Immune-Stimulatory Effects of a Bacteria-Based Probiotic on Peripheral Leukocyte Subpopulations and Cytokine mRNA Expression Levels in Scouring Holstein Calves

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ABSTRACT. Subpopulations of peripheral leukocytes and cytokine mRNA expression levels were evaluated in scouring and healthy Holstein calves (age 10 ± 5 days; n=42) treated with a probiotic consisting of *Lactobacillus plantarum*, *Enterococcus faecium* and *Clostridium butyricum*. The calves were assigned to the scouring or healthy group and then subdivided into pathogen-positive treated (n=8), pathogen-positive control (n=8), pathogen-negative treated (n=6), pathogen-negative control (n=6), healthy treated (n=6) and healthy control (n=8) groups. A single dose of the probiotic (3.0 g/100 kg body weight) was given to each calf in the treatment groups for 5 days. Blood samples were collected on the first day of scour occurrence (day 0) and on day 7. In the scouring calves, smaller peripheral leukocyte subpopulations and cytokine mRNA expression levels were noted on day 0. The numbers of $CD3^+$ T cells; $CD4^+$, $CD8^+$ and $WC1^+$ γδ T cell subsets; and $CD14^+$, $CD21^+$ and $CD282^+$ (TLR2) cells were significantly increased in the scouring and healthy treated calves on day 7. Furthermore, interleukin-6, tumor necrosis factor-alpha and interferon-gamma mRNA expression was elevated in the peripheral leukocytes of the scouring and healthy treated calves on day 7. The scouring calves given the probiotic recovered on day 7. A significantly smaller number of peripheral leukocytes and lower cytokine mRNA expression level might be induced by scouring in calves. Repeated probiotic administration might stimulate cellular immunity and encourage recovery from scouring in pre-weaning Holstein calves.
KEY WORDS: cytokine, leukocyte, probiotic, scouring calf, toll-like receptor 2.

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Calves are born with a naive immune system, and appropriate development of the gastrointestinal tract (GIT) microbiota in the early weeks of life is crucial for a functional immune system [25, 27, 39]. GIT diseases are characterized by acute intestinal inflammation, much of which is thought to be due to inappropriate activation of the immune system in calves [19, 22]. Greater vulnerability to invasive infections and the development of GIT disorders, such as scouring, are associated with the immaturity of the immune system of calves at birth, although the specific mechanism is unclear. Regulation by the immune-stimulatory effects of probiotics may contribute to the treatment of scouring in calves [26]. Probiotics contribute to the homeostasis of the bacterial flora in the GIT, improving animal health and protecting calves against infection [35, 36]. Lactic acid bacteria (LAB) probiotics are known to help with GIT colonization via the competitive exclusion of undesirable microorganisms and the development of a homeostatic gastrointestinal environment in calves [1, 12, 18]. LAB probiotics are also known to have a beneficial impact on intestinal infections and are commonly used to treat calf scouring [19]. More recently, *in vivo* studies have examined their immune-stimulatory effects in calves [26, 35, 36]. In cases of inadequate initial bacterial colonization, probiotics can be used to achieve a balanced immune response [17]. Therefore, the immune-modulating effect of probiotics is likely more appropriate for young calves, when the intestinal bacterial inhabitants are less well-established. The phagocytic activities of leukocytes have been reported to be increased in the peripheral blood of calves following the administration of a probiotic consisting of *Lactobacillus plantarum* [18]. A number of systemic immune markers, including $CD8^+$ cells, increased in response to treatment with a probiotic in scouring calves [26]. In adult cattle, the immune-stimulatory effect of a probiotic containing *Enterococcus faecium* and *Saccharomyces cerevisiae* has been recognized in feedlot steers and dairy cows [7, 20]. In the intestine, *Clostridium butyricum* as a probiotic may increase the resistance of the gut to pathogen invasion by inducing the secretion of anti-inflammatory cytokines [11]. The stimuli provided by probiotics are essential for the development of a fully functional and balanced immune system, including the homing of B and T cells to the lamina propria [4]. It has generally been assumed that some probiotic strains predominantly induce cytokine mRNA expression in leukocytes, promote T cell development and augment immune defense to prevent infections, thereby ameliorating inflammatory diseases [7, 20, 33, 34]. Recently, we studied the effects of a probiotic on peripheral leukocytes and their mRNA expres-

Table 1.   Monoclonal antibodies used for flow cytometry

Item	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

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

sion in healthy weaned calves (in press). However, the effect of probiotics on subpopulations of peripheral leukocytes in scouring and healthy pre-weaning calves is unknown. In the present study, the immune-stimulatory effects of a probiotic on peripheral blood mononuclear cells (PBMCs) and their mRNA expression levels were investigated in scouring and healthy pre-weaning Holstein calves.

