Immunomodulation in gut-associated lymphoid tissue of neonatal chicks by immunobiotic diets

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ABSTRACT Developmental changes in immunocompetent cells of the gut during the first week posthatch were determined in broiler chicks fed immunobiotic lactic acid bacteria in the form of *Lactobacillus jensenii* TL2937-, *Lactobacillus gasseri* JCM1131^{T}-, *Lactobacillus delbrueckii* ssp. *bulgaricus* NIAIB6-, or *L. gasseri* TL2919-supplemented diets. The relative weights of spleen and bursa of Fabricius in chicks fed the immunobiotic diets were slightly higher than the control valued at 1 and 3 d of age, with the exception of spleen weight in the *L. gasseri* JCM1131^{T} at 3 d of age, the bursa of Fabricius weight in the *L. gasseri* JCM1131^{T} at 1 and 3 d of age, and bursa of Fabricius weight in the *L. gasseri* TL2919 group at 1 d of age. There were no significant differences in body and liver weights among the treatments. When chicks were fed the *L. jensenii* TL2937- or *L. gasseri* TL2919-supplemented diets, expression of T cell-related mRNA [cluster of differentiation 3 (CD3), interleukin-2 (IL-2), and interferon-γ (IFN-γ)] in the foregut was significantly higher than that of control chicks at 3 or 7 d of age. Expression levels of toll-like receptor (TLR) mRNA tended to increase in the foregut of chicks fed the immunobiotic diets, except for the *L. delbrueckii* ssp. *bulgaricus* NIAIB6, compared with expression levels in control chicks. The Bu-1 mRNA expression levels in the bursa of Fabricius were not affected by the supplementations with immunobiotic lactic acid bacteria. These results show that immunobiotics, particularly *L. gasseri* TL2919, might be useful as immunomodulators to stimulate the gut-associated immune system in neonatal chicks, and thereby protect them from disease without decreasing growth performance as a possible substitution of antibiotics.

Key words: immunobiotic, lactic acid bacteria, gut immune cell, immunomodulation, newly hatched chick

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INTRODUCTION

A recent trend in the meat production industry is to decrease or stop the use of antibiotics, which are used to prevent disease and thereby promote growth in poultry (Ferket, 2004). When used for such purposes, antibiotics increase growth performance and edible meat yield in broiler chickens. To this extent, efficient procedures for disease prevention in the absence of antibiotic use must be found to maintain poultry meat production levels. Control of the immune system is an alternative way to avoid the use of antibiotics because immunomodulators to enhance humoral immunity in chickens could provide protection from diseases with decreasing growth performance (Klasing, 1998).

The development and immunological function of chick gut-associated lymphoid tissue (**GALT**) is critical for survival immediately after hatch because GALT is exposed to adult-type microflora from concomitant foraging and the environment (Bar-Shira et al., 2003). Our previous study on age-related changes in GALT of chickens indicated that GALT contains functionally immature T and B lymphocytes at hatch and that the function of these cells is attained during the first 2 wk of life (Miyazaki et al., 2007). As such, the rapid maturation of innate immunity in neonatal chicks is important to provide protection from disease.

There are many reports in the literature showing that dietary supplementation with probiotic bacteria, particularly lactobacilli, improves broiler BW gain (Nurmi and Rantala, 1973) and feed conversion rate (Jin et al., 1998). In addition, *Lactobacillus*-based probiotic culture (FM-B11) is efficacious for reducing the incidence of *Salmonella* Enteritidis in neonatal chicks (Higgins et al., 2008). These results indicate that effective probiotic

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products may provide a viable alternative to antibiotic use in broiler production. However, there is no information available on the effect of probiotic lactic acid bacteria on the development of immunological function in the chick GALT in the immediate posthatch period. We postulated that probiotic bacteria might enhance development of the immunological function of GALT in newly hatched chicks, thereby providing protection against bacterial attack and subsequent negative effects on growth. Within probiotics, Clancy proposed the concept of immunobiotics with reference to microorganisms that stimulate activation of mucosal immunity in the GALT (Clancy, 2003). On this basis, we have 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 (Tohno et al., 2006, 2007).

In the present study, we have investigated whether the in vitro-selected immunobiotic lactic acid bacteria enhance the expression of TLR mRNA and T lymphocyte function [cluster of differentiation 3 (**CD3**), interleukin-2 (**IL-2**), and interferon-γ (**IFN-γ**)] and improve the development of GALT immune function in posthatch chicks.

