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Effect of Milk Fermented with *Lactobacillus acidophilus* NCDC15 on Nutrient Digestibility, Faecal Biomarkers and Immune Response in Murrah calves

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HIGHLIGHTS

- Inclusion of Lactobacillus acidophilus NCDC15 in the form of fermented milk at 100, 200 and 300 mL/calf/day in Murrah buffalo calves.
- Fermented milk improved immunity and faecal biomarkers in Murrah buffalo calves without any adverse effect on nutrient utilization
- Responses were more evident in 200 and 300 mL probiotic fermented milk-fed groups as compared to 100 mL.

Abstract: In neonates, rapid change in diet imbalances gut health allowing colonization of opportunistic pathogens that confer harmful effects on animal health causing reduced digestion and malabsorption of nutrients. In this milieu, probiotic feeding can be a promising approach in promoting animal health and stabilization of gastrointestinal microbiota. Hence, the present study was designed to investigate the effect of *Lactobacillus acidophilus* NCDC15 enriched fermented milk on nutrient digestibility, faecal biomarkers and immune response in Murrah buffalo calves. Twenty-four, neonatal calves (5-7 days) were randomly allocated into four groups for 90 days. The control group (CT) was provided a basal diet of calf starter and green fodder (maize and jowar), without any probiotic fermented milk (PFM) supplementation. Basal diet was supplemented with probiotic fermented milk at 100, 200 and 300 mL/calf/day, in PFM100, PFM200 and

PFM300 groups, respectively. Nutrient digestibility remained similar among the groups. Faecal acetate was higher (P<0.05) in PFM200 and PFM300, while, faecal butyrate was increased (P<0.05) with all levels of probiotic supplementation than control. Faecal *Lactobacillus* and *Bifidobacterium* count were increased (P<0.05) with a concomitant reduction in coliform population (P<0.05) among all the treatments. Cell-mediated and humoral immune response were higher (P<0.001) in PFM200 and PFM300 than CT. Overall, it can be concluded that inclusion of *Lactobacillus acidophilus* NCDC15 in the form of fermented milk upto 300 mL/calf/day improved immunity and faecal biomarkers in Murrah buffalo calves without any adverse effect on nutrient utilization which may positively impact growth performance in Murrah buffalo calves.

Keywords: Buffalo calves; Fermented milk; Faecal biomarkers; Immunity; *Lactobacillus acidophilus* NCDC15.

INTRODUCTION

Gastrointestinal tract (GIT) of newborn calves is rapidly colonized by an array of microbiota during and after birth [1] and is a critical period for the gut formation. The immune system of neonates is immature in the initial weeks of life and often results in high morbidity and mortality, as results of inappropriate colostral supply or contact to pathogenic microbes [2]. Furthermore, rapid change in diet, environment and other stresses [3, 4] imbalances gut health and allows colonization of opportunistic pathogens that confer harmful effects on animal health [5, 6] particularly scours [7] accompanied by reduced digestion and malabsorption of nutrients [8]. Hence, it becomes tough to diminish the occurrence of such gastrointestinal infections in young calves to gain optimum growth and succeeding productivity in later life.

Antibiotics have been used to treat and prevent intestinal illnesses since the 1940s, and these practices have resulted in the accumulation of antimicrobial residues in animal products as well as the emergence of microbial drug resistance [9]. Therefore, the European Union banned the application of antibiotics in food animals since 2006 [10] and this necessitated a worldwide consciousness to discover possible alternatives to replace antibiotics while not negotiating animal safety and consumer health perspectives. In this milieu, probiotics can be a promising approach in promoting animal health and stabilization of gastrointestinal microbiota. Probiotics are live microorganisms when administrated in adequate amount confers beneficial health effects to host. Microorganism most extensively studied in these aspects are Lactobacilli species [11]. It is well known that feeding probiotics to calves [12, 13, 14] enhanced gut health, digestive ability and growth performance [15, 16, 17]. Probiotics can improve immunity by inducing serum immunoglobulin secretion in early-weaned calves [18]. Humoral immunity also increased due to the combined effects of probiotic and prebiotic in calves [19]. Although these studies well proved the effectiveness of probiotic administration in cattle calves but till date studies on indigenous Murrah buffalo calves are limited. Furthermore, apart from host species the response of probiotic differs upon the type and efficacy of microorganisms administered in host.

In support of the earlier findings, it was assumed that the administration of fermented milk with probiotic to Murrah buffalo calves will improve the gut microbiota and may possibly enhance their performance along with advantageous repercussions on gut health and faecal characteristics of Murrah buffalo calves. This also believed that fortified milk supplemented with probiotics can have beneficial effects on the immune response in calves.

