i>Bacillus subtilis</i> and <i>Bacillus licheniformis</i> ; and <i>Saccharomyces cerevisiae</i>	Improved carcass and meat quality in broilers
Liu et al. (2012)	<i>Bacillus licheniformis</i>	Enhanced growth promotion and meat quality
Bai et al. (2013)	<i>Lactobacillus fermentum</i> and <i>Saccharomyces cerevisiae</i>	Stimulated intestinal T cell immune system
Gao et al. (2008)	Yeast culture	Improved immune function, growth performance and intestinal mucosal morphology
Haghighi et al. (2006)	<i>Lactobacillus</i>	Produced natural antibodies like intestinal IgA, serum IgG and IgM
Samanya and Yamauchi (2002)	<i>Bacillus subtilis</i>	Improved growth performance as well as intestinal morphology
Higgins et al. (2010)	<i>Lactobacillus</i> cultures	Developed normal microflora in chicken gut and reduced incidence of <i>Salmonella</i>
Dalloul et al. (2003)	<i>Lactobacillus</i>	Improved innate and adaptive response against <i>Eimeria</i>
Stern et al. (2001)	<i>Klebsiella pneumoniae</i> , <i>Citrobacter diversus</i> , and <i>E. coli</i>	Reduced number of <i>Campylobacter jejuni</i>
Dalloul et al. (2005)	<i>Lactobacillus</i> based probiotic	Reduced fecal oocyst shedding of <i>Eimeria acervulina</i>
Hofacre et al. (2003)	Lactic acid bacteria	Reduced mortality due to <i>necrotic enteritis</i>
Jayaraman et al. (2013)	<i>Bacillus subtilis</i>	Reduced FCR and intestinal lesions in broilers challenged with <i>Clostridium</i> and <i>Eimeria</i>

Some studies that used probiotics of *Bacillus* and LAB complex were able to improve egg production and other traits like reduction of serum and egg cholesterol level in laying hens (Li et al., 2006; Kurtoglu et al., 2004). Combination of humate and probiotics (*Lactobacillus*, *Bifidobacterium*, *Streptococcus*, and *Enterococcus* spp.) in

late laying age of hens improved egg quality and feed conversion whereas decreased mortality (Yörük et al., 2004). Growth performance as well as immune modulation by production of mucosal IgA were the best with yeast culture supplemented diets at level of 2.5 g/kg among the various levels provided (0, 2.5, 5.0 and 7.5 g/kg) (Gao et al., 2008). Similarly, probiotics containing LAB and *Saccharomyces cerevisiae* supplemented at 0.2% enhanced growth performance as well as T-cell function in broilers (Bai et al., 2013). Probiotics supplementation increased production of natural antibodies like intestinal IgA, serum IgG and IgM that are the indicators of enhanced immunity (Haghighi et al., 2006). Chickens fed dietary *B. subtilis* for 28 days had a tendency to display greater growth performance as well as pronounced intestinal morphology, including prominent villus height, extended cell area and consistent cell mitosis compared to those fed a control diet (Samanya and Yamauchi, 2002).

Probiotic strains differentially modulate, and especially balance pro- and anti-inflammatory cytokines (Foligné et al., 2010). Pro-inflammatory cytokines like TNF α , IL-1 β and IL-6 released from monocytes and macrophages are augmented by LAB and *Bifidobacteria* (Helwig et al., 2006; Miettinen et al., 1998). Anti-inflammatory cytokine like IL-10 is also released from cells like dendritic cells and monocytes due to LAB or *Bifidobacteria* feeding (Braat et al., 2004; Smits et al., 2005). It has been shown that LAB increased production of anti- and pro-inflammatory cytokines such as IL-12, IFN- γ , IL-10, and TNF- α from the intestinal epithelium of broiler chicken (Arvola et al., 1999).