MATERIALS AND METHODS

Animals and treatment: The experimental design was approved by the Laboratory Animal Care and Use Committee of Iwate University, Iwate, Japan. Forty-two scouring and healthy pre-weaning Holstein calves (age 10 ± 5 days) housed in two commercial dairy farms were used in the experiment. All calves were examined clinically, and the scouring status of each calf was inspected. The scour condition was watery to muddy yellow, and the appetite of the scouring calves was almost normal. The calves were assigned to treatment and corresponding control groups based on the type of scouring (pathogenic or non-pathogenic) and clinically healthy (non-scouring) status. The treatment groups included scouring pathogen-positive treated (PPT; n=8), scouring pathogen-negative treated (PNT; n=8) and healthy treated (HT; n=6) calves. The corresponding control groups included scouring pathogen-positive control (PPC; n=6) and pathogen-negative control (PNC; n=6) calves, and healthy control (HC; n=8) calves without probiotic treatment were used as the main control group. All calves received colostrum at birth and were fed a milk replacement twice daily.

Treatment of the calves began on the first day of scour occurrence (day 0). After a clinical examination and the collection of blood and stool samples, a probiotic (Miyarisan Pharmaceutical Co., Ltd., Tokyo, Japan) that included *L. plantarum* strain 220 (9 × 10^6 colony forming units [CFU]/g), *E. faecium* strain 26 (9 × 10^5 CFU/g) and *C. butyricum* strain Miyari (9 × 10^4 CFU/g) was administered to each of the calves in the treatment groups once daily at a dose of 3.0 g/100 kg body weight (BW) for 5 days. The probiotic was mixed with a milk replacement and fed to each calf in the morning. The scouring calves were not treated by any other medicine or electrolyte therapy, although all calves had access to fresh water *ad libitum*.

Blood collection and PBMC isolation: Blood samples were collected on days 0 and 7. A blood specimen (6 m*l*) was collected in a heparinized tube (BD Vacutainer Systems, Plymouth, U.K.) and used to isolate PBMCs for flow cytometric analysis. Blood (4 m*l*) was also collected in two EDTA tubes (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ, U.S.A.) and used for total RNA extraction from peripheral leukocytes and for white blood cell (WBC) counts. The 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 the hemogram method with the Dif-Quick staining protocol. PBMCs were isolated as described by Ohtsuka *et al.* [27]. To purify PBMCs and to lyse red blood cells, 2 m*l* of the heparinized blood was mixed with 8 m*l* of 0.83% ammonium chloride solution. After 5 min at room temperature, the samples were centrifuged (2,000 rpm, 10 min and 4°C). The leukocytes were then purified by washing twice in phosphate-buffered saline (PBS; pH 7.4) followed by centrifugation (2,000 rpm, 5 min and 4°C). After the final wash, the cell pellet was diluted in 500 μl of PBS, and the cells were counted. The cell concentration was then adjusted to 1 × 10^7/m*l* in PBS, and the cells were kept at 4°C until they were used for flow cytometry.

Flow cytometry: The primary monoclonal antibodies used in the present study are shown in Table 1. Flow cytometry was performed as described by Ohtsuka *et al.* [27]. PBMCs were labeled with the primary antibody and incubated for 60 min at 4°C. $CD3^+$ T cells and each of the $CD4^+$ and $CD8^+$ T cell subsets were additionally labeled with a monoclonal antibody against CD45R to mark pan-leukocytes. After incubation, the cells were washed twice to remove unbound antibodies. The labeled cells were then stained with specific purified IgG conjugated to a 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, the stained cells were diluted in 500 μl of PBS. Flow cytometry was performed using a FACScan analyzer (Becton Dickinson) equipped with a computer running Cell Quest software (Becton Dickinson).

