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Effects of Oral Probiotic Feeding on Toll-Like Receptor Gene Expression of the Chicken's Cecal Tonsil

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Abstract

Background: It was proposed that probiotics may influence immune system through direct or indirect exposure. Direct exposure is mostly mediated by surface receptors. Toll-like receptors (TLRs) are conserved molecular sensors which could be triggered via some pathogen associated structures, hence, modulate the immune responses. This study was conducted to elucidate the impact of lactobacillus acidophilus as a common probiotic on the expression level of TLRs in the chicken's cecal tonsil.

Methods: Thirty one-day-old chicken were selected and separated into three groups as probiotic-fed, dairy-fed and control. In addition to commercial powder supply, each chicken in the probiotic-fed group received 109 CFU/Kg of L. acidophilus daily. While, chickens in the dairy-fed group were provided with commercial powder feed and sterile dairy milk. After 14 and 21 days of oral feeding the cecal tonsil was removed and the expression of TLR2, TLR4 and TLR5 were examined by real-time PCR.

Results: At the age of 14-day, there was a slight upregulation in the expression levels of TLR2 (118.9%), TLR4 (129.6%) and TLR5 (123.7%) of the cecal tonsil in the probiotic-fed group; however, these alterations were not statistically significant. At the age of 21-day, a non-significant downregulation was observed in TLR expression level of both dairy-fed (TLR2, 85%; TLR4, 79.5%; and TLR5, 86.5%) and probiotic-fed (TLR2, 88.8%; TLR4, 81%; and TLR5, 87.2%) groups in comparison to controls.

Conclusions: The findings revealed that although the probiotic supplementation could be useful but it did not significantly affect innate immunity state through alteration of TLRs.

Keywords: Cecal tonsil, Chicken, Probiotic, TLR

Introduction

Recently, probiotic bacteria have become a great interest of research due to their health benefits. Among microorganisms that can be classified as a probiotic, Lactic acid bacteria (LAB) are considered as the most preferred groups with various genus (*Lactococcus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, and *Lactobacillus*) (1). Probiotics are involved in gastrointestinal mucosal barrier system and protect us against pathogenic microorganisms via regulating innate and adaptive immunity. Several beneficial effects of probiotics including control of gastrointestinal microbiota, control of serum cholesterol level, lactose tolerance, anti-carcinogenic activity and immune-potentiation properties have been reported (2). Their therapeutic effects in some infections as well as allergic and inflammatory diseases have been shown (3). They could also play a role in the

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prevention of irritable bowel syndrome (4), inflammatory bowel disease (IBD), and necrotizing enterocolitis (NEC) (5). Moreover, considering the increased resistance of pathogenic microorganisms to chemotherapeutics medications in the last decades, it seems that probiotics could be an interesting replacement to antibiotic therapies and prevention of infectious diarrheal and nosocomial infections (6). Probably the functionality of probiotics is mediated via epithelial cells stimulation and dendritic cells activation which is mainly conducted by the means of toll-like receptors (TLRs) and cytokines (7).

As a group of transmembrane proteins, TLRs exist in various kinds of immune and non-immune cells including dendritic cells (DCs), B-cells, natural killer cells (NK-cells), macrophages and epithelial cells (8). They are a family of pattern recognition receptors (PRRs) that recognize conserved molecules on microorganisms (9). Signaling of TLRs play a role at least in three physiologic phenomena including the proliferation of epithelial cells, secretion of antimicrobial factors and regulation of immune response which all together lead to maintenance of the epithelial barrier integrity (10).

TLRs have a primary role in the initiation of innate immune responses to the microbial components and subsequent activation of adaptive immunity (11).

In addition, activation of TLRs triggers different signaling pathways which lead to production of pro-inflammatory cytokines, expression of co-stimulatory molecules on antigen presenting cells and subsequently activation of T cells (12). Molecular patterns which are expressed on probiotics could also be recognized by TLRs on gastrointestinal lumen dendritic cells (2). Unlike pathogenic bacteria which initiate pro-inflammatory cascade after TLR triggering; probiotics hinder inflammatory responses by induction of gut homeostasis through regulating of nuclear factor-κB (NFκB) activation (13, 14). Although many studies have reported the beneficial effects of probiotics on the regulation of immune responses, still the precise mechanism is not clear. Herein, we investigated the expression of three kinds of TLRs in the gut section of chicken after oral administration of these useful bacteria.