MATERIALS AND METHODS

Preparation of Immunobiotic Lactic Acid Bacteria

Four selected strains of lactobacilli were used (Tohno et al., 2006, 2007). *Lactobacillus delbrueckii* ssp. *bulgaricus* NIAIB6 was obtained from the National Institute of Animal Industry (Tsukuba, Japan). *Lactobacillus gasseri* JCM1131^T was purchased from the Japan Collection of Microorganisms (JCM, Saitama, Japan). *Lactobacillus jensenii* TL2937 and *L. gasseri* TL2919 isolated from human feces were from the library of Meiji Dairies Co. (Odawara, Kanagawa, Japan). All strains were cultured at 37°C for 16 h in de Man, Rogosa, Sharpe broth (Difco, Detroit, MI), harvested by centrifugation at 6,000 × *g* for 5 min at 4°C, and washed by sterile saline solution. After washing, cells were freeze-dried for 3 d.

Birds, Diets, and Tissue Sampling

Two hundred fifty fertile eggs of the Cobb broiler strain were incubated at 37°C and 65% RH. Eighty male hatched chicks were obtained and 75 chicks were allocated to 1 of 5 groups (15 chicks/group). Birds were kept in electrically heated cages at 34°C and the temperature was gradually lowered to 27°C at 7 d of age. The chicks were provided a corn-soybean meal-based diet (control, Murakami et al., 1995) or a similar diet supplemented with 1 of 4 strains of immunobiotic lactic acid bacteria, *L. jensenii* TL2937, *L. gasseri* JCM1131^T, *L. delbrueckii* ssp. *bulgaricus* NIAIB6, or *L. gasseri* TL2919, to achieve the desired concentration of 10^9 cfu/kg of food. Diet and water were provided freely. At posthatch (i.e., within 4 h of hatching), 1, 3, and 7 d of age, 5 chicks in each dietary group were killed by decapitation. Gut and bursa of Fabricius were removed and flushed with a cold phosphate buffer containing 154 m*M* NaCl, 3 m*M* KCl, 12 m*M* Na_2HPO_4, and 2 m*M* KH_2PO_4 (pH 7.4, 0°C). Foregut (about 1 g, from the end of the duodenum to the middle section of the jejunum; Miyazaki et al., 2007) and bursa of Fabricius were frozen in liquid nitrogen and stored at −80°C until analysis. The Animal Care and Use Committee of the Graduate School of Agriculture of Tohoku University approved all procedures.

Quantitation of mRNA Using Real-Time PCR

Total RNA was extracted from chick gut and bursa of Fabricius using Trizol reagent (15596-018, Invitrogen, Carlsbad, CA). To study the expression of particular chick immune genes, real-time reverse transcription-PCR analysis was performed using the iCycler Real Time Detection System (Bio-Rad Laboratories, Hercules, CA). The reverse transcription, amplification, and detection methods used were as described previously (Miyazaki et al., 2007; Sato et al., 2008). Primer sequences are shown in Table 1. At the end of each run, melting curve profiles were recorded. Analysis of the standard curve from each product allowed calculation of the mRNA levels of the respective genes. Results are presented as the ratio of each gene to 18S ribosomal RNA, to correct for differences in the amounts of template DNA used.

Statistical Analysis

The SAS applications software package was used for statistical calculations (SAS Institute Inc., Cary, NC). Group data for multiple comparisons were analyzed by ANOVA using a GLM procedure followed by Tukey's test. For analysis of the age × bacteria species interaction, the data of body and organ weights and gene expression levels were analyzed using a 3 (ages) × 5 (diets) ANOVA. Results are expressed as mean ± SD of the data from 5 chicks from each age and diet group. Statistical significance was interpreted as values of $P < 0.05$.

