Effects of a Bacteria-Based Probiotic on Subpopulations of Peripheral Leukocytes and Their Cytokine mRNA Expression in Calves

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ABSTRACT.   Eight Holstein calves (10 ± 3 weeks) were used to examine the interaction between a bacteria-based probiotic agent (probiotic) and the function of peripheral blood mononuclear cells (PBMCs). The probiotic, consisting of *Lactobacillus plantarum*, *Enterococcus faecium* and *Clostridium butyricum*, was administered orally at 3.0 g/100 kg body weight to calves once daily for 5 consecutive days. Calves given the vehicle alone with no probiotic served as the control. In the treatment group, increases in numbers of $CD282^+$ (TLR2) monocytes, $CD3^+$ T cells and $CD4^+$, $CD8^+$ and $WC1^+$ γδ T cell subsets were noted on day 7 post-placement compared to predose day and the control group. Expression of interleukin (IL)-6, interferon-gamma (INF-γ) and tumor necrosis factor-alpha (TNF-α) was elevated in peripheral leukocytes on days 7 and 14. These results suggest that peripheral blood leukocytes in healthy calves may be stimulated via the gastrointestinal microbiota, which was increased by the oral probiotic treatment, with overall stability of the rumen bacterial flora. The 5-day repeated administration of a bacteria-based probiotic may enhance cellular immune function in weaned calves.
KEY WORDS: calf, cytokine, leukocyte, probiotic, toll-like receptor 2.

Enhancement of the immune system is an important trait following administration of bacteria-based probiotics in human and animals. Certain multi-strain and multi-species probiotics have been reported to have immune- stimulating effects in cattle [6, 14, 15], and beneficial effects of probiotics have been recognized in improving animal health and protecting calves against infection [29, 30]. *Lactobacillus* strains, alone or in combination with other probiotics, may reduce scouring in neonatal calves, increase weight gain and maintain health [13, 14]. However, the interaction between probiotics and the function of host peripheral blood mononuclear cells (PBMCs) is not fully understood.

It has been hypothesized that bacteria-based probiotics might impact the host immune system in a number of ways, such as upregulation of cell-mediated immunity, increased antibody production and epithelial barrier integrity, enhanced dendritic cell-T cell interactions, heightened T cell association and increased Toll-like receptor (TLR) signaling [14, 17, 35]. One valuable effect of probiotics is that they contribute to homeostasis of bacterial flora in the gastrointestinal tract, especially in calves [2]. Thus, the immune-modulating effect of probiotics in ruminants is likely more appropriate at a young age, when the intestinal bacterial inhabitants are less well established. Phagocytic activities of leukocytes have been reported to be increased in peripheral blood of calves following administration of a probiotic consisting of *Lactobacillus plantarum* [14]. Increases in acute-phase proteins, such as serum amyloid A and plasma lipopolysaccharide-binding protein (LBP), have been recognized in feedlot steers receiving a probiotic containing *Enterococcus faecium* and *Saccharomyces cerevisiae* [6]. Furthermore, LBP binds to bacterial lipopolysaccharide (LPS) to elicit immune responses by presenting the LPS to the important cell-surface proteins CD14 and TLR2 [22]. Recently, it was also reported that T cell subsets in peripheral blood of scouring calves were stimulated by a bacteria-based probiotic [24]. Likewise, a probiotic consisting of lactic acid bacteria (LAB) has been shown to enhance non-specific immunity in infants, including T cell responses and increased levels of interferon-gamma (IFN-γ) and interleukin (IL)-4 [17, 35].

The effects of probiotics on ruminal components and possible interactions with resident bacterial populations in cattle have been reported [2, 4, 34]. Furthermore, variation in bacterial communities and subsequently immune stimulatory effects, such as the expression of TLR, is observed in steers [4]. The immune-modulating effects of some probiotics have been evaluated during intestinal disorders in newborn calves [24]. However, few studies have described the effects of probiotics on the peripheral leukocytes of healthy weaned calves. In the present study, lymphocyte subpopulations, monocytes and cytokine mRNA expression in peripheral leukocytes were investigated using healthy weaned calves to confirm the effects of a bacteria-based probiotic on the

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immune system.