Materials and Methods

Animal Housing

Thirty-one-day old ROSS chicken were randomly selected and kept in separate cages. Prior to housing, the cages, drinkers and feeders were cleaned. Drinking water and commercial powder feed without antibiotic or other additives were available ad libitum. During the experimental period, no adverse events were observed in the chickens. The experimental animals were handled under the regulation of Iran University of Medical Sciences animal care committee, based on Helsinki guidelines.

Experimental Design

Chickens were randomly assigned into six groups so that five chickens were placed in each group. In the control group a basal diet composed of commercial feed was used for feeding. The dairy group received 0.5 ml of sterilized milk every day from hatching time, along with the basal diet. In addition to basal diet, the probiotic group orally intake 10^9 CFU/Kg *Lactobacillus acidophilus* LA5 in 0.5 ml sterilized milk, daily.

Probiotic

Lactobacillus acidophilus strain LA-5 was chosen as a common probiotic in this study. Briefly, the L. acidophilus strain was subcultured and grown in fresh MRS broth (DeMann, Rogosa, and Sharpe medium) under anaerobic conditions at 37 °C for 48h. Each day, 100 µL of the bacterial suspension was added to 10 ml of fresh medium and incubated for another 16 h to reach to an optical density at 600nm which was equivalent to a bacterial concentration of approximately 109 CFU/ml. The probiotic were then washed three times with sterile 0.9% saline and was diluted in 0.5 ml of sterile milk to an expected concentration of 109cfu/Kg to be used for daily oral gavage of chickens. Actual colony-forming units which were administered for daily routine experiments were also determined retrospectively by spread-plating on MRS agar.

Tissue Collections

At days 14 and 21 of study, three animals from each group were euthanized by decapitation, the

abdominal cavity was opened and cecal tonsils (CT) from the midpoint of cecum were excised. Samples were washed with saline and immediately frozen in liquid nitrogen.

RNA Extraction and cDNA Synthesis

Chicken's cecal tonsils were chopped into small pieces and homogenized by vibration and total RNA was extracted by GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA). The quantity of RNA was determined by measurement of absorbance, and samples with A260/A280 ratio between 1.8 and 2.0 were chosen for further study. Approximately two micrograms of each total RNA sample was used for cDNA synthesis by RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions.

RT PCR

Initial setup of the PCR reaction was performed on ABI PCR machine (Applied Biosystems, Rotkreuz, Switzerland) in 20 µl volumes using 1µl of cDNA, 1 µl of each of the forward and reverse primers (Table-1) using EvaGreen qPCR Mix Plus as the master mix. The samples were denatured at 95°C for 3 min and amplified using 30 cycles of 94 °C for 30 sec, 58 °C for 30 sec, and 72 °C for 30 sec and the final elongation at 72 °C for 3 min. Five microliters of the PCR products were analyzed by electrophoresis on a 2% agarose gel. This step was only used for initial evaluation of the purity and singularity of the PCR product. However, the later steps were determined by real-time PCR method.

Real-time PCR

Gene-specific primers for target genes (TLR2, TLR4 and TLR5) were used for real-time-PCR reaction (Table1). The qPCR analysis was performed on an ABI StepOne™ real-time PCR machine (Applied Biosystems, Rotkreuz, Switzerland), using EvaGreen qPCR Mix Plus, with ROX. Similar amounts of reagents which were used in conventional RT-PCR method were also applied in this reaction. Amplification was achieved using the following cycle settings: 5 min at 95°C followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. The melting curve was analyzed to ensure the specificity of the amplification. The expression level of TLR genes in cecal tonsil was quantified as cycle threshold by deducting the cycle threshold values of β-actin as the reference gene to those of the samples. Finally, the mean of results was presented as percentage of control (15).

Table 1. Oligonucleotide sequences of primers used in real-time PCR

Gene of target		Primer sequences
β-Actin	Forward	5′-TGCTGTGTTCCCATCTATCG-3′
	Reverse	5′-TTGGTGACAATACCGTGTTCA-3′
TLR2	Forward	5′-AGGCACTTGAGATGGAGCAC-3′
	Reverse	5′-CCTGTTATGGGCCAGGTTTA-3′
TLR4	Forward	5′-AGTCTGAAATTGCTGAGCTCAAAT-3′
	Reverse	5′-GCGACGTTAAGCCATGGAAG-3′
TLR5	Forward	5′-TGCACATGTTTTCTCCTAGGT-3′
	Reverse	5′-CCACATCTGACTTCTGCCTTT-3′

Statistical Analysis

The expression level of TLR genes was normalized to beta-actin as the internal reference control. The significance of the difference among the six groups was analyzed by variance analysis (ANOVA), confirmed with Tukey posthoc using Graph Pad Prism 6.