Total RNA extraction from leukocytes and cDNA synthesis: Total RNA was extracted from 1 m*l* of whole blood leuko-

Table 2. Primers used for real-time PCR

Gene	GenBank acc. no.	Sequence (5′→3′)	Annealing temperature (°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

Table 3A. Numbers of peripheral blood mononuclear cells (PBMCs) and CD45R$^-$/CD45R$^+$ T cells in the scouring probiotic-treated and scouring control calves

	PPC[a]		PPT[b]		PNC[c]		PNT[d]	
	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7	Day 0	Day 7
PBMCs	4,231 ± 430	5,658 ± 952	3,822 ± 313	5,947 ± 554*	4,427 ± 495	5,126 ± 425	4,140 ± 422	5,610 ± 393*
CD3$^+$CD45R$^-$	1,088 ± 103	2,656 ± 565*	1,064 ± 85	2,342 ± 282*	1,185 ± 79	2,558 ± 401*	1,375 ± 127	2,228 ± 268*
CD3$^+$CD45R$^+$	361 ± 28	1,305 ± 288*	564 ± 27	1,394 ± 150*	678 ± 85	968 ± 160*	552 ± 60	1,419 ± 131*#
CD4$^+$CD45R$^-$	133 ± 18	377 ± 53*	73 ± 14	320 ± 47*	111 ± 17	287 ± 75*	155 ± 41	205 ± 25*
CD4$^+$CD45R$^+$	177 ± 29	978 ± 144*	264 ± 23	963 ± 151*	450 ± 98	654 ± 58*	344 ± 63	1,049 ± 130*#
CD8$^+$CD45R$^-$	67 ± 15	284 ± 41*	80 ± 43	201 ± 22*	58 ± 7.0	96 ± 15	44 ± 12	113 ± 32*
CD8$^+$CD45R$^+$	238 ± 41	409 ± 73*	204 ± 16	710 ± 142*#	290 ± 51	305 ± 60	328 ± 46	505 ± 52*#

The values (cells/μl) represent the means ± SE. a) Pathogen-positive control (n=8). b) Pathogen-positive treated (n=6). c) Pathogen-negative control (n=8). d) Pathogen-negative treated (n=6). * Compared with day 0 in the same group ($P<0.01$). # Compared with the values of the corresponding control groups ($P<0.05$).

Table 3B. Numbers of peripheral blood mononuclear cells (PBMCs) and CD45R$^-$/CD45R$^+$ T cell subpopulations in the healthy probiotic-treated and healthy control calves

	HC[a]		HT[b]	
	Day 0	Day 7	Day 0	Day 7
PBMCs	4,500 ± 238	5,148 ± 476	3,808 ± 258	7,218 ± 365*
CD3$^+$CD45R$^-$	1,621 ± 116	1,725 ± 198	1,498 ± 98	2,003 ± 491*
CD3$^+$CD45R$^+$	979 ± 71	1,114 ± 152	1,042 ± 45	1,815 ± 105*#
CD4$^+$CD45R$^-$	128 ± 34	219 ± 51	108 ± 21	284 ± 22*
CD4$^+$CD45R$^+$	482 ± 81	439 ± 81	501 ± 71	1,307 ± 96*#
CD8$^+$CD45R$^-$	52 ± 12	68 ± 11	50 ± 7.5	101 ± 14*
CD8$^+$CD45R$^+$	409 ± 50	502 ± 70	421 ± 22	693 ± 66*

The values (cells/μl) represent the means ± SE. a) Healthy control calves without treatment (n=8). b) Healthy treated calves (received 3.0 g/100 kg of probiotic for 5 days; n=6). *Compared with day 0 in the same group ($P<0.01$). # Compared with the healthy controls on the same day ($P<0.05$).

cytes 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 U of DNase I per 100 mg of 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 the purity was determined using a NanoDrop 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). The cDNA

was stored at −80°C until it was used for real-time PCR.