RESULTS

Body and Organ Weight of Chicks Fed Immunobiotic Lactic Acid Bacteria-Supplemented Diets

Body and liver weights did not differ significantly in response to immunobiotic lactic acid bacteria supplementation in diets, although the BW of chicks fed *L. gasseri* JCM1131^T-, *L. delbrueckii* ssp. *bulgaricus* NI-

Table 1. Oligonucleotide sequences of sense and antisense primers for real-time PCR products determined

Gene[1]		Primer sequences	Accession number	Product size (bp)
18S rRNA	Sense	5′-TAGATAACCTCGAGCCGATCGCA-3′	AF173612	312
	Antisense	5′-GACTTGCCCTCCAATGGATCCTC-3′		
CD3	Sense	5′-CAGGGATTGTGGTCGCAGAT-3′	AJ250458	164
	Antisense	5′-TACTGTCCATCATTCCGCTCAC-3′		
IL-2	Sense	5′-ACTGCCATGATGTGCAAAGTACTGATCT-3′	AF017645	428
	Antisense	5′-ATTTTTGGCCAAGATATCTCACAAAGTTGGT-3′		
IFN-γ	Sense	5′-ACTGAGCCAGATTGTTTCGATGT-3′	X99774	288
	Antisense	5′-TGCCATTAGCAATTGCATCTCCT-3′		
TLR2	Sense	5′-CATTCACCATGAGGCAGGGATAG-3′	AB046533	157
	Antisense	5′-GGTGCAGATCAAGGACACTAGGA-3′		
TLR4	Sense	5′-TTCAGAACGGACTCTTGAGTGG-3′	AY064697	131
	Antisense	5′-CAACCGAATAGTGGTGACGTTG-3′		
TLR7	Sense	5′-TTGCTGCTGTTGTCTTGAGTGAG-3′	AJ627563	182
	Antisense	5′-AACAACAGTGCATTTGACGTCCT-3′		
Bu-1	Sense	5′-GGCTGTTGTGTCCTCACTCATCT-3′	X92865	106
	Antisense	5′-CACCACCGACATTGTTATTCCAT-3′		

[1]CD3 = cluster of differentiation 3; IL-2 = interleukin-2; IFN-γ = interferon-γ; TLR2 = toll-like receptor 2; TLR4 = toll-like receptor 4; TLR7 = toll-like receptor 7.

AIB6-, and *L. gasseri* TL2919-supplemented diets was slightly lower than control chicks at 7 d of age (Table 2). Although there was no significant differences between the lactic acid bacteria-fed birds on the weight of spleen and bursa of Fabricius compared with the control group, in general, the relative weights of those organs were slightly higher than the control valued at 1 and 3 d of age, with the exception of spleen weight in the *L. gasseri* JCM1131^T at 1 and 3 d of age and bursa of Fabricius weight in the *L. gasseri* TL2919 group at 1 d of age. There were no differences among the treatments for chicks at 7 d of age (Table 3).

Changes in the T Cell-Related and TLR mRNA Expression in the Foregut of Chicks Fed Immunobiotic Lactic Acid Bacteria-Supplemented Diets

Figure 1 shows developmental changes in T cell-related mRNA expression in the foregut sections of male broiler chicks fed an immunobiotic lactic acid bacteria-supplemented diet after hatching. The expression of CD3, IL-2, and IFN-γ mRNA tended to increase during d 3 to 7 of age in control chicks. The level of CD3 mRNA in the foregut of chicks fed a *L. jensenii* TL2937-supplemented diet was found to be significantly higher than that in response to other treatments for chicks at 3 d of age, although this difference was not found in chicks at other ages. The levels of IL-2 and IFN-γ in the foregut of chicks fed lactic acid bacteria-supplemented diets tended to be higher than those of control chicks at d 1, 3, and 7 of age. The levels of the mRNA for IL-2 in chicks fed a *L. gasseri* TL2919-supplemented diet were significantly higher than those of control chicks at 3 and 7 d of age and were significantly higher in the *L. jensenii* TL2937 at 3 d of age compared with the control. The levels of IFN-γ were significantly higher than the controls in the *L. jensenii* TL2937-fed birds at 3 d of age, and in the *L. gasseri* TL2019-fed birds at 7 d of age. However, the IL-2 and IFN-γ mRNA levels were not changed by the supplementation with *L. delbrueckii* ssp. *bulgaricus* NIAIB6 compared with the levels in chicks fed the control diet.

