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Leukocyte Phagocytic Activity with or Without Probiotics in Holstein Calves

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Abstract: Calves are frequently affected by diarrhea in their early stage of lactation period which would be caused by immaturity of immune system of young animals. The previous reports showed that addition of two selected strains *Lactobacillus plantarum* Chikuso-1 and *Candida* sp. CO119 to milk replacer significantly suppressed diarrhea in Holstein calves. It remains unknown, however, whether Chikuso-1 and CO119 could improve immune function of calves. In the present study, changes with day and effects of the microbe feeding on leukocyte phagocytic activities of Holstein calves were examined. Blood samples were collected on day 0 (a day before beginning of the microbe feeding), 9 and 16 from 6.3±1.5 days old control and microbe-fed calves and phagocytic activities of granulocytes and monocytes were determined by flow cytometer. Phagocytic activities were significantly increased with day but the effect of microbe treatment was non-significant. These suggest that calf diarrhea in the early lactation period would be caused partly due to immaturity of leukocyte innate immunity and the effect of Chikuso-1 and CO119 on suppression of calf diarrhea is not likely to be mediated by the system.

Key words: Lactic acid bacteria, yeast, probiotics, leukocyte, phagocytosis, Holstein calves

INTRODUCTION

Diarrhea is one of the main causes of calf mortality and morbidity and also of economic loss in the cattle industry. It is known that calves are affected by diarrhea more severely at early stage of lactation period (Virtala *et al*., 1996), it is assumed that at the time, calves are suffering from certain unhealthy conditions caused mainly by immaturity of immune system. Phagocytosis by granulocytes and monocytes is one of the major innate immune systems against exogenous bacterial and fungal pathogens (Kantari *et al*., 2008), previous investigations have focused on the maturity of phagocytic activities of calf peripheral blood leukocytes after birth. LaMotte and Eberhart (1976) showed that phagocytic activity of calf neutrophils was low at birth and increased after 6 h. Menge *et al*. (1998) also reported that phagocytic activities of calf polymorphonuclear leukocytes and monocytes were higher after 4 h of birth than those after 1 h. However, overall time course of the activities in both granulocytes and monocytes of calves during lactation period is not reported yet. The previous reports have shown that addition of Lactic Acid Bacteria (LAB) and yeast strains, *Lactobacillus plantarum* Chikuso-1 (Cai *et al*., 2003) and *Candida* sp. CO119, to milk replacer significantly decreases fecal scoring in Holstein calves (Kawakami *et al*., 2010). Probiotics are generally known to modulate gut-associated lymphoid and epithelial tissue response to enhance the activities of intestinal and systemic immune system (Madsen, 2001). These suggest that feeding of Chikuso-1 and CO119 could stimulate immune system to suppress calf diarrhea. However, the effect of feeding of the microbe on calf immunity is unknown.

In the present study, therefore, changes with day and effect of the probiotics on the phagocytic acitivities of granulocytes and monocytes were examined in Holstein calves. The results suggest that calf phagocytic activities increase with day during lactation period and Chikuso-1 and CO119 do not enhance the activities.

MATERIALS AND METHODS

Animals, feeding and treatment: All animal experiments in the present study were conducted according to the animal care and use guidelines of the National Institute of Livestock and Grassland Science of Japan. Animals, feeding and treatment were described previously

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(Kawakami *et al*., 2010). Eight Holstein calves at 6.3±1.5 days of age were divided into 2 groups, control (n = 4) and microbe-fed (n = 4). Microbe-fed group received milk replacer containing Chikuso-1 (3.7×10^{11} Colony Forming Unit (CFU head^{-1}) and CO119 (2.6×10^{9} CFU head^{-1}) in the every morning for 28 days whereas no microbe treatment in control group.

Phagocytic activities of granulocytes and monocytes: On day 0 (a day before beginning of the microbe feeding), 9 and 16 blood samples were taken from the jugular vein of each calf before feeding. The blood was collected in 10 mL vacuum tubes containing Na-heparin (TERUMO Co. Ltd., Tokyo, Japan). Phagocytic activities of granulocytes and monocytes of the calves were assayed by PHAGOTEST (ORPEGEN Pharma, Heidelberg, Germany). Briefly, 100 μL of heparinized whole blood from each sample was dispensed in 3 assay tubes, 2 for replicates and 1 for negative control (without incubation procedure for 30 min at 37°C).