Real-time PCR: Real-time PCR was performed using an iQ SYBR Green Supermix Kit (Bio-Rad) as described previously [23]. Specific individual cytokine primer sequences and the internal standard gene used (β-actin) are shown in Table 2. Amplification was performed using a Mini Opticon (Bio-Rad) thermal cycler with the following profile: 1 cycle of 95°C for 2 min followed by 45 cycles of 95°C for 30 sec, and 65 or 59°C for 30 sec and 74°C for 30 sec followed by 74°C for 7 min. A melting curve analysis was performed from 50 to 95°C with readings every 0.5°C (holding for 5 sec).
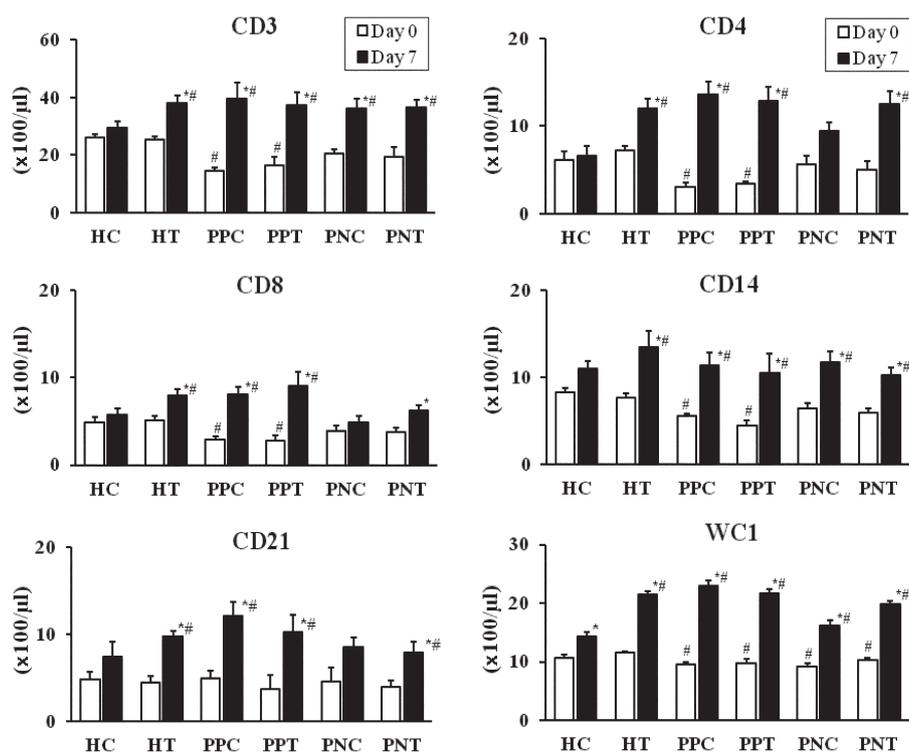
Fig. 1.   The numbers of CD3, CD4, CD8, CD14, CD21 and WC1 cells in the peripheral blood of the scouring PPT (n=8), PPC (n=8), PNT (n=6), PNC (n=6), HT (n=6) and HC (n=8) groups. Calves in the treated groups were given 3.0 g/100 kg BW of the probiotic once daily for 5 days, and calves without probiotic treatment served as corresponding controls. The values represent the mean ± SE. * Compared to day 0 in the same group. # Compared to the HC group (*P*<0.05). The first day of scouring occurrence and probiotic treatment was designated as day 0.

Relative gene expression was calculated using the comparative threshold cycle number ($2^{-\Delta Ct}$) method, as described by Livak and Schmitge [23]. The 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.

Stool analysis: The common pathogens considered essential for calf scouring in the field were examined using stool samples from the calves in the scouring groups. On the first day of scouring, stool samples were obtained from each calf by mechanical stimulation of the anus and preserved in a sterilized tube. Deoxycholate hydrogen sulfide-lactose agar (Elken Chemical Co., Tokyo, Japan) medium was used for the recognition of *Salmonella* and *Escherichia coli* according to the methods of Ishihara *et al.* [15]. The *Cryptosporidium parvum* test strip BIO K 155 (Bio-X Diagnostics, Jemelle, Belgium) was used according to the manufacturer's instructions with 0.1 g of stool sample and was evaluated visually. For the detection of coccidia spores, a previously described microscopic method was used [22]. For rotavirus, a specific test strip (SA Scientific, San Antonio, TX, U.S.A.) was used following the product use recommendations. Among the scouring calves diagnosed as pathogen-positive (n=16), the pathogens found were rotavirus (43%), coccidian *Eimeria bovis* and/or *Eimeria zuernii* (25%), *C. parvum* (19%), *E.*

coli (13%) and *Salmonella* (0%). Those calves with negative stool tests were assigned to the non-pathogenic scouring groups, and clinically healthy calves without scouring were placed in the healthy groups. The scouring status of each calf was inspected every day. Reduced occurrence of scouring and an improvement in clinical status were noted in the probiotic-treated calves on day 7.

