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REVIEW PAPER

A review on the influence of dietary immunobiotics on the performance, intestinal morphology and immune-related gene expression in post-hatched broiler chicks

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ABSTRACT

The use of antibiotics in the broiler industry is continuously increasing for promoting growth performance, improving the edible meat yield, and preventing microbial infections in the chicks. Due to the extreme misuse of antibiotics, antimicrobial resistance is developing among the broilers and simultaneously to their consumers. Keeping in view these facts current review was planned to understand the effect of different dietary immunobiotics on the performance, intestinal morphology, and immune-related gene expression in post-hatched broiler chicks. The review of the literature indicated that the application of immunobiotics as functional foods and its biological value have been reported by many scientists worldwide. In addition, to develop immunologically functional foods, immunobiotics also help in regulating intestinal immunity. The current review further explored that the immunobiotics regulate intestinal immune homeostasis, cellular and molecular mechanisms. It was also interesting to note that immunobiotics concerning microorganisms stimulate the activation of mucosal immunity in the Gut Associated Lymphoid Tissues (GALT). In vitro studies on the toll-like receptor (TLR) 2-transfected cells showed that immunobiotics can potentially be used to enhance the immune system in the GALT. Keeping in view reviewed studies on immunobiotics it could be concluded that immunobiotics positively influence the performance, intestinal morphology, and immune-related gene expression in post-hatch chicks. They could be used as the best alternative to antibiotics.
Keywords: Gut Associated Lymphoid Tissues; Immune system; Prebiotics; Receptors.

INTRODUCTION

Antibiotics are broadly used to prevent microbial infections, promote growth performance, and increase the edible meat yield of broilers that results in antimicrobial resistance among the broilers and simultaneously among the consumers (Tako, 2004). Although, antibiotics are extensively used due to various benefits, keeping in view their negative impacts it seems necessary to overcome their extensive use in the poultry industry. Reducing the misuse of antibiotics and increasing the performance of broilers is a major goal of the poultry industry nowadays. The alternative to antimicrobial growth promoters, no doubt multiple supplements have been explored for maintaining the growth performance of broilers (Biggs and Parsons, 2008; Chowdhury *et al.*, 2009); however, still, there is need of certain other agents to be explored though can be used as the best alternative of antibiotics and antimicrobial growth promoters. Controlling and regulating the immune system of broiler chicks is also an option for broiler producers to get rid of the use of antibiotics. Immunomodulators are assumed to enhance the humoral immunity of broiler chicks against different diseases (Gao *et al.*, 2009).

It has also been reported that gut-associated lymphoid tissues (GALT) of broiler chicks contain T and B lymphocytes which are functionally immature till the first two weeks of life. In such cases, rapid maturation of innate immunity is very necessary to protect the chicks against different diseases (Miyazaki *et al.*, 2007). Immunological functions of GALT can be a critical point for reducing the incidence of enteric disorders, improving the performance and intestinal health of broiler chicks. Because GALT is prone to the various microflora from feed and environment. Studies on the biological functions of immunobiotics have reported their worldwide application as functional foods and supplements (Clancy, 2003). Although, the beneficial effects of immunobiotics in the regulation of intestinal immunity are very important for developing immunologically functional foods but the details regarding the cellular and molecular mechanisms by which immunobiotics regulate intestinal immune homeostasis have not been elucidated yet. For producing the health-promoting effects, firstly several structural components of these immunobiotics are recognized by specific receptors on innate immune cells that mediate their beneficial properties. This hypothesis is supported by the observation that a wide range of bacteria shares a conserved pattern of cell surface components, which interact with pattern recognition receptors of the innate immune system (Sato *et al.*, 2009). It is assumed that these immunobiotics might influence the performance, intestinal morphology and immune-related gene expression in post-hatched broiler chicks because Clancy, (2003) has already proposed the concept of immunobiotics concerning microorganisms that stimulate the activation of mucosal immunity in the GALT and on that basis has performed in vitro studies with toll-like receptor (TLR) 2-transfected cells to select possible immunobiotic lactic acid bacteria, which may potentially be used to enhance the immune system in the GALT. Therefore, the present review was planned to investigate the influence of different dietary immunobiotics on the performance, intestinal morphology, and immune-related gene expression in post-hatched broiler chicks.