The primary concern of this experiment was to determine the effects of fermented milk with probiotic *Lactobacillus acidophilus* (*L. acidophilus*) NCDC15 on apparent nutrient digestibility, faecal biomarkers and immune responses in Murrah buffalo calves.

MATERIAL AND METHODS

The present study of 90 days was conducted in the Livestock Research Centre of ICAR-National Dairy Research Institute (NDRI), Karnal, Haryana, India. Experimental protocol involving handling and management of animals were carried out in compliance with applicable rules and guidelines laid down by the Institutional Animal Ethics Committee (IAEC) constituted under Committee for the Purpose of Control and Supervision of Experiments in Animals (CPCSEA), New Delhi, Government of India.

Preparation of probiotic enriched fermented milk for administration to calves

Pure, freeze-dried culture of *L. acidophilus* NCDC15 was procured from the National Collection for Dairy Culture (NCDC), Dairy Microbiology Department, ICAR-NDRI, Karnal and revived in MRS broth. *L. acidophilus* NCDC15 selected for milk fermentation had proven probiotic potential [20]. The 1 mL of inoculum was added to 9 mL phosphate-buffered saline (PBS) and vortexed thoroughly. Serial 10-fold dilutions were then made in PBS. A 100-µL volume of each dilution was inoculated onto MRS agar. The MRS plates were incubated anaerobically at 37°C for 48 h for isolation of lactic acid bacteria. Lactic acid bacteria were identified by colony morphology and colony counts were recorded. All testing was performed in triplicate. Based on previous studies we select the 10^8 cfu/ml and to make probiotic enriched fermented milk, a loop-full of inoculum was added to the milk and incubated for 24 hours at 37 °C. At every alternate day, colony-forming unit (CFU) per mL of fermented milk was counted to test the viability of bacterial cells and maintained at a level of 10^8 CFU/mL throughout experimental feeding.

Animal distribution, housing, treatments and feeding regime

Twenty-four neonatal Murrah buffalo calves aged 5-7 days and 31 ± 2.0 kg BW were randomly allocated into four groups of six animals in each. Buffalo calves were weaned from their dams and housed individually in well-ventilated calf pens having adequate access to sunlight. Before accommodating the experimental calves, all of pens were cleaned with detergent, disinfected with potassium permanganate solution and washed with diluted phenyl to ensure appropriate preventive measures against various contagious and infectious diseases. Individual pens were well equipped with detached feeder and waterer to allow free access to feed and water, respectively, during the entire experimental period. Calves were dewormed as per the standard deworming schedule.

The control (CT) group received a basal diet consisting of calf starter and green fodder as well as whole milk without any probiotic supplementation. In PFM100, PFM200 and PFM300 groups, the basal diet remained same except that the probiotic fermented milk was provided at 100, 200 and 300 mL/calf/day, respectively. Fermented milk was prepared from milk offered to the animals as per their schedule, to make sure that no additional intake by animals in treatment groups. The calf starter containing maize, bajra, groundnut cake (GNC), soybean meal (SBM), mustard oil cake (MOC), wheat bran, rice polish, mineral mixture was formulated (Table 1) using quality ingredients and offered from the second week onwards. The animals were given *ad libitum* freshly harvested green forage comprising maize and jowar. All the calves had access to clean water *ad libitum* 24h. Whole milk was fed to the calves at 1/10th of actual BW up to the 1st two weeks followed by 1/15th, 1/20th and 1/25th of actual BW in the 3rd to 4th week, 5th to 6th week and 7th to 8th week of respectively, after morning and evening diet distribution.

Table 1. Gross and chemical composition of the basal diet.

Ingredients	Parts (%)
Maize	28
Bajra	5
Ground nut cake	10
Soybean meal	15
Mustard oil cake	13
Wheat Bran	15
Rice polish	11
Mineral Mixture	2
Salt	1

Chemical composition of the basal diet

Nutrients (%)	Calf starter	Maize green	Jowar green	Milk	Skim milk
DM	89.6	24.5	27.2	15	9
OM	91.2	90.4	93.5		
CP	22.1	9.8	8.6		
EE	4.3	2.3	1.6		
NDF	24.3	63.1	61.3		
ADF	14.2	30.4	32.4		

Structural growth measurements, digestion trial and proximate analysis of samples

Structural growth measurements were monitored by assessing body length, wither height, hip-height and heart girth using “tape measures". Following 60 days of experimental feeding, a digestion trial of seven days was conducted, in which daily dry matter intake (DMI) and total faeces voided were listed. For estimation of N, faecal samples ($1/50^{th}$ fraction of the total voiding) were pooled and conserved in 25% sulphuric acid for 7 days from each animal. Representative samples (feeds, orts and faeces) were oven-dried at 60°C for 48 h and grounded in a hammer mill of 1 mm sieve size and tested for proximate principles [21] such as total ash (942.05), ether extract (920.39), Kjeldahl nitrogen (984.13), neutral detergent fibre (2002.04), acid detergent fibre (973.18).