Statistical analysis: The number of CD-positive or -negative cells was calculated from the percentages of leukocytes gated by flow cytometry and total PBMCs. The values are expressed as means ± standard error (SE). GraphPad Prism ver.5.01 software (La Jolla, CA, U.S.A.) was used for the statistical calculations and one-way ANOVA, and the Kruskal-Wallis test was subsequently used to evaluate differences among the groups. A *P*-value<0.05 was considered significant.

RESULTS

Subpopulations of peripheral leukocytes: Compared with the values of the HC and HT groups on day 0, a significantly lower (*P*<0.05) number of CD3$^+$ T cells; CD4$^+$, CD8$^+$ and WC1$^+$ γδ T cell subsets; and CD14$^+$ cells were noted in the peripheral blood of scouring PPT and PPC calves. On day 0,
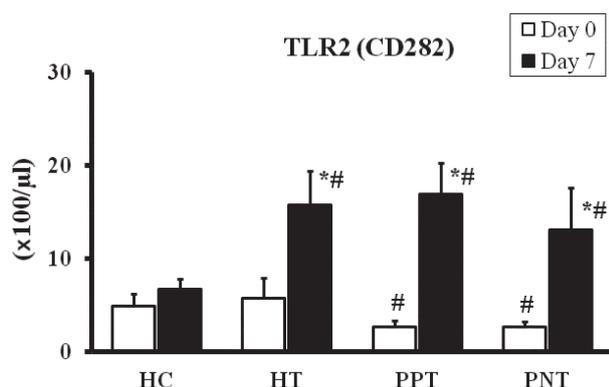
TLR2 (CD282)

□ Day 0
■ Day 7

Fig. 2.   Number of TLR2 (CD282) cells in the peripheral blood of the scouring PPT (n=8), PNT (n=6), HT (n=6) and HC (n=8) groups. Calves in the treated groups were given 3.0 g/100 kg BW of the probiotic once daily for 5 days, and calves without probiotic treatment served as corresponding controls. The values represent the mean ± SE. * Compared to day 0 in the same group. # Compared to the HC group ($P<0.05$). The first day of scouring occurrence and probiotic treatment was designated as day 0.

the numbers of CD3$^+$ T cells; the CD4$^+$, CD8$^+$ and WC1$^+$ γδ T cells subsets; and CD14$^+$ cells were lower ($P<0.05$) in the PPT and PPC groups than in the other groups. The number of CD21$^+$ cells was similar among the scouring and healthy groups on day 0 (Fig. 1). Compared to the HC group, CD3$^+$ T cells; the CD4$^+$, CD8$^+$ and WC1$^+$ γδ T cell subsets; and CD14$^+$ and CD21$^+$ cells were increased ($P<0.05$) in the scouring PPT, PNT and PPC groups on day 7. However, the numbers of CD8$^+$ and CD21$^+$ cells were unchanged in the scouring PNC group on day 7. The values among the scouring PPT, PNT, PPC and PNC groups were not significantly different on day 7. Moreover, compared with the values on day 0 and those of the HC group, an increased ($P<0.05$) number of CD3$^+$ T cells; the CD4$^+$, CD8$^+$ and WC1$^+$ γδ T cell subsets; and CD14$^+$ and CD21$^+$ cells were noted in the HT group on day 7. Compared with the value on day 0, WC1$^+$ γδ T cells were increased ($P<0.05$) in the HC group on day 7. Likewise, compared with the values on day 0, PBMCs were increased ($P<0.05$) in the scouring PPT, PNT and HT groups on day 7. A discrete analysis of the data indicated that the numbers of CD45R$^+$ and CD45R$^-$ T cells were increased ($P<0.05$) in the proportion of CD3$^+$ (CD3$^+$D45R$^+$/CD45R$^-$) T cells, CD4$^+$ (CD4$^+$CD45R$^+$/CD45R$^-$) and CD8$^+$ (CD8$^+$CD45R$^+$/CD45R$^-$) T cell subsets in the scouring PPT, PNT, PPC and HT groups on day 7, compared to the values on day 0 in the same group or the HC group. However, the values were increased ($P<0.05$) only in the number of CD3$^+$ (CD3$^+$CD45R$^+$) T cells and CD4$^+$ (CD4$^+$CD45R$^+$) T cell subset in the scouring PNC group on day 7. The values were not significantly different among the scouring PPT, PNT, PPC and PNC groups (Table 3A and 3B). Peripheral CD282$^+$ [toll-like receptor 2 (TLR2)] monocytes were evaluated only in the scouring PPT, PNT, HT and HC groups. Similar to that of the T cell subpopulations, a significantly lower ($P<0.05$) number of peripheral TLR2$^+$ monocytes were noted in the

scouring calves on day 0. However, the number of TLR2$^+$ monocytes was increased ($P<0.05$) in the scouring PPT, PNT and HT groups on day 7, compared with the values on day 0 and that of the HC group (Fig. 2).