Immunobiotics and performance of post-hatch broiler chicks

The evolution of immunobiotic was started when the word probiotic was first time used in the food industry for describing the microbes which are beneficial to health. The scientific criticism, absence of a mechanistic framework, and soft clinical targets limited the value of this concept (Tako, 2004). Recently, studies by clinical trials and animal models supported the role of certain immunobiotics which activate the intestinal mucosal system by stimulating the gut antigen-presenting cells for promoting the protection and switching regulatory mechanisms. For bacteria, which promote health by driving the mucosal immune mechanisms a new term was required thereafter the term immunobiotics was suggested as appropriate to fulfill this need (Clancy, 2003).

Immunobiotics such as Lactobacillus plantarum P-8 have been proved to be used as the best alternative to antibiotics in the broiler chicks for promoting the growth performance. Like antibiotics, P-8 also provides similar benefits in feed intake, feed efficiency, and weight gain. In addition, P-8 activates defensive immune responses of the broilers, however, such response cannot be achieved by antibiotics (Gao *et al.*, 2009). Furthermore, P-8 induces higher fecal secretory IgA (sIgA) levels on day 42 and IgA+ lymphocytes in the jejunum and Peyer's patches (PP), whereas CD3+ T cells in the small intestinal tissues are also increased. It also increases CD4+ T cells significantly in the intestinal tissues while, Th1 and Th2 cytokine expressions are also enhanced by P-8 (Wang *et al.*, 2015). Administration of immunoprobiotic bacteria by oral route may be helpful for broilers' health and performance. Appropriate selection is necessarily required for the species specificity of such immunobiotic microorganisms. Nowadays, for the selection of immunoprobiotic bacteria, an in vitro assay has been developed in chicken which allows testing of large numbers of individual strains. In vitro and in vivo immune stimulation correlates well and no false-negative results have been reported yet. Therefore these assays can be an appropriate selection tool for immunomodulating properties of immunobiotic bacteria in broiler chicks (Marjorie *et al*., 2004).

Immunobiotics like Lactobacillus species are widely used in broilers feeding nowadays due to their health-promoting property and positive influence on the performance. These microbiotas are important inhabitants of the gastrointestinal tract of the animals including broilers chicks. Induction of probiotic Lactobacillus in poultry has been proved to improve the health, growth performance, and microbial food safety. It is considered an important substitute for the sub-therapeutic use of antibiotics (Tohno *et al*., 2006; Tohno *et al*., 2007). No doubt, colonization of lactobacillus in the gastrointestinal tract is thought to be critical due to their probiotic ability but the mechanisms of Lactobacillus colonization in poultry are not well understood still yet. Advancement in omics technologies including gene expression profiling, proteomics, genome sequencing, and analysis has allowed the identification of microbial factors such as efflux transporters, adhesive bacterial surface proteins, and stress responses in poultry. As the selection and application of probiotics in poultry can only be performed by understanding their basic functional mechanisms; therefore, additional research is still required to assess the role of these factors in gastrointestinal colonization and their contribution for health benefits of Lactobacillus in poultry (Broderick *et al*., 2016). Granulopoiesis plays a vital role in the innate immune response of broiler chicks. The balance between granulopoiesis in the bone marrow and clearance or destruction in peripheral tissues are main strategies whereby granulocytes are maintained in the body. The factors influencing the normal granulopoiesis result in alterations in innate immune response and lessen the resistance against the infections (Tohno *et al*., 2006). The role of microbiota in maintaining granulopoiesis and the immunological mechanisms has been studied. It has been reported that the addition of immunobiotics in the diet is an interesting option to improve the steady-state, resistance against respiratory pathogens, emergency granulopoiesis, and respiratory innate immune response in the host (Salva *et al*., 2017).