Changes in the mRNA expression of TLR in the foregut are shown in Figure 2. The TLR2/18S mRNA was significantly increased at 1 and 7 d of age in the *L. gasseri* TL2919, and at 3 d of age in the *L. jensenii* TL2937-fed birds compared with the control group. The TLR4/18S mRNA was significantly increased at 3 and 7 d of age, and at 3 d of age in the *L. gasseri* TL2919 and *L. jensenii* TL2937, respectively, compared with the control group. The TLR7/18S mRNA was significantly increased in *L. jensenii* TL2937- and *L. gasseri* JCM1131^T-fed birds at 3 d of age, and the *L. gasseri* TL2919-fed group at 7 d of age compared to the control group.

Changes in B Cell-Related Gene Expression in the Bursa of Fabricius of Chicks Fed an Immunobiotic Lactic Acid Bacteria-Supplemented Diet

Changes in Bu-1, a B cell marker, mRNA expression levels in the bursa of Fabricius are shown in Figure 3. The Bu-1 mRNA expression levels tended to be modestly higher at 3 d of age in chicks fed immunobiotic lactic acid bacteria-supplemented diets, but these increases were not significantly different from chicks fed a control diet.

DISCUSSION

There are many reports that lactobacilli have beneficial effects, such as augmentation of mitogenic activity (Kitazawa et al., 1992; Takeda et al., 1997; Kirjavainen et al., 1999), anti-infectious effects (Perdigon et al., 1986), and induction of cytokine production (Kitazawa et al., 1994; Hessle et al., 1999) in mammals. These

Table 2. Body and liver weights of broiler chickens fed immunobiotic lactic acid bacteria-supplemented diets (n = 5)

	BW (g)				Liver (g/100 g of BW)			
Treatment	0 d	1 d	3 d	7 d	0 d	1 d	3 d	7 d
Control	41.4 ± 3.8	58.8 ± 2.9	85.1 ± 9.1	188.1 ± 16.8	2.762 ± 0.046	3.457 ± 0.285	3.562 ± 0.465	3.847 ± 0.526
Lactobacillus jensenii TL2937	—	56.6 ± 2.7	79.0 ± 7.4	187.0 ± 12.4	—	3.316 ± 0.283	3.848 ± 0.486	3.976 ± 0.816
Lactobacillus gasseri JCM1131^{T}	—	59.8 ± 4.5	81.8 ± 3.5	178.8 ± 4.8	—	3.205 ± 0.379	4.179 ± 0.342	3.118 ± 0.244
Lactobacillus delbrueckii ssp. *bulgaricus* NIAIB6	—	57.6 ± 6.1	76.9 ± 5.9	170.2 ± 8.6	—	3.252 ± 0.389	4.002 ± 0.527	3.595 ± 0.125
L. gasseri TL2919	—	56.0 ± 3.7	75.8 ± 16.0	172.2 ± 11.0	—	3.727 ± 0.420	3.687 ± 0.790	3.408 ± 0.720
Probability								
Age			$P < 0.01$				$P < 0.01$	
Supplementation			NS				NS	
Age × supplementation			NS				NS	

Table 3. Spleen and bursa of Fabricius weights of broiler chickens fed immunobiotic lactic acid bacteria-supplemented diets (n = 5)

	Spleen (g/100 g of BW)				Bursa of Fabricius (g/100 g of BW)			
Treatment	0 d	1 d	3 d	7 d	0 d	1 d	3 d	7 d
Control	0.038 ± 0.006	0.046 ± 0.005	0.076 ± 0.013^{ab}	0.098 ± 0.010	0.105 ± 0.017	0.142 ± 0.035^{ab}	0.161 ± 0.020^{b}	0.199 ± 0.063
Lactobacillus jensenii TL2937	—	0.049 ± 0.015	0.088 ± 0.016^{a}	0.101 ± 0.020	—	0.152 ± 0.021^{ab}	0.186 ± 0.041^{ab}	0.198 ± 0.064
Lactobacillus gasseri JCM1131^{T}	—	0.056 ± 0.015	0.054 ± 0.008^{b}	0.109 ± 0.020	—	0.112 ± 0.033^{b}	0.149 ± 0.029^{b}	0.211 ± 0.029
Lactobacillus delbrueckii ssp. *bulgaricus* NIAIB6	—	0.060 ± 0.013	0.089 ± 0.019^{a}	0.093 ± 0.009	—	0.185 ± 0.025^{a}	0.238 ± 0.017^{a}	0.191 ± 0.021
L. gasseri TL2919	—	0.054 ± 0.014	0.085 ± 0.016^{ab}	0.100 ± 0.040	—	0.139 ± 0.021^{ab}	0.165 ± 0.032^{b}	0.209 ± 0.044
Probability								
Age			$P < 0.01$				$P < 0.01$	
Supplementation			$P < 0.05$				$P < 0.01$	
Age × supplementation			NS				NS	

[a,b]Mean ± SD with different superscripts within a column are significantly different ($P < 0.05$).