MATERIALS AND METHODS

Animals and treatment: The experimental design was approved by the Laboratory Animal Care and Use Committee of Iwate University, Iwate, Japan. Eight clinically healthy Holstein bull calves, aged 10 ± 3 weeks, were purchased from a government farm (National Livestock Breeding Center, Morioka, Japan) and were housed in a 4×5 m^2, open-sided straw bed and naturally ventilated barn at the Cattle Research Center, Iwate University. The calves had been weaned within 4 weeks after birth and fed starter pellets containing ground corn (standard diet) and had access to fresh water *ad libitum*.

Calves were assigned to a 4×2 experimental design for the probiotic and control groups. A probiotic (Miyarisan Pharmaceutical Co., Ltd., Tokyo, Japan), which included *Lactobacillus plantarum* strain 220 [9×10^6 colony forming units (CFU)/g], *Enterococcus faecium* strain 26 (9×10^5 CFU/g) and *Clostridium butyricum* strain Miyari (9×10^4 CFU/g), was administered daily at 3.0 g/100 kg body weight (BW) to the calves once daily for 5 consecutive days. The probiotic was mixed with 50 m*l* tap water in a beaker, and the probiotic suspension was administered orally to each calf in a 50 m*l* drencher in the morning, prior to feeding. Calves given tap water alone, with no probiotic, served as the control. Each calf was examined clinically for normal health and growth. No abnormal changes in the health of any calf were observed during the experimental period.

Blood collection and PBMC isolation: Blood was drawn by jugular venipuncture from each calf at predose and on days 7 and 14. The first administration day was considered day 1. A blood specimen (6 m*l*) was collected in a heparinized tube (BD Vacutainer, Belliver Industrial Estate, Plymouth, U.K.) and used for PBMC isolation for flow cytometry analysis. Blood (4 m*l*) was also collected in two EDTA tubes (BD Vacutainer, Franklin Lakes, NJ, U.S.A.) and used for total RNA extraction from peripheral leukocytes and white blood cell (WBC) counts. WBC count was determined using an automatic hematology analyzer device equipped with software for bovine samples (pocH-100iV Diff, Sysmex, Kobe, Japan). The percentage of PBMCs was determined using a hemogram method with the Dif-Quick staining protocol. PBMCs were isolated as described previously [26]. Briefly, to purify PBMCs and to lyse red blood cells, 2 m*l* heparinized blood was mixed with 8 m*l* 0.83% ammonium chloride solution. After 5 min at room temperature, samples were centrifuged (2,000 rpm, 10 min, 4°C). Leukocytes were purified by washing twice in phosphate-buffered saline (PBS, pH 7.4) followed by centrifugation (2,000 rpm, 5 min, 4°C). After the final wash, the cell pellet was diluted in 500 μl PBS, and cells were counted. Then, the cell concentration was adjusted to 1×10^7/m*l* in PBS, and the cells were kept at 4°C until used for the flow cytometry assay.

Flow cytometry assay: The primary monoclonal antibodies used in the present study are shown in Table 1. Flow cytometry analysis was performed as described previously [25]. Briefly, PBMCs were labeled with primary antibody and incubated for 60 min at 4°C. CD3$^+$ T cells and each of CD4$^+$ and CD8$^+$ T cell subsets were additionally labeled with CD45R monoclonal antibody to mark pan-leukocytes. After incubation, cells were washed twice to remove unbound antibodies. Labeled cells were stained with specific purified IgG conjugated to phycoerythrin (PE) polyclonal secondary antibody (goat anti-mouse IgG$_1$ RPE, AbD Serotec, Kidlington, Oxford, U.K.) or fluorescein isothiocyanate (FITC)-conjugated antibody (goat anti-mouse IgG or IgM FITC, Southern Biotech, Birmingham, AL, U.S.A.) and incubated for 30 min at 4°C. After washing twice, stained cells were diluted in 500 μl PBS. Flow cytometry was performed using a FACScan analyzer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), equipped with a computer running the Cell Quest software (Becton Dickinson, Milan, Italy).