mRNA expression of cytokines: Compared with the value for the HC calves, the mRNA expression levels of interleukin (IL)-6 and interferon-gamma (IFN-γ) were lower ($P<0.05$) in the scouring PPT, PNT, PPC and PNC groups on day 0. The mRNA expression level of tumor necrosis factor-alpha (TNF-α) was lower ($P<0.05$) in the scouring PPT and PPC groups, but the values were greater in the scouring PNT, PNC, HT and HC groups on day 0 (Fig. 3). IL-6, IFN-γ and TNF-α mRNA expression was increased ($P<0.05$) in the PPT, PNT, PPC, PNC and HT groups on day 7. Furthermore, compared to day 0, the TNF-α mRNA expression level was higher ($P<0.05$) in the HC group on day 7. The mRNA expression level of IL-8 was similar between the scouring and healthy calves on day 0, but was elevated ($P<0.05$) in the scouring PPT and PPC groups on day 7. Compared to the HC group, the expression of IL-8 mRNA was slightly higher in the HT group on day 7, but the differences were not statistically significant.

DISCUSSION

We recently studied the effects of a probiotic consisting of *L. plantarum*, *E. faecium* and *C. butyricum* on the peripheral leukocyte subpopulations and cytokine mRNA expression levels in healthy weaned calves (in press). In the present study, peripheral leukocyte subpopulations and their cytokine mRNA expression levels were evaluated in the field in scouring and healthy pre-weaning calves to clarify the immune-stimulatory effects of the same probiotic agent.

Some reports have indicated that a probiotic including LAB reduced the rate of GIT disorders in calves [1, 26, 35, 36]. Increased intestinal LAB might stimulate the gut epithelial and associated lymphoid tissues and may activate local immune responses in the intestine [12]. It was also suggested that a decreased number of peripheral leukocytes, including CD8$^+$ and WC1$^+$ γδ T cells, might result in a high risk of calf scouring [27]. Similarly, a lower number of CD4$^+$ and CD8$^+$ cells and reduced cytokine mRNA expression levels were noted in neonates with diarrhea, compared with healthy children [9, 37]. In agreement with a previous report by Ohtsuka *et al*. [27], the numbers of CD4$^+$, CD8$^+$ and WC1$^+$ γδ T cell subsets and of CD14$^+$ and TLR2$^+$ cells were significantly lower in scouring calves in the present study. Probiotics are known to affect the host immune system in different ways, including inducing greater antibody production and increasing epithelial barrier integrity, the upregulation of cell-mediated immunity, increased TLR signaling, enhanced dendritic cell (DC)-T cell interactions and heightened T cell associations [5, 18, 32]. The immune-stimulating properties of a *Bacillus*-based probiotic were suggested by the increase in peripheral blood CD8$^+$ cells in scouring calves compared with controls [26]. Furthermore, the number of CD4$^+$ cells increased in response to a probiotic consisting of *C. butyricum* in dairy cows [20]. In a study of calf PBMCs, the
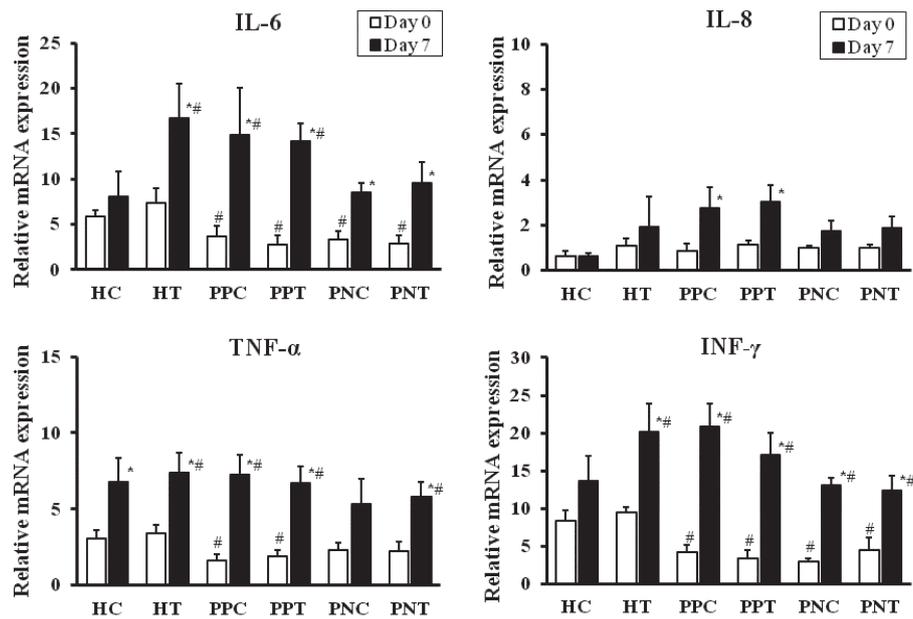
Fig. 3.   Relative mRNA expression of IL-6, IL-8, TNF-α and IFN-γ in the peripheral blood of the
scouring PPT (n=8), PPC (n=8), PNT (n=6), PNC (n=6), HT (n=6) and HC (n=8) groups. Calves
in the treated groups were given 3.0 g/100 kg BW of the probiotic once daily for 5 days, and calves
without probiotic treatment served as corresponding controls. The values represent the mean ± SE.*
Compared to day 0 in the same group. [#] Compared to the HC group (*P*<0.05). The first day of scouring
occurrence and probiotic treatment was designated as day 0.