Influence of immunobiotics on intestinal morphology of post-hatched broiler chicks

Intestinal epithelial cells (IECs) detect bacterial and viral associated molecular patterns through germline-encoded pattern-recognition receptors (PRRs). These cells have the ability to mount immune responses against pathogens and maintaining the immune tolerance to communities of resident commensal bacteria. Toll-like receptors (TLRs) are the major class of PRRs expressed on IECs and immune cells (Sato *et al*., 2009). TLRs play a very important role in bringing on immune tolerance, prevent and treat several gastrointestinal inflammatory disorders when probiotics with the immunoregulatory property are provided in the diet. Intestinal epithelial (BIE) cell line derived from intestinal epitheliocytes has been used to evaluate the influence of immunobiotics on TLR-mediated inflammation. The role of TLRs and their negative regulators in both the inflammatory response and other beneficial effects of immunobiotics have also been investigated. Specific emphasis on the cellular and molecular interactions of immunobiotics with BIE cells through TLRs was given and that revealed the scientific basis for the development of immunomodulatory feed for animals including broilers (Villena *et al*., 2014). Immunobiotic products containing *Lactobacillus fermentum* and Saccharomyces cerevisiae have been studied to check their influence on the growth performance and immune status of the intestine in broiler chicks. These immunobiotic products stimulate the immune system by enhancing the intestinal T-cells production and their activity in broilers. Furthermore, immunobiotics also help in improving the feed efficiency, ADG, CD3+; CD4+; CD8+, and T-lymphocytes. Regarding the effect of immunobiotics feeding on the mRNA expression level of B-cell marker chB6 (Bu-1) in the foregut of chicks, no significant influence has been reported yet. It has also been reported that probiotic supplementation increases the RNA expression levels of Toll-like receptor (TLR) 2 and TLR 4 at day 21^{st}, and only the TLR2 mRNA level at day 42^{nd} in the foregut of chicks, but do not have any influence on TLR7 mRNA expression (Bai *et al.*, 2013).

Immunobiotic lactic acid bacteria such as *L. jensenii* TL2937, *L. gasseri* JCM1131T-, *L. delbrueckii* ssp. Bulgaricus NIAIB6-, and *L. gasseri* TL2919 have been studied in broilers to examine the developmental changes in immunocompetent cells of the gut during the first week. The relative weight of bursa of fabricius and spleen in chicks remain slightly higher at day 1^{st} and 3^{rd}, when chicks are feed with above, said probiotics excepting *L. gasseri* JCM1131T. Feeding chicks with *L. jensenii* TL2937 or *L. gasseri* TL2919 causes better expression of T cell-related mRNA in the foregut of chicks at age of day 3^{rd} or 7^{th}. Expression levels of toll-like receptor (TLR) mRNA increase in the foregut of chicks with immunobiotic diets, except for the *L. delbrueckii* ssp. Bulgaricus NIAIB6. The Bu-1 mRNA expression level in the bursa of fabricius is usually not affected by the supplementation of immunobiotic lactic acid bacteria. Immunobiotics, especially *L. gasseri* TL2919 is considered as more useful to protect the chicks from diseases without decreasing growth performance by immunomodulation and stimulating the gut-associated immune system (Sato *et al.*, 2009).

It has been reported that prebiotics and synbiotic have a significant influence on the specific immune cell composition, distribution in the cecal tonsils (CT), bursa of fabricius, and ileum of broilers. The adverse effects of prebiotics and synbiotics on the GALT development in chicks have not been reported yet. The number of B-cells in bursa decrease with the increased colonization rate of the peripheral lymphoid organs. Cecal tonsils show more potent colonization of the GALT by T cells and B cells with pre and synbiotic supplementation. With the increase of age, symbiotic results increase in T-cell numbers in the ileum with faster colonization of the cecal tonsils by B cells (Madej *et al.*, 2016). Bifidobacterium breve MCC-117 possesses the ability to modulate the inflammatory response triggered by enterotoxigenic Escherichia coli (ETEC) in intestinal epithelial (PIE) cell line. While the ability of this strain to influence PIE and immune cell interactions which ultimately lead to the stimulation of regulatory T cells are two major factors that influence the immunoregulatory effects of B. breve MCC-117. About 23 strains of bifidobacteria taxonomically belonging to five species have been tested for their potent immunomodulatory effects. Two methods have been indicated to be used such as the NF-κB-reporter assay using a toll-like receptor 2-expressing transfectant (HEKpTLR2 system) and the mitogenic assay using Peyer's patches immunocompetent cells (Fujie *et al.*, 2011).