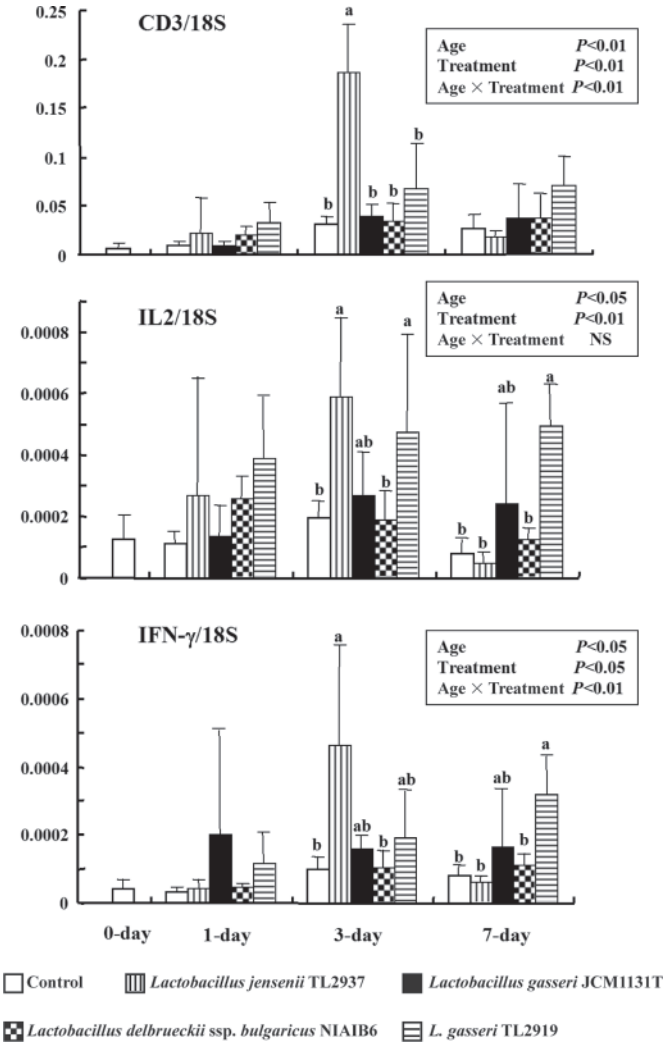

Figure 1. Developmental changes in T cell-related mRNA [cluster of differentiation 3 (CD3), interleukin-2 (IL-2), and cluster of differentiation 30 (IFN-γ)] expression in the gut of male broiler chicks fed an immunobiotic lactic acid bacteria-supplemented diet after hatch. The expression of each gene was determined by real-time reverse transcription-PCR (iCycler iQ Real Time Detection System, Bio-Rad Laboratories, Hercules, CA) as described in the Materials and Methods and was expressed as a ratio to 18S levels. Bars indicate the SD of the mean (n = 5). The statistical letters serve only for comparisons within a time period (e.g., 1, 3, or 7 d) for each specific gene. Different letters indicate significant differences ($P < 0.05$).