phagocytic activities of leukocytes were increased by a pro-
biotic consisting of *L. plantarum* [18]. In addition, selective
effects of a probiotic including *Lactobacillus* were reported
for T cell activation, CD8^{+} cell activity and cytokine produc-
tion [5]. Most peripheral T cell subpopulations have CD3
expressed on their surface, which might appear as a signal-
transducing unit for the T cell receptor (TCR) [32]. Similar
to our recent study using the same probiotic agent in healthy
weaned calves, the proportion of CD3^{+}CD45R^{+} T cells was
marginally higher in the systemic circulation of pre-weaning
scouring and HT calves. Similar to previous reports, a great-
er presence of CD45R^{+} T cells might facilitate the regular
activation of T cell subsets in probiotic-treated calves [26].
In contrast, bovine WC1^{+} γδ T cells are known to be immune
suppressors and are involved in secreting IFN-γ [13, 28, 29].
WC1[+] γδ T cells can respond to stimulation via the TCR and
through CD3 stimulation, leading to the induction of IFN-γ
expression in these cells [32]. Early production of IFN-γ by
γδ T cells may have a role in linking innate and adaptive im-
mune responses [29]. Furthermore, bovine γδ T lymphocyte
subsets are activated indirectly through cytokines secreted
by T cells, antigen-presenting cells and other accessory cells
[13, 28, 31]. Our results show that the number of WC1^{+} γδ
T cells increased in both the scouring and HT groups and
was associated with increased IFN-γ expression. In addi-
tion, WC1^{+} γδ T cells were also greater in number in the HC
group. This is in accordance with a previous report, which
noted that γδ cell subsets were significantly larger in fetal
and maturing calves than in mature animals [39].

Probiotics including *Lactobacillus* and *C. butyricum*
induce the release of IL-6 and TNF-α, which are involved
in nonspecific immune responses [11]. It was previously
reported that pre-weaning calves given a probiotic had
increased serum IgG and IFN-γ levels [35]. Furthermore,
probiotics including *E. faecium* and *C. butyricum* or
Lactobacillus induced the production of pro- and anti-
inflammatory cytokines, including IL-6, IFN-γ and TNF-α,
in human PBMCs [5, 14]. In the present study, increased
IL-6, INF-γ and TNF-α mRNA expression was observed in
the scouring treated, HT and HC groups. In contrast, IL-8
mRNA expression was unchanged, but it was elevated in
the scouring groups. Greater expression of IL-8 is likely
related to neutrophil function and/or inflammatory stimula-
tion in bovines [2]. Therefore, IL-8 mRNA expression was
changed only in scouring calves, but not in healthy calves.
It was reported that TNF-α mRNA expression was related to
age-related changes in intracellular cytokine production [9].
In the present study, a higher TNF-α mRNA expression level
was also noted in the healthy control calves.