Influence of immunobiotics on immune system of post-hatch broiler chicks

The immune system of newly hatched chicks is functionally immature during the first week of age. The humoral response provides an effective defense mechanism to combat pathogens until sufficient antibody titers are attained. While, innate immune system (i.e., heterophils) has the capability to respond more quickly against pathogens (Bar-Shira *et al.*, 2003). Influence of probiotic bacteria to upregulate heterophil's function has been studied. Degranulation and oxidative burst are two bactericidal mechanisms which are mostly used by heterophils to destroy the pathogens. These mechanisms can be used as the best indicators for the function of heterophils. Immunobiotics bear the ability to increase the degranulation and heterophil oxidative burst. Probiotics like *L. lactis lactis*

(G6), *Bacillus subtilis* (G3) and *L. acidophilus* (G8) have been proved to elicit better heterophil response under in vivo and in vitro conditions (Farnell *et al.*, 2006).

Various researches on the biological functions of dairy lactic acid bacteria (LAB) have made LAB to be applicable in functional foods and supplements in the global market. Recently, a term is known as "immunobiotics" has been proposed for identifying the probiotic bacteria which promote health by activation of intestinal immunity. Specific effectors molecules and their receptor targets have also been studied (Pasare and Medzhitov, 2004). Currently, immunostimulatory AT oligonucleotide (AT-ODN) has been found but CpG ODN from *L. gasseri* JCM 1131T triggered immune responses via Toll-like receptor 9 (TLR9) has not been observed as these are particular receptor for the bacterial DNA containing the specific sequence pattern of unmethylated CpG dinucleotide. After the discovery of TLR9, possible molecular mechanisms in immune responses through bacterial DNA have rapidly been reported. Recently, it has been indicated that ATODN from Lactobacillus gasseri, induces immune activation in Peyer's patches (Pps) through TLR9, while TLR9 is basically a receptor for CpG and non-CpG AT ODN as a result of the induction of nuclear factor-κB (NF-κB) activation by gene reporter assay (Kitazawa *et al.*, 2005).