effects are mediated via TLR, which recognize various kinds of pathogen-associated molecular patterns (i.e., peptidoglycan, lipoproteins, double-stranded viral RNA, lipopolysaccharide, and unmethylated bacterial CpG DNA (Hawlisch and Köhl, 2006). As such, TLR play a crucial role in activating the immune system in the gut, especially via the MyD88-dependent TLR/IL-1R signaling pathway (Takeuchi et al., 2000). In neonatal chicks, the functions of T and B lymphocytes in GALT are not developed, but are obtained during the first 2 wk of life (Miyazaki et al., 2007). It is, therefore, likely that the control and development of the immune system during this stage in chicks might be susceptible to protection from disease by the use of products that do not decrease growth performance. In this manner, in the present study, we investigated whether in vitro-selected immunobiotic lactic acid bacteria (Tohno et al., 2006, 2007) could improve the developmental alterations of GALT immune function in posthatch chicks. Here, we provide evidence that the supplementation of neonatal chick diets with selected lactic acid bacteria, especially *L. jensenii* TL2937 and *L. gasseri* TL2919, enhances the expression of TLR and T cell-related mRNA expression levels in the gut (Figures 1 and 2). In contrast, BW and Bu-1 mRNA expression levels of chicks fed immunobiotic lactic acid bacteria were not different compared with those of control chicks (Table 2 and Figure 3). These results show that selected immunobiotic lactic acid bacteria stimulate the T cell immune system via TLR in the gut, but do not affect BW or the B cell-related immune system. As such, in the present study, immunobiotic lactic acid bacteria (i.e., *L. jensenii* TL2937 and *L. gasseri* TL2919 in particular) are appropriate immunomodulators to stimulate the gut-associated immune system in chicks. These selected immunobiotic lactic acid bacteria might therefore be useful supplements for improving the immune system without decreasing growth performance in neonatal chicks.

The dietary supplementation of probiotic bacteria improves broiler production, such as BW gain and feed conversion rate (Nurmi and Rantala, 1973; Jin et al., 1998). Our results revealed, however, that the BW of chicks fed immunobiotic lactic acid bacteria-supplemented diets were unaltered compared with those of control chicks (Table 2). In addition, the feed efficiency of chicks fed immunobiotic lactic acid bacteria-supplemented diets was unaltered compared with those of control chicks (average 0.86 ± 0.09, data not shown). It is not clear, however, whether these differences can be accounted for by the *Lactobacillus* strain or the specific response of neonatal chicks. Further experiments involving dietary supplementation with these selected lactobacilli during the growth stage of broiler chickens may help to elucidate the effects of lactobacilli supplementation in relation to BW alterations and feed efficiency.

In this study, the relative weights of spleen and bursa of Fabricius in chicks fed the immunobiotic diets were slightly higher than the control valued at 1 and 3 d of age, except for spleen weight in the *L. gasseri* JCM1131^T at 3 d of age, the bursa of Fabricius weight in the *L. gasseri* JCM1131^T at 1 and 3 d of age, and bursa of Fabricius weight in the *L. gasseri* TL2919 group at 1 d of age (Table 3). In a similar manner, the T cell-related mRNA and TLR mRNA expression levels in the foregut of chicks fed *L. jensenii* TL2937- or *L. gasseri* TL2919-supplemented diets tended to be increased in 3-d-old chicks (Figures 1 and 2). These results suggest that the probiotic bacteria used in the present study might be most effective in the first 3 d after feeding of hatch. However, the mRNA expression levels in the gut of chicks fed diets supplemented with *L. jensenii* TL2937, *L. gasseri* JCM1131^T, or *L. delbrueckii* ssp. *bulgaricus*