Production of cytokines leads to the overall immune modulation in the chicken. LAB has shown the modulating effects on the immune system of both layer- and meat-type chickens. The ability of LAB to modulate chicken cytokines, toll-like receptors and chemokine gene expression has been demonstrated (Haghighi et al., 2008; Brisbin et al., 2011). Increase in the antibody secretion due to increase in B-lymphocytes (humoral immunity) is a potential mechanism by LAB in boosting the immunity in broiler chicks (Apatá, 2008). The increase in the population of white blood cells may be attributed to the presence of LAB in the diet stimulating the production of lymphocytes, particularly the B-cells that are responsible for forming antibodies that provide humoral immunity. Enhancement of gut barrier function through modulation of the cytoskeleton and epithelial tight junctions in the intestinal mucosa is one of the mechanisms of probiotics in preventing pathogens (Ng et al., 2009). SCFA production due to probiotics helps to promote intestinal health and integrity by directly stimulating epithelial cell proliferation, acts as the epigenetic regulators of the gene expression of multiple genes that help in growth and overall health of poultry (Kang et al., 2014; Meimandipour et al., 2010; Wu et al., 2009).

Pathogens like *Salmonella*, *Campylobacter*, *Clostridium* and *E. coli* are displaced or reduced by probiotic bacteria supplemented in chickens (Table 2). Supplementation of probiotics in feed helps in reducing *Salmonella* colonization in ceca and other internal organs either by the mechanism of CE (Nurmi and Rantala, 1973) or reduction of the colonization of opportunistic bacteria in the GI tract (Patterson and Burkholder, 2003; Callaway et al., 2008; Vicente et al., 2008). However, the idea behind using probiotic cultures as CE in chickens was that the chickens should be *Salmonella* free and the CE cultures should be given at the earliest period of age (Mead, 2000).

Early intestinal colonization with beneficial bacteria not only prevents pathogenic bacteria but also improves maturation of the gut and its integrity (Lan et al., 2005). Development of the broiler intestinal microbiota starts at the hatching. Therefore, the type of microbes provided in the initial days of chickens helps in establishing the gut microbial community (Rinttilä et al., 2013). Also, there is a lifetime stable community of microbiota from the first inoculum that leads to developed immune system (Apajalahti et al., 2004). LAB culture has shown accelerated development of healthy and beneficial microflora in broiler chickens, providing increased resistance against *Salmonella* sp. infections (Higgins et al., 2010; Vicente et al., 2008). The mucosal flora is an important component to limit *Salmonella* colonization, and microbial attachment to the mucosal surface is the key to *Salmonella* exclusion (Mead, 2000). When birds are stressed, probiotics strains like LAB and *Bifidobacterium* populate the GIT overcoming the stress produced by pathogens. They compete directly or indirectly to outnumber pathogens (Patterson and Burkholder, 2003; Callaway et al., 2008). Innate and adaptive responses against broilers infected with *Eimeria* and treated with *Lactobacillus*-based probiotic were also observed where surface markers like cluster of differentiations; CD3, CD4, CD8, and $\alpha\beta$ T-cell receptor (TCR) were increased in pronounced numbers (Dalloul et al., 2003).

Oral administration of *Klebsiella pneumoniae*, *Citrobacter diversus*, and *E. coli* significantly reduced *Campylobacter jejuni* colonization of chickens (Stern et al., 2001). Downregulation of some flagellar genes like *flaA* by LAB supplementation was able to reduce pathogenesis due to the *Campylobacter* in chicken (Ding et al., 2005). Similarly, a study reported that dietary probiotics were able to provide the better cell-mediated immunity and the reduction in shedding of fecal oocysts of *Eimeria acervulina* (Dalloul et al., 2005). The authors further demonstrated that the probiotic continued to afford some measure of protection through immune modulation despite a fairly overwhelming dose of *E. acervulina*. Mortality due to necrotic enteritis was reduced from 60 to 30% due to lactic acid bacteria added in feed (Hofacre et al., 2003). Dietary supplementation of *Bacillus subtilis* reduced FCR as well as reduced intestinal lesions in broilers challenged with *Clostridium* and *Eimeria* (Jayaraman et al., 2013). The effect of *Bacillus* on *Eimeria maxima* infection in broiler chickens was studied and it was found that *Bacillus subtilis* reduced the clinical signs of experimental avian coccidiosis and increased various parameters of immunity in broiler chickens (Lee et al., 2010 b).