Total RNA extraction from leukocytes and cDNA synthesis: Total RNA was extracted from 1 m*l* whole blood leukocytes using the SV Total RNA Isolation System (Promega, Tokyo, Japan). Trace genomic DNA in the crude total RNA samples was removed by incubation with 4–6 units DNase I per 100 mg total RNA (Ambion, Austin, TX, U.S.A.) for 30 min at 37°C. The concentration of total RNA was verified on a 1% agarose gel, and purity was determined using a Nano-Drop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, U.S.A.). cDNA was prepared using an oligo(dT) primer and a thermal cycler (MyCycler; Bio-Rad, Tokyo, Japan). cDNA synthesis was performed using the Im-Prom Reverse Transcription System (Promega). cDNA was stored at −80°C until used for real-time PCR measurements.

Real-time PCR assay: Real-time PCR analysis was carried out using the iQ SYBR Green Supermix kit (Bio-Lad) as described previously [18]. Specific individual cytokine primer sequences and the internal standard gene β-actin used are shown in Table 2. Amplification was carried out using a Mini Opticon (Bio-Rad) thermal cycler with the following profile: one cycle of 95°C for 2 min, followed by 45 cycles of 95°C for 30 sec, 65 or 59°C for 30 sec and 74°C for 30 sec, followed by 74°C for 7 min. Melting curve analysis was performed from 50 to 95°C, read every 0.5°C holding for 5 sec. Relative gene expression was calculated using the comparative threshold cycle number ($2^{-\Delta\Delta Ct}$) method, as described by Livak and Schmitge [19]. Results are summarized as ΔCt values, where ΔCt is the difference in the threshold cycle for the target and β-actin, as an internal control.

Statistical analysis: The number of CD-positive or CD-negative cells was calculated from the percentages of leukocytes gated by flow cytometry analysis and total PBMCs. Values are expressed as means ± standard error (SE). Prism-Pad software (ver. 5.01) was used for statistical calculations, and one-way repeated-measures analysis of variance followed by the Tukey-Kramer multiple comparison method was used to evaluate differences in values among the groups. A P-value<0.05 was considered to indicate statistical significance.

Table 1.　Monoclonal antibodies used for flow cytometry assay

Items	Target molecule	Specificity	mAb clone	Isotype	Supplier
CD3	TcR complex	pan-T cells	MM1A	IgG$_1$	VMRD
CD4	CD4 molecule	T-helper cells	CACT183A	IgG$_1$	VMRD
CD8	CD8β	cytotoxic T cells	BAT82A	IgG$_1$	VMRD
CD45R	PTPR	pan-leukocytes	GC6A	IgM	VMRD
WC1	Unknown	γδ T cells	IL-A29	IgG$_1$	VMRD
CD14	LPS receptor	monocytes, macrophages	CAM36A	IgG$_1$	VMRD
CD21	CR2	B cells	BAQ15A	IgM	VMRD
CD282	TLR2	monocytes	HCA151F	IgG	AbD-Serotec

TcR, T cell receptor; PTPR, protein tyrosine phosphatase receptor; LPS, lipopolysaccharide; CR2, complement component receptor 2; TLR2, toll-like receptor 2.

Table 2.　Primers used for real-time PCR

Gene	GenBank access. no.	Sequence (5'–3')	Annealing temp (°C)	Concentration (nM)
β-actin	AY141970	F: GGCCGAGCGGAAATCG R: GCCATCTCCTGCTCGAAGTC	65	750
IL-6	NM173923	F: GTCTTCAAACGAGTGGGTAAAG R: TGACCAGAGGAGGGAATGC	65	250
IL-8	NM173925	F: CACTGTGAAAATTCAGAAATCATTGTTA R: CTTCACAAATACCTGCACAACCTTC	59	250
TNF-α	Z48808	F: CGGTGGTGGGACTCGTATG R: GCTGGTTGTCTTCCAGCTTCA	65	750
IFN-γ	NM174086	F: TAGGCAAGTCTATGGGATTTC R: GCATTCATTACATCATCAAGTG	59	250