TLR expression is associated with bacterial immune
stimulators, including LAB, in the GIT of calves [25]. In
our preliminary study, it was revealed that calves given the
probiotic had a stable ruminal pH compared to the controls.
It was also reported that a significant increase in ruminal
pH was correlated with increasing taxonomic units of
Clostridia in the gastro-intestine of cattle [30]. Therefore,
improved diversity and bacterial immigration might affect
intestinal bacterial populations. However, the bacterial flora

was not evaluated in the present study. In bovines, the expression of TLR2 was obvious on antigen-presenting cells with a high level expression on peripheral monocytes and gastrointestinal macrophages [19, 21, 25]. Moreover, bovine TLR2 cell surface molecules on monocyte-derived DCs and intestinal macrophages are most responsible for the recognition of Gram-positive bacteria [3, 6, 10]. In human PBMCs, monocytes presenting TLRs played an important role in *Lactobacillus*-induced immunological responses [5]. Mechanisms indicating a direct role for TLR2 include bacterial reorganization by epithelial macrophages in calves [25]. However, CD14^{+} cells are involved with the TLR4 and MD2 complex, which is essential for the response to most Gram-negative bacteria [19, 25]. Among probiotics, *Lactobacillus* strains have a rigid cell wall that is resistant to intracellular digestion and can stimulate intestinal macrophages presenting TLR2 to secrete a large quantity of cytokines [33]. In the present study, the numbers of CD14^{+} and TLR2^{+} cells were increased in the scouring and HT calves compared to the controls. A B-cell receptor complex including CD21 is present on mature B cells and ileal Peyer's patch-derived B lymphocytes in young calves [16]. The stimulation of CD21^{+} cells is related to intestinal immune stimuli, and probiotics are known to trigger naive plasma cells to become IgA-producing CD21^{+} B cells [12, 24, 38]. In our study, the number of CD21^{+} cells was increased in the scouring and healthy probiotic-treated calves on day 7.

An interaction between probiotics and the host immune system leads to the interface of intestinal immune cells, such as CD14^{+} and TLR^{+} cell macrophages or DCs and to the release of cytokine stimulators by T and B cells [6, 8, 14]. The activation and balanced response of T cells is an important component of the recovery from GIT disease in cattle [25, 38]. Probiotics contribute to homeostasis of the intestinal bacterial flora and result in scouring recovery in young calves [26, 35, 36].

In the present study, a probiotic was administed to pre-weaning scouring calves for 5 days, and stool samples were examined to discover the underlying cause of scouring. The scouring PPT and PPC or PNT and PNC groups revealed a greater number of peripheral leukocytes and higher cytokine mRNA expression levels on day 7, although the immune marker values were slightly higher in the PPT and PPC groups compared with PNT and PNC groups. Higher immune marker values on day 7 in the both pathogen-positive and pathogen-negative scouring groups (PPT and PNT) might be induced by probiotic administration and scouring causers. Furthermore, the increased numbers of circulating immune cells in HT group indicated the immune-stimulatory effects of the probiotic, as suggested by previous studies [7, 20]. On the other hand, the scouring calves given no probiotic (PPC and PNC groups) exhibited greater expression of immune markers on day 7, which might be associated with the usual reaction of immune system through the GIT disorders [37]. The recovery of scouring in the calves given probiotic was in agreement with that in previous reports [1, 12, 19, 26]. From these findings, probiotic administration to the scouring calves might be effective in both pathogen-positive and pathogen-negative cases. In this study, serum IgG was not analyzed. However, it was previously suggested that the serum IgG concentration was increased by probiotic administration in pre-weaning calves [36].

In conclusion, the administration of a bacteria-based probiotic resulted in increased numbers of CD3^{+} T cells; CD4^{+}, CD8^{+} and WC1^{+} γδ T cells; and CD14^{+}, CD21^{+} and TLR2^{+} cells in scouring PPT, PNT and HT calves, compared with healthy controls. Moreover, higher IL-6, INF-γ and TNF-α mRNA expression levels were noted in peripheral leukocytes, and the probiotic-treated calves recovered from scouring. These findings demonstrate that the administration of a probiotic elicits immunological changes in the systemic circulation of scouring and healthy calves. Considering the effects of probiotics on immune markers in healthy calves, the repeated treatment of healthy pre-weaning calves with a 5-day probiotic regimen could elevate the levels of reduced immune markers, thereby reducing the incidence of scouring in young calves.

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