However, some of the recent studies have evaluated the effects of the combination of both probiotics and prebiotics in chickens. A study that combined *Enterococcus* mixture with MOS showed alleviating negative effects of heat stress in broilers (Sohail et al., 2012). There was growth improvement in broilers by supplementing both isomalto oligosaccharides and LAB mixture (Mookiah et al., 2014). Increase in the lactic acid production by the combination treatment resulted in elimination of pathogens like *Clostridium* from the ileum and ceca, and the growth performance was better in chickens fed both prebiotics and probiotics (Abudabos et al., 2015). Similarly, combination of *Bacillus subtilis* and MOS showed improved growth performance, small intestine morphology and LAB population in male broilers (Wang et al., 2016).

***In ovo* feeding of prebiotics and probiotics in chicken**

Apart from in-feed and in-water supplementation, *in ovo* feeding of both prebiotics and probiotics have gained more attention recently. *In ovo* technology involves administration of a solution of a given substance directly to incubating eggs (Madej et al., 2015). The idea behind *in ovo* supplementation is to provide food to animal as early as possible to combat the possible pathogens that would be colonized at hatching (Bednarczyk et al., 2016). Chicks can be exposed to pathogens during hatching, sexing, vaccination and transportation before they consume their first feed. Therefore, introducing the poultry embryo's digestive tract to the external environment is essential to establish healthy gut microbial community at earlier ages (de Oliveira et al., 2014). Villaluenga et al. (2004) and Pilarski et al. (2005) previously reported that d 12 incubation is an optimal time of prebiotic injection and resulted in high levels of *Bifidobacteria* in the colon of chicken. At 12 d incubation, embryo is totally immersed in amniotic fluid allowing the transfer of solution from air cell to embryonic GIT (Bednarczyk et al., 2016). Other than maintaining healthy bacteria in the gut, *in ovo* feeding of prebiotics also resulted in higher growth rate, nutrient digestibility and immune system development (Sławińska et al., 2014). Different sites of *in ovo* administration has been performed including the amnion, allantois, embryo or the yolk sac (Cheled-Shoval et al., 2011; Pilarski et al., 2005; Salmanzadeh, 2012; Uni et al., 2005). *In ovo* feeding of prebiotics (inulin) and synbiotics (inulin with *Lactococcus lactis*) resulted in improved immune responses related to stimulation of Peyer's patch, cecal tonsil colonization by T-cells as well as developed immune organs like spleen and thymus (Madej et al., 2015; Sławińska et al., 2014).

Similarly, probiotics have been administered mostly at d 18 incubation. Lactic acid bacteria cultures were administered at d 18 incubation (Cox et al., 1992). Chickens injected with probiotics at d 18 incubation had increased microbiota diversity but decreased *Enterobacteriaceae*, the family to which several enteropathogenic bacteria belong, including *Salmonella* spp. and *E. coli* (Pedroso et al., 2016). Probiotics bacteria like LAB, *Bacillus subtilis*, *B. licheniformis* and *B. amyloliquefaciens*, *Enterococcus faecium* and their combinations were inoculated at 17.5 d of incubation, followed by a grow-out *Salmonella* challenge study (de Oliveira et al., 2014). The study reported an improved growth performance as well as complete elimination of *Salmonella* in market-age broilers. However, Yamawaki et al. (2013) reported no protection against *Salmonella* when eggs were inoculated with *Lactobacillus acidophilus*, *Lactobacillus fermentum*, and *Lactobacillus salivarius* at 18 d of incubation into the air cell.