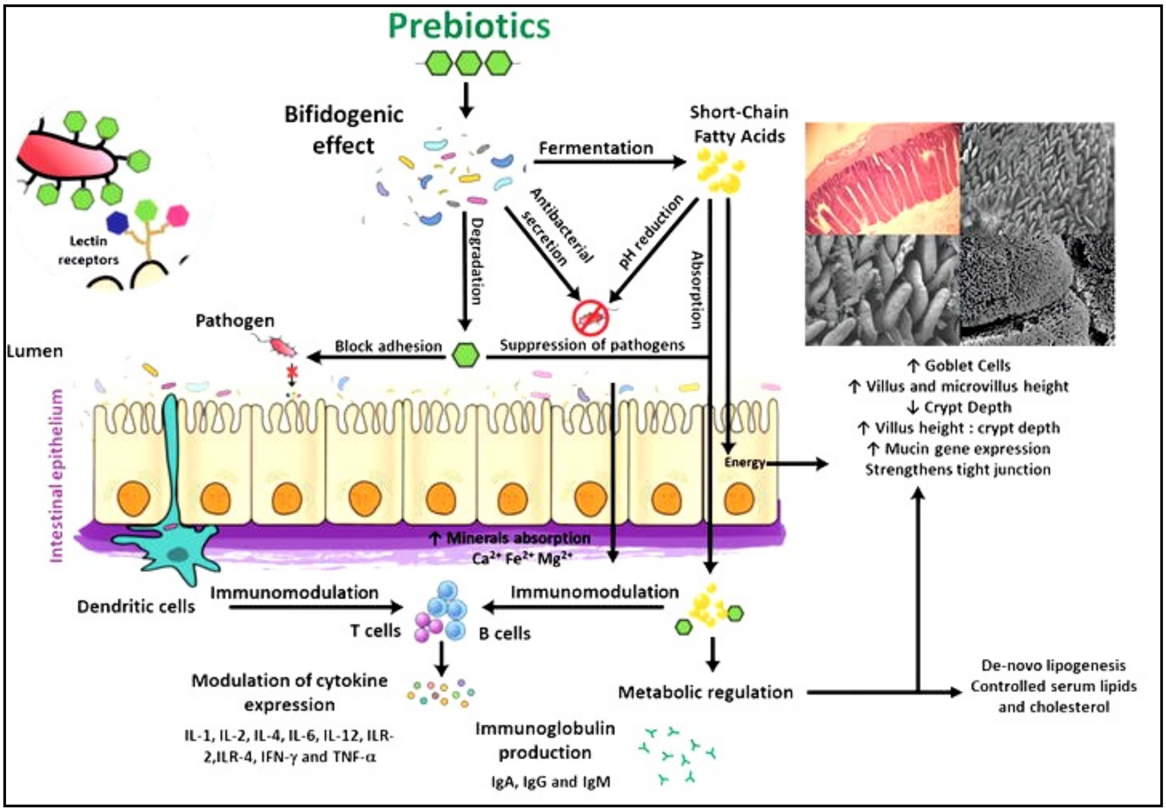

Figure 1. Some of the potential mechanisms of action of prebiotics in poultry species, showing how they are metabolized by the host microbiota and have positive effects on immunity, gut health, metabolic activity, and pathogen colonization

The cells containing interleukin-6 (IL-6) and IgA in the intestine of broiler chicks is significantly influenced by the use of probiotics in the diet. Probiotics like *Streptococcus faecalis, Clostridium buthricum* and *Bacillus mesentericus* have been investigated by multiple scientists. For the immune system, IL-6 is considered as multifunctional cytokine and increased level of IL-6 secretion may influence the immune functions in the mucosal tissues of intestine. The IL-6 immunoreaction product has been identified in the leukocytes in the subepithelial tissues, mucosal and crypt epithelium and lamina propria. In duodenum, the density of immunoreactive (ir) IL-6 cell population in the mucosa is usually lower and in colon it is highest (Figure 1). However, with probiotics feeding this density remains greater (Yoshimura *et al.*, 2010). In another study it was reported that Toll-like receptors (TLR) are major pattern recognition receptors that play an important role in the induction of innate immunity through recognition of exogenous pathogen-associated molecular patterns. TLR-mediated signaling primarily activates nuclear factor-kB (NF-kB), which plays critical role in regulating the expression of genes involved in immune and inflammatory responses (Janssens and Beyaert, 2003). A total of 11 members of the TLR family have been till to date. TLR2 being a one member of TLR family is very important for the recognition of components from Gram-positive bacteria, such as peptidoglycans and lipoteichoic acid (Wetzler, 2003). As interest in the broilers' immune system is increasing nowadays due to its broad economic importance therefore; broilers expert are in plan to produce immunologically functional foods containing immunobiotics that specifically target immune responses in the GALT of broilers. Such developments will definitely require selecting a specific immunobiotics that have an ability to activate the immune system via TLR2 using sTLR2 transfectant (Hochrein and Wagner 2004).