Results

In order to examine immunomodulatory effects of probiotics, the expression of TLRs were evaluated in chicken after administration of *lactobacillus acidophilus* for 14 and 21 days.

At the age of 14-day, the level of mRNA encoding for TLR2 (118.9%), TLR4 (129.6%) and TLR5 (123.7%) tended to increase in cecal tonsil of

probiotic-fed chicken; but in comparison with dairy-fed and control group, these alterations were not significant.

Analyzing cecal tonsil of chickens at 21-d showed that the treatments did not affect the mRNA expression significantly at this period, too. A slight decrease in both dairy-fed (TLR2 85%, TLR4 79.5%, and TLR5 86.5%) and probiotic-fed (TLR2 88.8%, TLR4 81%, and TLR5 87.2%) groups were seen as compared with control. Comparison of results between two different age groups revealed that although there was no significant difference between them, the expression of TLRs were slightly downregulated at 21-day. The results were shown as relative percent of control in figure 1.

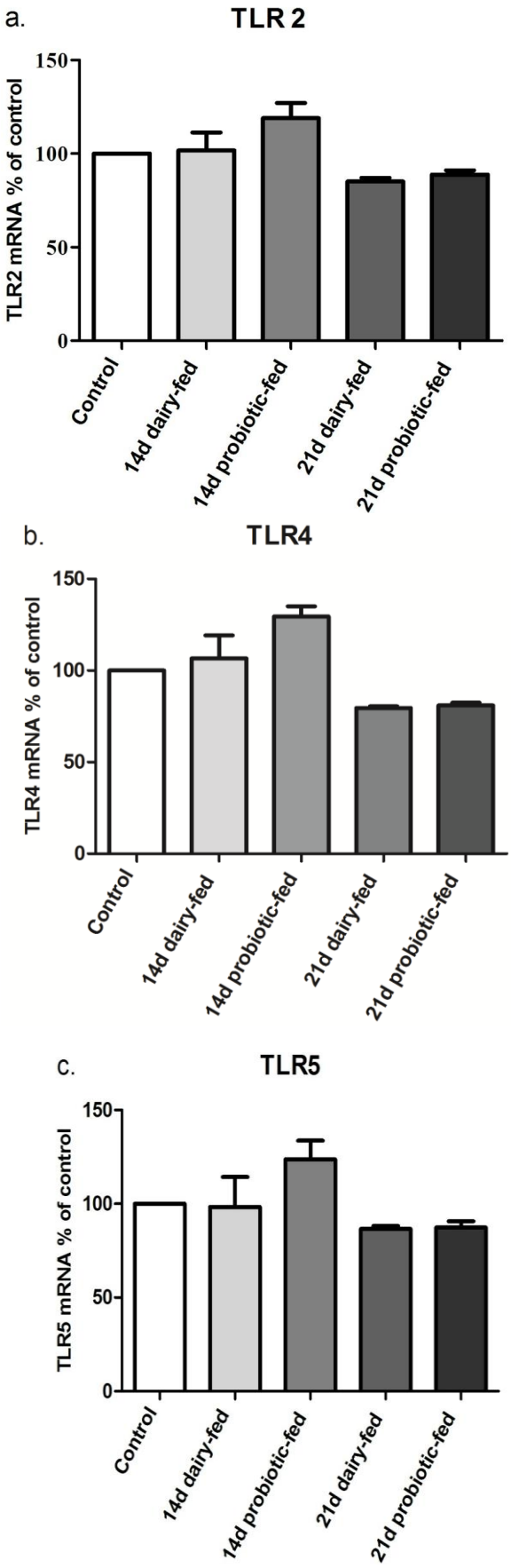

Fig. 1. The expression level of TLRs. One old day chickens separated into three groups of five each, as a probiotic-fed, dairy-fed and control group. After 14 and 21 days of the experiment, the cecal tonsil was removed and gene expression of a.) TLR2, b.) TLR4, and c.) TLR5 were evaluated by Real-time PCR and normalized to the levels of β-actin. Bar indicates the mean of three experiments. TLR= toll-like receptor.