RESULTS

Subpopulations of peripheral leukocytes: The number of CD-positive cells is shown in Figs. 1 and 2. Compared to the value on the predose day and the control value on the same day, increased ($P<0.05$) numbers of CD3$^+$ T cells (CD3$^+$ CD45R$^+$/CD45R$^-$), WC1$^+$ γδ T cells and CD282$^+$ monocytes were noted in the treatment group on day 7. The values remained higher on day 14; however, the difference was not statistically significant. Compared to the value on the predose day, CD8$^+$ T cells were increased ($P<0.05$) in the treatment group on day 7. Compared to the control value on the same day, the CD4$^+$ T cell subset was also increased ($P<0.05$) in the treatment group on day 7. The values of CD3$^+$, CD4$^+$ and CD8$^+$ cell subsets decreased slightly from day 7 to day 14, and the differences were not statistically significant on day 14. Discrete analysis of data indicated that the numbers of CD45R$^+$ and CD45R$^-$ T cells were increased ($P<0.05$) in the CD3$^+$ cells in the treatment group. However, the values were increased ($P<0.05$) only in the number of CD4$^+$CD45R$^+$ and CD8$^+$CD45R$^+$ T cells on day 7 compared to the values on the predose day and/or the control; they were not significantly different among the groups on day 14 (Table 3). The numbers of CD14$^+$ and CD21$^+$ cells changed slightly in the treatment group on days 7 and 14, but there was no statistically significant difference between the treatment and control groups.

mRNA expression of cytokines: Expression of IL-6 and IFN-γ mRNA was increased slightly in the treatment group on day 7 compared to the control, and the values were increased ($P<0.05$) on day 14 compared to the predose day (Fig. 3). Expression of IL-8 mRNA was similar between the treatment and control groups on days 7 and 14. Expression of tumor necrosis factor-alpha (TNF-α) was slightly higher on days 7 and 14 in the treatment group, but the differences were not statistically significant ($P=0.07$) between the treatment and control groups.

DISCUSSION

Certain probiotics have been suggested to have immune-stimulating properties in pre-weaned calves and adult cattle [14, 15, 24]. In the present study, Toll-like receptor 2 (TLR2; CD282) cell surface molecule antibody was used as a marker to distinguish monocytes in peripheral blood of healthy weaned calves receiving probiotic and a control group. Thus, in addition to CD282$^+$ cells, the numbers of CD3$^+$ T cells, CD4$^+$, CD8$^+$ and WC1$^+$ γδ T cell subsets and CD14$^+$ and CD21$^+$ cells were evaluated. Likewise, the expression of cytokine mRNA, such as IL-6, IL-8, INF-γ and TNF-α, was examined to assess the possible immune-stimulating effects of the probiotic on the activation of peripheral leukocytes.