The blood was mixed with 20 μL of opsonized FITC-labelled *E. coli* suspension. After incubated for 30 min at 37°C, 100 μL of Quenching Solution was added. The samples were centrifuged, washed with Washing Solution, lysed and fixed with 2 mL of Lying Solution and stained with propidium iodide-containing DNA Staining Solution. Finally, the samples were analyzed on an EPICSXL flow cytometer (Beckman Coulter, Miami, USA) and a live gate was set on the granulocyte and monocyte population, acquired by the forward scatter/side scatter profile. Ten thousands lymphocytes were studied in each sample. Expo32 software (Beckman Coulter) was used to analyze the data. Results were presented as gate (%): a percentage of granulocytes and monocytes showing phagocytosis (ingestion of one or more FITC-labelled *E. coli* per phagocyte) and as Xmean: the number of *E. coli* ingested per phagocyte.

Statistical analysis: Statistical analyses were performed using SAS (2001). Differences of the phagocytic activities were evaluated by repeated measurements ANOVA using the Mixed procedure of SAS. The statistical model included fixed effects for treatment, day and treatment x day interaction, with calf as random effect. If the interaction was significant, simple effects were calculated by using the slice option for the LSMEAN statement. The level of significance was set at $p<0.05$ and at $p<0.1$ for a trend.

RESULTS AND DISCUSSION

Phagocytic activities of granulocytes and monocytes in control and microbe-fed Holstein calves were shown in Table 1 and Fig. 1. There were no significant effects of microbe treatment on granulocyte and monocyte phagocytic activities (Table 1). But significant effect of day was observed (Table 1). Granulocyte gate (%) was significantly increased on day 9 (Fig. 1a, $p<0.01$) and day 16 (Fig. 1a, $p<0.01$) compared with that of day 0. Granulocyte Xmean was significantly increased on day 16 (Fig. 1c, $p<0.01$) compared with that of day 0 and day 9. Monocyte gate (%) was significantly increased on day 16 (Fig. 1b, $p<0.05$) compared with that of day 0. Monocyte Xmean was significantly increased on day 16 (Fig. 1d, $p<0.01$) compared with that of day 0 and 9.

Table 1: Phagocytic activities of control and microbe-fed Holstein calves. Data was presented as gate (%): a percentage of granulocytes and monocytes showing phagocytosis and as Xmean: the number of *E. coli* ingested per phagocyte

	Treatment			p-value		
Activities	Control	Microbe-fed	SE	Treatment	Day	Treatment x day
Granulocytes						
Gate (%)	70.90	81.24	5.36	NS	**	NS
Xmean	9.14	11.00	1.01	NS	**	NS
Monocytes						
Gate (%)	53.00	52.14	2.49	NS	*	NS
Xmean	4.800	5.290	0.28	NS	**	NS

*Significant difference ($p<0.05$). **Significant difference ($p<0.01$). SE: Standard Error of the mean; NS: Not Significant

Immaturity of immune system in young animals is thought to contribute to increased susceptibility to infectious diseases in their early life (Burgio *et al*., 1989). Calves are known to have lower concentrations of immunoglobulin (Adams *et al*., 1992) and lower proportions of circulating B cells (Senogles *et al*., 1978) than those of adult cows, suggesting the immaturity of humoral immunity in calves. However, the information about the maturity of calf innate immunity was limited. LaMotte and Eberhart (1976) showed that phagocytic activity of calf neutrophils was low at birth, increased after 6 h, decreased after 12 h and held constant until 6 days after birth.