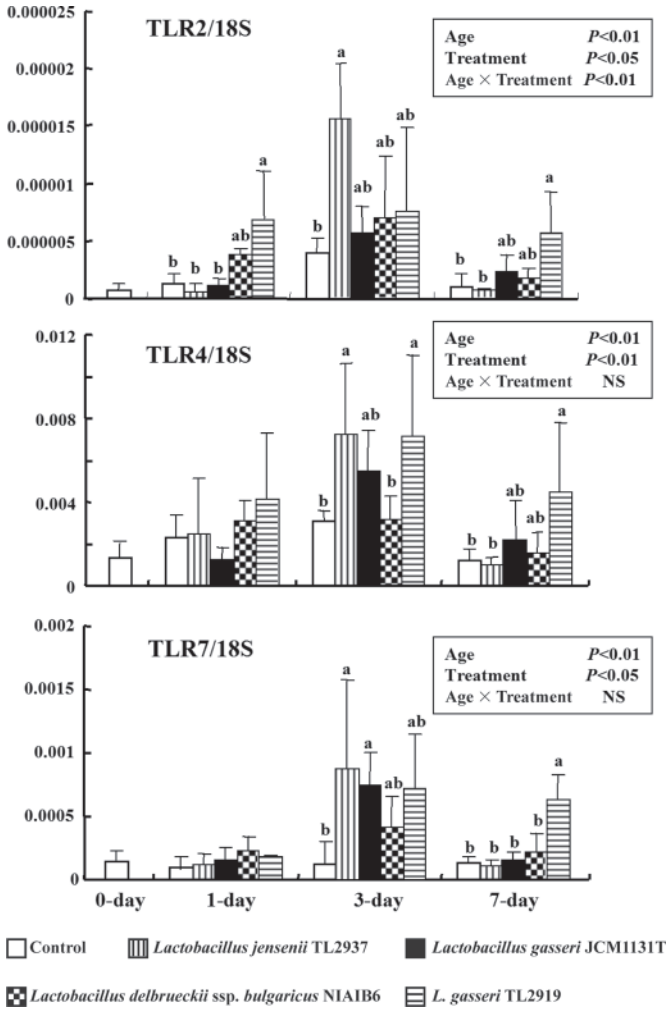

Figure 2. Developmental changes in toll-like receptor (TLR) mRNA (TLR2, TLR4, and TLR7) expression in the gut of male broiler chicks fed an immunobiotic lactic acid bacteria-supplemented diet after hatch. The expression of each gene was determined by real-time reverse transcription-PCR (iCycler iQ Real Time Detection System, Bio-Rad Laboratories, Hercules, CA) as described in the Materials and Methods and was expressed as a ratio to 18S levels. Bars indicate the SD of the mean (n = 5). The statistical letters serve only for comparisons within a time period (e.g., 1, 3, or 7 d) for each specific gene. Different letters indicate significant differences ($P < 0.05$).

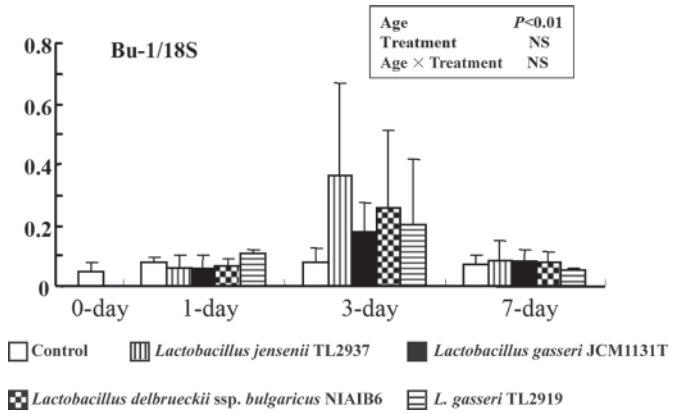

Figure 3. Developmental changes in Bu-1 mRNA expression in the bursa of Fabricius of male broiler chicks fed an immunobiotic lactic acid bacteria-supplemented diet after hatch. The expression of each gene was determined by real-time reverse transcription-PCR (iCycler iQ Real Time Detection System, Bio-Rad Laboratories, Hercules, CA) as described in the Materials and Methods and was expressed as a ratio to 18S levels. Bars indicate the SD of the mean (n = 5).

NIAIB6 were similar to those of control chicks at 7 d of age. In contrast, the mRNA expression levels in the gut of chicks fed a *L. gasseri* TL2919-supplemented diet were higher than those of control chicks, even at 7 d of age. It is, therefore, likely that *L. gasseri* TL2919 is the bacteria that induces the best immune response in the neonatal chick gut. In in vitro experiments to select immunobiotic lactic acid bacteria, nuclear factor-κB reporter activities (which are indicative of the activation of immune signaling via TLR), of *L. jensenii* TL2937 were approximately 2 times higher than those of *L. gasseri* TL2919 (Tohno et al., 2007). In those experiments, swine TLR2-transfected cells were used as a model for the selection of lactobacilli to potentially enhance the immune system in GALT. The present study, however, provides the first report that in vitro-selected lactobacilli, particularly *L. gasseri* TL2919, stimulate the T cell-related immune system; may be activated via TLR signaling, in the gut of neonatal chicks; and that our *L. gasseri* TL2919 is suitable for activating the underdeveloped GALT in chicks posthatch, suggesting that it may improve the bacterial colonization.

In conclusion, the in vitro-selected lactobacilli used in this study, particularly *L. gasseri* TL2919, enhanced development of the gut immune system in neonatal chicks, suggesting that they could be useful as immunomodulators to enhance the gut-associated immune system and therefore might protect the chicks from disease without decreasing growth performance as one of the possible substitutions for antibiotics.

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