In a previous study, the number of CD4$^+$ cells was higher after repeated administration of a probiotic consisting of *Clostridium butyricum* strain Miyairi in dairy cows [15]. In another study, CD8$^+$ cells were stimulated in the peripheral blood of pre-weaned calves by administration of a bacteria-based probiotic [24]. Furthermore, a number of *in vitro* and
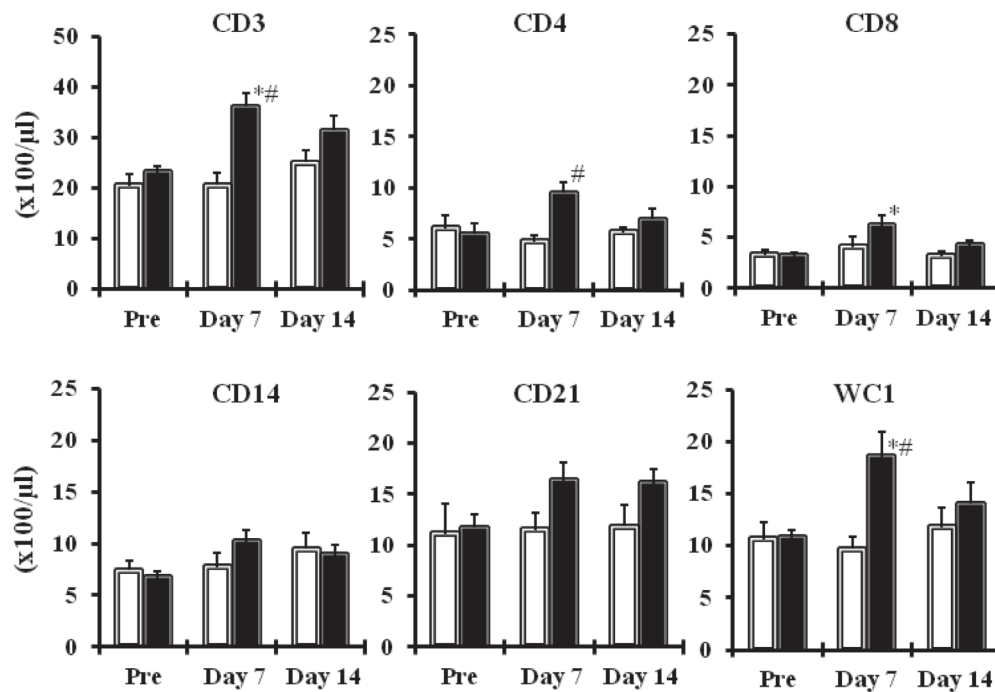
Fig.1. Number of CD3, CD4, CD8, CD14, CD21 and WC1$^+$ cells in the peripheral blood of calves given 3.0 g/100 kg BW of probiotic once daily for 5 consecutive days (black columns). Calves receiving the vehicle alone served as a control (white columns). Numbers were counted at predose (Pre) and on days 7 and 14. Values represent means ± SE (n=8). * Values in the treatment group compared to the predose day in the same group (*P*<0.05). # Values compared to the control on the same day (*P*<0.05). The first administration day of the probiotic was deemed day 1.
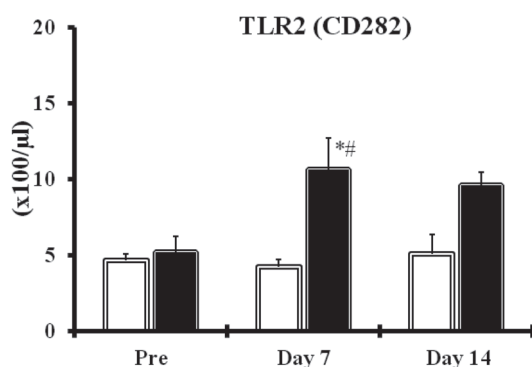

Fig. 2. Number of TLR2$^+$ (CD282) cells in the peripheral blood of calves given 3.0 g/100 kg BW of probiotic once daily for 5 consecutive days (black columns). Calves receiving the vehicle alone served as a control (white columns). Numbers were counted at predose (Pre) and on days 7 and 14. Values represent means ± SE (n=8). * Values in the treatment group compared to the predose day in the same group (*P*<0.05). # Values compared to the control on the same day (*P*<0.05). The first administration day of the probiotic was deemed day 1.

in vivo studies on human PBMCs have suggested that a probiotic containing *E. faecium*, *C. butyricum* or *Lactobacillus* subspecies shows enhancing effects on T cell subsets and monocytes [12, 35]. In young mammals, most CD3$^+$, CD4$^+$ and CD8$^+$ cells express the high-molecular-weight isoform of CD45R [31], and an increase in the number of T lymphocytes expressing CD45R facilitates regular activation of T cells [8]. In the present study, the increase in the number of CD3$^+$CD45R$^+$ T cells in the treatment group, compared to the predose day or control, may indicate a nonspecific, but enhanced immune state with possibly multiple causes after the 5-day repeated administration of the probiotic. Similar to CD3$^+$ cells, increased numbers of CD4$^+$CD45R$^+$ and CD8$^+$CD45R$^+$ T cells were observed. They may be involved in immune system responses, along with direct or indirect immune-stimulatory effects of the probiotic in the calves, as suggested by previous studies in cattle [6, 15, 24].