The present results showed that calf phagocytic activities of peripheral granulocytes and monocytes increased with day from experimental day 0-9 and 16, which are equivalent to days 6, 15 and 22 after birth, respectively. On the whole, changes of phagocytic activity of calf granulocytes estimated from previous and present results are likely as follows: the activity is low at birth, increased after 6 h and decreased after 12 h, held constant until 6 days and increased again on day 15 and 22 after birth. The time course of changes of monocyte phagocytic activity in calves is however, not known exactly since there are no reports showing the activity during 6 days after birth. More detailed researches are required to verify the change of phagocytic activity in calf monocytes. The previous report showed that addition of Chikuso-1 and CO119 to milk replacer significantly

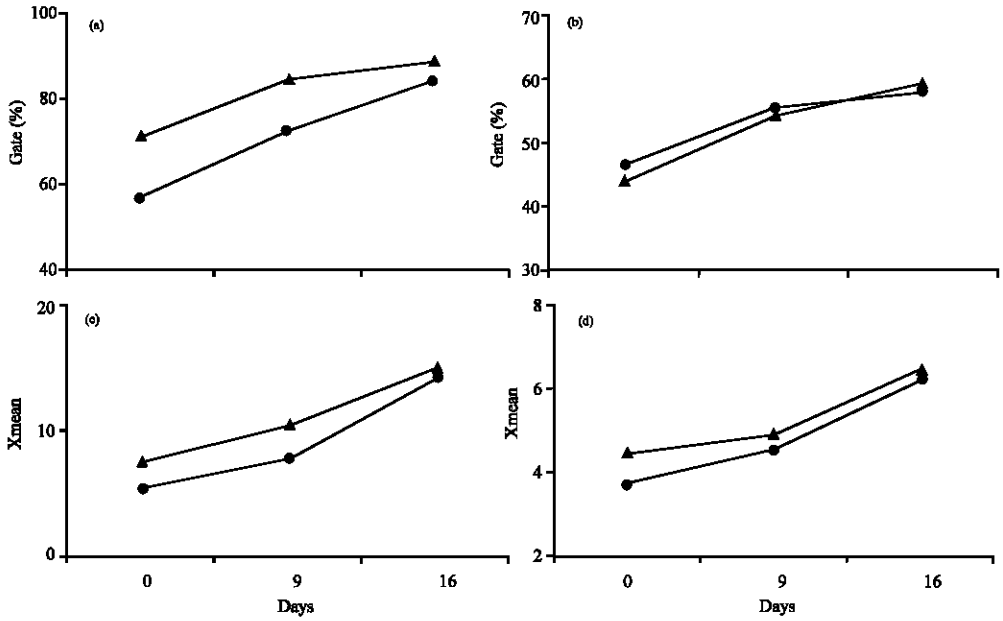

Fig. 1: Phagocytic activities of granulocytes (a, c) and monocytes (b, d) of Holstein calves. Data was presented as gate (%) (a, b) and as Xmean (c, d). ●: control group; ▲: microbe-fed group

decreased fecal scoring of Holstein calves (Kawakami *et al*., 2010) suggesting that feeding of the microbe suppressed calf diarrhea.

The researchers assumed, therefore that the microbe could suppress diarrhea by improving calf immune system. In the present study however, feeding of Chikuso-1 and CO119 did not enhance phagocytic activities of granulocytes and monocytes in Holstein calves. Magalhaes *et al*. (2008) also reported that feeding of *Saccharomyces cerevisiae* suppressed diarrhea but did not significantly increased the number of phagocytized bacteria and proportion of phagocytized bacteria killed in neutrophils of Holstein calves.

These suggest that innate immunity do not mediate the effect of probiotics on suppression of diarrhea at least in calves. The mechanisms remain unknown, probiotics might exert their beneficial effects by modifying intestinal microbial flora of host animals.

The data showed that feeding of Chikuso-1 and CO119 significantly increased the number of fecal LAB in early stage of lactation period in Holstein calves (Kawakami *et al*., 2010). Chikuso-1 and CO119 might increase the number of intestinal LAB which could antagonize pathogenic bacteria causing calf diarrhea. Alternatively, the increased LAB might stimulate gut associated lymphoid and epithelial tissues to activate local immune responses in intestine (Heczko *et al*., 2006). Further studies are needed to clarify the mechanisms for suppression of calf diarrhea by the microbe.

CONCLUSION

The present results concluded that calf phagocytic activities of granulocytes and monocytes increase with day during lactation period and feeding of Chikuso-1 and CO119 do not enhance the activities in Holstein calves. These suggest that calf diarrhea in the early lactation period would be caused partly by immaturity of leukocyte innate immunity and the effect of the probiotics on suppression of calf diarrhea is not likely to be mediated by the system.

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