Discussion

The gastrointestinal tract contains diverse commensal microbiome which play a crucial role in its functionality and integrity. It is well documented that intestinal microbiota intercede a symbiotic relationship with their host (16) (17) and could improve digestion, absorption, and storage of nutrients (18) and promote immune responses, as well (19). Probiotics are live non-pathogenic microorganisms that when administered in adequate amounts may confer health benefits to the host (4). They control the growth of intestinal pathogens through harnessing the antimicrobial mechanisms including competitive exclusion and production of a variety of biological products such as bacteriocins, organic acids, hydrogen peroxide and carbon dioxide (20).

Studies showed that probiotic can raise immune responses in chickens (21). In our previous study, we found that Lactobacillus acidophilus can influence the distribution of lymphocyte subpopulations(22) According to the immunomodulatory effect of Lactobacillus family on avian, in the present study, we decided to examine the effect of Lactobacillus acidophilus on the expression of TLR molecules which play a crucial role in innate immunity. Hence, Lactobacillus acidophilus was administered to the chickens at 14 and 21-days of age. We found that in comparison with control group, probiotic administration decreased the expression of TLR2, TLR4, and TLR5 in chicken's cecal tonsil at age 21; however, the differences were negligible. Although, it seems that probiotics may confer their tolerogenic effects through inhibition of the expression level of TLRs or their signaling pathways, in this study we did not find a significant difference between the expression level of TLRs in the studied groups.

Several species of LAB including *Lactobacillus*, *Enterococcus*, and *Bifidobacterium* may be colonized in the gut, and play a role as probiotic (23). After localization, they supply a source of ligands for TLRs which are highly expressed on intestinal epithelial dendritic cells (24), hence they may play a role in the control of inflammation in the gut tissue.

TLRs are a highly conserved family of pattern recognition receptors and capable of binding to the pathogen-associated molecular patterns (PAMPs) such as peptidoglycans, lipoproteins, lipopolysaccharides, and unmethylated bacterial CpG DNA (25). Studies have proven the presence of 10 types of TLRs in avian tissues; among them, TLR2, TLR4, TLR5, and TLR7 are orthologues with those of mice and human (26, 27).

The cecal tonsil is a gut-associated lymphoid tissue of the chicken (28) in which *Lactobacillus* comprise approximately 50% of the whole bacterial population at 25-d of age (29). Previous studies showed that *Lactobacillus*-based probiotics could reduce the level of pro-inflammatory cytokines in the intestine of *S. Enteritidis*-infected chickens and increase the expression of TLR2 in their cecal tonsils (30). In this study, we showed slight up regulation of TLRs at 14-d, while those expressions were slightly decreased at the 21-d chicken.

Castillo showed that administration of *L. Casei* to BALB/c mice can modulate the inflammatory response to pathogenic bacteria such as *S. Typhimurium* via affecting TLR expression. They reported that the continuous administration of probiotics could upregulate the expression of TLR2, TLR4, and TLR9 (31).

Bermudez-brito reported that administration of *Lactobacillus paracasei* and its cell-free culture supernatant may alter innate immunity responses through diminishing the secretion of pro-inflammatory cytokines in *Salmonella*-challenged DCs. They suggested that the possible mechanism of this decrement might be the modulation of TLR activation (32). Interaction of microbiota with TLR9 may inhibit the nuclear internalization of NF-κB and subsequently, prevent the production of pro-inflammatory cytokines in gut. As mentioned, in this study we showed a non-significant decrease of TLR expression. In addition, Aumeunier et al. found that a probiotic mixture containing *bifidobacterium*, *lactobacillus* and *streptococcus* can suppress both allergic and autoimmune responses in NOD mice through TLRs stimulation (33). Interestingly, the activation of TLRs on DCs could induce the secretion of TGF-β which is a crucial cytokine for deviation and development of the regulatory T cells (32). Therefore, understanding of the possible mechanisms which underlies the anti-inflammatory properties of microbiota needs more investigations (28, 34, 35).

Although there are some contradictory reports with our results, we propose that the mechanism of immunomodulatory properties of probiotics could differ according to type and species of the probiotic, as well as the age and tissue type of the host. Taken together, our results along with other studies showed that *Lactobacillus acidophilus* may influence the innate immune system of the mucosal system by immunomodulation of TLR expression.

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