The WC1 molecule is a member of the scavenger receptor cysteine-rich (SRCR) family found on γδ T cells, which are negative for CD4 and CD8 [33]. In cattle, the regulatory potential of WC1$^+$ γδ T cell subsets is comparable to that of CD14$^+$ cells [11], and the WC1$^+$ T cell subpopulation is larger in calves [1]. Bovine γδ lymphocyte subsets are activated indirectly through cytokines secreted by T cells, antigen-presenting cells (APCs) and other accessory cells [26]. As our results show, the higher number of WC1$^+$ γδ
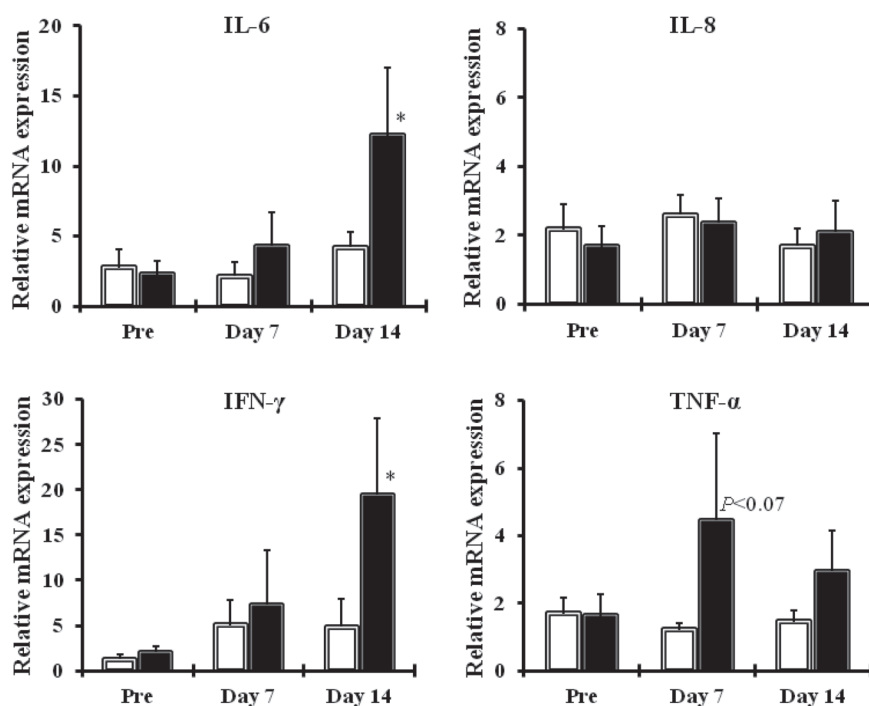
Fig. 3. Relative mRNA expression of IL-6, IL-8, IFN-γ and TNF-α in peripheral leukocytes of calves given 3.0 g/100 kg BW of probiotic once daily for 5 consecutive days (black columns). Calves receiving the vehicle alone served as a control (white columns). Expression was measured at predose (Pre) and on days 7 and 14. Values represent means ± SE (n=8). * Values in the treatment group compared to the predose day in the same group (P<0.05). The first administration day of the probiotic was deemed day 1.

Table 3. The numbers of peripheral blood mononuclear cell (PBMC) and T cell subpopulations (cell/μl) in probiotic-treated and control calves

	Treatment [a]			Control		
	Pre [b]	Day 7	Day 14	Pre	Day 7	Day 14
PBMC	5,283 ± 193	6,323 ± 318	5,650 ± 260	4,836 ± 346	4,467 ± 415	5,303 ± 513
CD3+CD45R−	1,425 ± 117	2,199 ± 123*#	1,940 ± 136	1,321 ± 157	1,431 ± 206	1,777 ± 202
CD3+CD45R+	727 ± 60	1142 ± 158*#	998 ± 82	668 ± 92	729 ± 76	857 ± 106
CD4+CD45R−	191 ± 47	315 ± 44	244 ± 52	201 ± 35	171 ± 37	228 ± 42
CD4+CD45R+	326 ± 62	625 ± 79*#	394 ± 39	328 ± 47	305 ± 59	333 ± 49
CD8+CD45R−	53 ± 80	77 ± 10	65 ± 12	68 ± 13	65 ± 28	69 ± 13
CD8+CD45R+	209 ± 41	526 ± 89*	340 ± 41	207 ± 28	304 ± 84	280 ± 43

a) Calves received 3.0 g/100 kg of probiotic for 5 consecutive days, and additional calves given the vehicle alone served as the control. b) Predose day (day before administration). Values represent the means ± SE (n=8). * Compared to the predose (Pre) values in the same group (P<0.05). # Compared to the values in the control on the same day (P<0.05).