Some inconsistencies among studies

The use of prebiotics as possible alternative to antimicrobial growth promoters, has given contradictory results, while their use in the modulation of the gut microbial population has been promising. Oligosaccharides, esp. raffinose series that are naturally present in feed ingredients have shown imprecise results with respect to broilers performance (Iji and Tivey, 1998). Broiler growth performance was negatively affected when FOS was supplemented at higher level (8 g/kg) (Xu et al., 2003). Feed intake and FCR both were increased upon either *in ovo* or water administration

of prebiotics like GOS (Bednarczyk et al., 2016). Another study that used GOS as a prebiotic source found neither positive nor negative effects on growth performance but observed increased intestinal anaerobic bacteria and LAB (Jung et al., 2008). Biggs et al. (2007) supplemented GOS at 4 g/kg and did not observe any significant growth performance in broiler chicks. The authors have also found depressed growth performance and a negative impact on amino acid digestibility as well as metabolizable energy when supplemented with higher level of inulin (8 g/kg). Promotion of *Bifidobacterium* without any effect on BW, FI and FCR has been observed in studies that used GOS in broilers. Addition of 0.025% beta-glucan did not improve broiler performance including FI, FCR and BWG in a starter period (Józefiak et al., 2008). Supplementation of inulin had no effect on villus height and crypt depth of jejunum (Rebolé et al., 2010). This also demands for repetitive researches with different levels or concentrations of FOS, MOS or GOS supplementing in the diets of poultry.

Such inconsistencies exist among the studies with probiotics. Beneficial effect of *Lactobacillus* spp. bacteria on chicken livability was observed without any effect on BWG and FCR (Brzóska et al., 2012). Feeding DFM containing *Bacillus* did not affect the growth performance (Lee et al., 2010 a). The use of probiotics did not influence the performance of the birds challenged with *Salmonella enteritidis*, neither the production of anti-*Salmonella* antibodies and intestinal morphology were observed (Ribeiro et al., 2007). The effects of probiotics on chickens also depend on rearing system (cage vs. floor pen) especially *Salmonella* challenge condition and this can be due to differences in hygienic conditions (Pirgozliev et al., 2014; Santos et al., 2008). A study has shown that the beneficial effects of additives like organic acids are pronounced in less hygienic housing conditions (Pirgozliev et al., 2014). Broilers raised in litter had lower cecal *Salmonella* count than in cages as litter birds may have more chance to get the modulated gut microbes due to CE and thus reduced *Salmonella* (Santos et al., 2008). Such results may question the effectiveness of similar feed additives as potential growth promoters. The factors behind the variability due to probiotics may include physiological state of bird, actual microbiota already present in the gut, dose and nature of strains used for probiotics culture, probiotics species, method of preparation of probiotic strains, route of administration and timing of application relative to any pathogen challenge (Brisbin et al., 2011; Ajuwon, 2015; Huyghebaert et al., 2011).

Conclusions

Improvement in gut health, immune system and performance parameters are the major areas to evaluate for the positive changes due to prebiotics and probiotics feeding. Both prebiotics and probiotics have a wide range of mechanism of action that eventually improve growth performance or eliminate the pathogens like *Salmonella* and *E. coli* in chicken. However, there needs to be adequate research regarding the mechanism of how the immune system is stimulated due to feeding both prebiotics and probiotics. Many factors need to be considered before we use prebiotics and probiotics to replace the antibiotics such as type of bacteria to use for CE, method of administration of the product, the active ingredients contained in the compound. Also, there should be enough research to show if the growth performance due to

prebiotics and probiotics feeding directly relates to the immune functions and gut health. Before deciding prebiotics and probiotics as the sole alternatives to antibiotics there should be enough knowledge on how they function in the animal intestine, particularly at the microbiota, host and the feed. The future studies should focus on evaluation of impact of prebiotics and probiotics on tissue specific effects (host tissues and microbial community) and intestinal digestive process thus improving utilization by these compounds by chickens. Nutrigenomics and metabolomics approaches may help elucidating the mechanisms of prebiotics and probiotics and interaction between the host and microbiome.

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