Immune-related gene expression and dietary immunobiotics

Influence of immunobiotics such as *Lactococcus lactis* has been studied to check their influence on immune-related gene expression in broiler chicks. It has been reported that *Lactococcus lactis* down-regulate the immune-related gene expression and magnitude of this down-regulation may be more prominent in the cecal tonsils compare to spleen. In addition, this magnitude also increases with the stabilization of the gastrointestinal microbiota and age of birds (Płowiec *et al.*, 2015). Studies on immunobiotics such as *Aspergillus oryzae* culture (CAO) have also been carried out to investigate their influence on the mRNA expression of immune-related mediators and systemic inflammatory response in the intestine of male broiler chicks. During the immunological stress induced by lipopolysaccharide and sephadex, CAO supplementation in the diet increases the early inflammatory response such as changes in the acute phase substance and growth performance (Reid and Friendship, 2002). The CAO and antibiotic feeding also lower the mRNA expression of interleukin (IL)-1β and interferon (IFN)-γ. The expressions of toll like receptor (TLR)-4 mRNA with the CAO feed has been reported to be much better at day 5th, and poor at day 14th. Furthermore, CAO has anti-inflammatory effect too. CAO and antibiotics produce similar kind of changes in the IFN-γ, IL-1β and TLR-4 mRNA in immune-related cells of the gut therefore these immune-related mediators in the intestine may be a useful marker to select immnunobiotics as best alternative to antibiotics (Takahashi, 2012). The family of toll-like receptors (TLR) play very essential role in host defense mechanism by recognizing the bacterial pathogen-associated molecular patterns. The molecular immunoassay system for immunobiotics, immunostimulatory DNA sequences from probiotics and high potential immunobiotic LAB strains via TLR signaling have been developed (Callaway *et al.*, 2008). These provide significant clues on the TLR signal transduction pathways and recognition mechanisms for the ligands. In addition to understanding intestinal immune regulation by cytokine, identifying immunoregulatory factors and immunogenics from LAB, networks may also be helpful for basic food immunology research for developing immunobiotic foods to prevent specific diseases (Kitazawa *et al*., 2008). For majority of microbial components, Toll-like receptor 2 (TLR2) is considered as an important receptor. It mediates activation signals in the cell relating to the innate immune system. sTLR2-expressing HEK293 cells are helpful in certain molecular immunoassay systems to produce physiologically functional foods having intestinal immunomodulatory abilities like maintenance of Th1/Th2 polarization etc (Zhang *et al*., 2004). Further, treatment of PIE cells with *L. plantarum* N14, *L. delbrueckii* TUA4408L or their APS and NPS induce the expression of TLR negative regulators TOLLIP, IRAK-M, SIGIRR, MKP-1, A20, and/or Bcl3 via TLR2, TLR4, and/or RP105. Treated-PIE cells showed a reduced inflammatory response in terms of chemokines and cytokines production after the subsequent challenge of with TLR4 agonists (Figure 2).