T cells in the calves receiving the probiotic was associated with an increased number of CD4+ cells, which might suggest stimulation of cellular immunity in the calves. Although WC1+ γδ T cells and CD14+ cells are generated comparably in bovines [11], we found that there were more WC1+ T cells than CD14+ cells. On the other hand, WC1+ cells are immune suppressors, secreting IFN-γ in bovines [11, 27], consistent with our results.

The expression of TLRs has been correlated with the population of total bacteria and LAB in the gastrointestinal tract, including the rumen of calves, during the first 6 months of life [20]. Moreover, the expression of TLR2 was evident on bovine antigen presenting cells with high level expression on peripheral blood monocytes and monocyte-derived macrophages [16]. The TLR2 cell surface molecule on bovine monocyte-derived dendritic cells (DCs) and gastrointestinal macrophages is most responsible for recognition of Gram-positive bacteria [4, 9, 10]. Mechanisms indicating a direct role of TLR2 include bacterial reorganization by epithelial macrophages in calves [20]. The transcytosis processing of

bacterial particles via ileal Peyer's patch M cells and the later appearance of TLRs on DCs in the lamina propria play a key role in the mechanism of probiotic immunomodulation in cattle [7, 20, 21]. Thus, DCs are the principal stimulators of T cells [7, 12]. The TLR signaling pathway is involved with the actions of a probiotic containing *C. butyricum*, and the absence of TLR2 expression might indicate the need for TLR2 in the recognition of *Clostridium* bacteria [9, 10]. In the present study, we provide evidence for an increased number of TLR2^{+} monocytes in the peripheral blood of calves receiving a probiotic consisting of three species of Gram-positive bacteria, including *C. butyricum*. The increased number of peripheral monocytes expressing TLR2 might be associated with the formal reaction of innate immunity in response to increasing and changing bacterial diversity in the gastrointestinal tract of the calves receiving the probiotic. Furthermore, the CD14 cell surface molecule is essential for the response to most Gram-negative bacteria, including lipopolysaccharides, via the TLR4 and MD2 complex [22, 28]. The TLR2 molecule recognizes microbial-associated molecule patterns independently [4, 7]. Although some differences were noted between the different antibodies assessed the expression of TLR2 and CD14^{+} monocytes in bovine [16]. Moreover, TLR2 is expressed on peripheral blood B lymphocytes [5]; however, a B cell receptor complex including the CD21 molecule is present on mature B cells and also ileal Peyer's patch-derived B lymphocytes in healthy calves [23]. Similar to these reports, the numbers of CD14^{+} and CD21^{+} B cells in the present study were slightly increased in the calves on days 7 and 14.

Probiotics including *Lactobacillus* and *C. butyricum* induce the release of proinflammatory cytokines, such as IL-6 and TNF-α, which are involved in nonspecific immune responses [9, 10]. A probiotic consisting of *E. faecium* and *C. butyricum* upregulates the level of IFN-γ and downregulates TNF-α in the supernatant of human PBMCs [12]. In our study, increased expression of IL-6, INF-γ and TNF-α mRNA was observed in the treatment group on days 7 and 14. Increased expression of IL-6, IFN-γ and TNF-α in peripheral leukocytes may be dependent on the period after administration and numbers of activated T lymphocytes or monocytes in the treatment group. Our observations are similar to those of Waldvogel *et al.* [32], in which increased expression of IFN-γ was observed only 2 weeks after trickle stimulation of cultured PBMCs from calves. On the other hand, expression of IL-8 is related to neutrophil function, especially inflammatory stimulation, in bovine [3]. In the present study, the expression of IL-8 was unchanged compared to the predose day and the control value on the same day, likely because the calves were healthy.

In conclusion, repeated 5-day administration of a bacteria-based probiotic resulted in increased numbers of CD3^{+} T cells, CD4^{+}, CD8^{+} and WC1^{+} T cell subsets and CD282^{+} monocytes in healthy Holstein calves. Moreover, higher mRNA expression of pro-inflammatory cytokines, such as IL-6, INF-γ and TNF-α, in peripheral leukocytes was noted. These results suggest that the peripheral blood leukocytes and cellular immune function in the healthy calves may have been stimulated via effects on the gastrointestinal microbiota, which generally increased, with overall stability of the rumen bacterial flora, by the oral probiotic treatment.

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