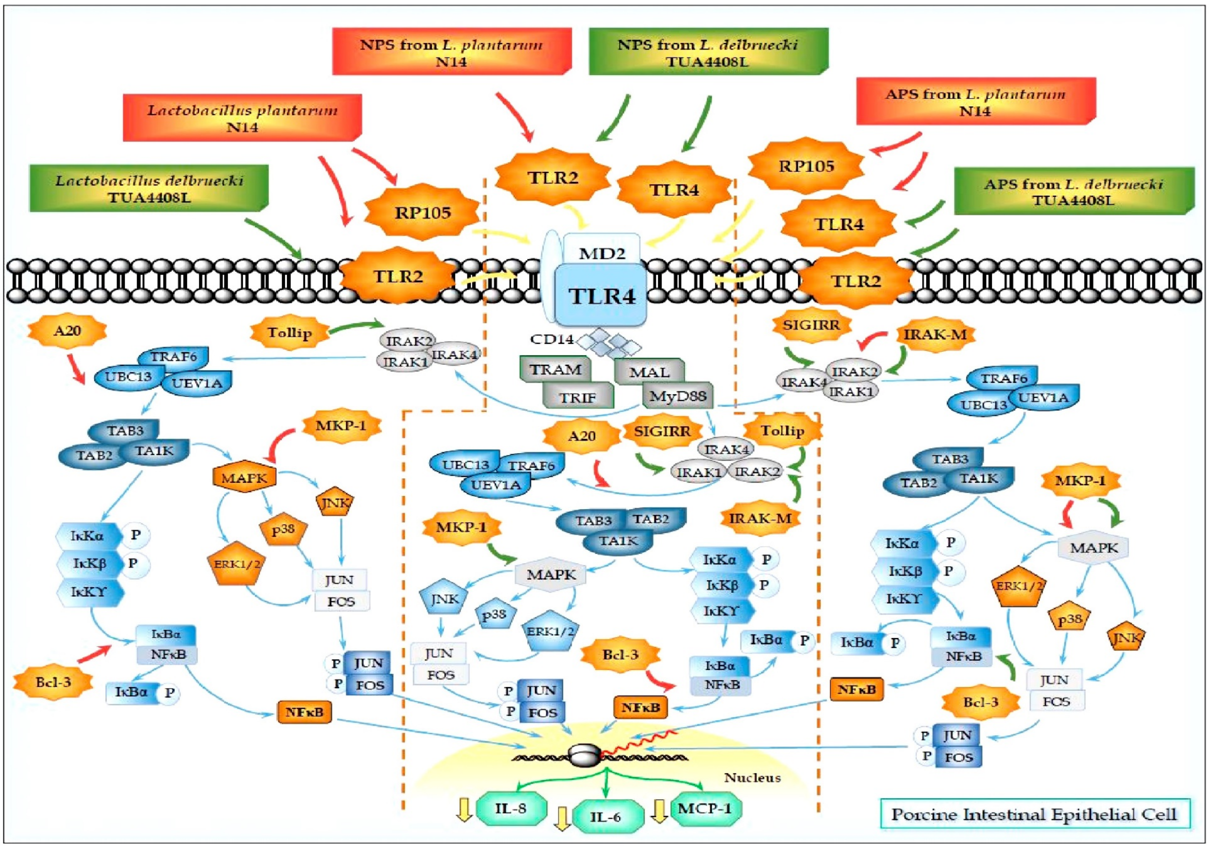

Figure 2. Modulation of Toll-like receptor 4 (TLR4) signaling pathway in porcine intestinal epithelial (PIE) cells by acidic (APS) and neutral (NPS) exopolysaccharides produced by Lactobacillus plantarum N14 and Lactobacillus delbrueckii TUA4408L

Various strains of lactic acid bacteria (LAB) via TLR2, the TLR2 (sTLR2)-expressing transfectant has been constructed by using embryonic kidney (HEK) 293 cells to evaluate the precise molecular immunoregulation. It has been reported that the intact immunobiotic LAB has the ability to induce immune responses by TLR2, while different nuclear factor-ϰB (NF-ϰB) activities of various strains can accurately be detected by sTLR2-expressing HEK293 cells. The cellular activation of NF-ϰB via TLR2 is reflected in enhanced binding and uptake of LAB. The sTLR2-expressing

HEK293 cells are also helpful to characterize the expression pattern of type I helper T (Th1) and type II helper T (Th2) cytokines by stimulating the immunobiotic LAB (Tohno *et al.*, 2007). TLR3 is also a member of TLR family which act as a receptor for double-strand RNA (dsRNA) and upon recognition of its ligand, TLR3 transmits signals that activate the transcription factors IRF-3, NF-kB, and AP-1, leading to the induction of type I interferons (IFN) (especially IFN-b) and cytokine/chemokine production (Matsumoto *et al.*, 2008). The production of cytokines and chemokines by IEC and immune cells after TLR3 activation can be a major innate immune response against dsRNA viruses like rotavirus etc. Since TLR3 responds to a synthetic dsRNA, poly(I:C) as well as viral dsRNA resulting in the induction of IFN-a/b and IFN- inducible gene transcription, it is thought that TLR3 plays a key role in antiviral immune responses (Matsumoto *et al.*, 2002). Immunobiotics might augment Toll-like receptor (TLR) signaling, regulate local mucosal cell-mediated immune responses, enhance dendritic cell-induced T cell hyporesponsiveness and promote epithelial barrier integrity in avian and mammalian species (Gao *et al.*, 2008; Ng *et al.*, 2009).

CONCLUSIONS

In conclusion, immunobiotics have a significant influence on the performance, intestinal morphology, and immune-related gene expression in post-hatched broiler chicks. The immunobiotics regulate intestinal immune homeostasis, cellular and molecular mechanisms. They stimulate the activation of mucosal immunity in the GALT, with reference to microorganisms. They also potentially enhance the immune system in the GALT. Keeping in view these facts immunobiotics could be suggested as a potential alternative to the antibiotics.

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