Faecal collection, sampling and biomarkers estimation

Faeces of the calves were scored for faecal consistency using a 1-4 point scale (1 = Normal and firm faeces, 2 = Soft or loose faeces, 3=Runny or very loose faeces and 4 = Watery faeces) [22]. Calves of fecal consistency 3 or 4 have been graded as diarrhoeal. Hydrated intervention was given when the calve had pale and dry mucous membranes along with diarrhea: 8 g of NaCl, 8 g of $NaHCO_3$, 2 g of KCl, 15 g of dextrose, and 2 L of warm water [23]. Faecal sample about 10-15 g was collected by a rectal massage from each animal following perianal cleansing with dilute betadine solution with sterile gloves at the monthly interval on d0, d30, d60, and d90 to evaluate faecal pH, ammonia (NH_3), lactate, volatile fatty acids (VFA) and microbiota populations. The samples were collected in sterile 50 mL falcon tubes at approximately 07:00h and transferred to the laboratory for further analysis. The pH of the samples was determined directly with a digital pH meter before aliquoting of the faeces (pH Spear, Eutech Instruments, Klang Selangor, DE, Malaysia, pH Range: -1.00 to 14.00 pH, Resolution: 0.01 pH, Accuracy: ±0.01 pH). The pH meter was specially designed to test the semi-solid samples by direct pH measurement [24]. Additional three faecal aliquots were prepared to evaluate fermentative end products *i.e.,* ammonia, lactate, and VFA [24]. Approximately 2.0g of freshly collected faeces was briefly mixed with 6 mL of 6.0 N HCl and processed at –20ºC for subsequent ammonia analysis [25]. A 2g fresh faecal aliquot was blended with 4 mL of freshly prepared 25% (w/v) metaphosphoric acid and centrifuged (10,000 rpm) for 10 min. The resultant supernatant was used for the analysis of total VFA [26]. The third aliquot of approximately 2g was diluted with 4 mL of distilled water and centrifuged for 10 min at 10,000 rpm, and the supernatant was processed for analysis of lactate [27].

Two sets of 10-fold (10^{-1} to 10^{-8}) serial dilutions with a combined 10 mL volume consisting of 1 g of homogenized fresh faeces and 9 mL of NS (normal saline: 0.9% NaCl) were enumerated for bacterial populations and plated in duplicate onto selective media [28]: for lactobacilli-MRS agar (Himedia), for coliforms-EMB Agar, Levine (Himedia), Clostridial agar (Himedia) for clostridia and Bifidobacteria agar

(Himedia) for bifidobacterium. Specific agar plates were aerobically incubated for lactobacilli and coliforms for 24 and 48 hours at 37°C; respectively. The bifidobacteria and clostridial agar were anaerobically incubated at 37°C. After incubation the agar plates were appraised for bacterial growth. The bacterial colonies were counted as CFU/g faeces and converted into $\log_{10}$ CFU/g. CFU were described as distinct colonies with a diameter of at least 1 mm [29].

Cell-mediated and humoral immune response

After 75 days of experimental feeding, all the calves were assessed for cell-mediated immune (CMI) response by measurement of skin indurations using *in vivo* DTH (delayed-type hypersensitivity) test against phytohemagglutinin-phaseolus vulgaris (PHA-p; Sigma, St Louis, MO, USA) as a mitogen [30]. Until conducting the DTH test, the skin area to be examined (both sides of the neck region) was cleaned and shaved before 24 h. An area of about one square cm was encircled on both sides of the neck region, with a black marker pen. Skin thickness was measured using an electronic digital Vernier caliper (measuring range 0-150mm), which reflected a basal value (0 h). All the animals were injected with 100 µL PHA-p intradermally (50 µg/100 µL in phosphate-buffered saline, PBS) solution on one side and normal saline solution on the other side of neck area as a negative control. Skin thickness was measured post-inoculation at 6, 12, 18, 24, 36, 48 and 72 h and was expressed as percentage of skin thickness increase relative to the value at 0^{th} h.

In case of humoral immune response (HIR), calves were injected intravenously (IV) with 10% suspension of 1 mL washed chicken red blood cell (CRBC) in 0.15M NaCl after 60 days of experimental feeding. Serum samples were collected before injection (0 days) and then on d7, d14, d21 and d28 and processed for antibody determination at -20°C. The sera samples were thawed, inactivated for 30 min at 56°C, and tested for antibody titre using the microtitre haemagglutination (HA) procedure [31]. The HA titers were read after 3 h at room temperature and expressed as $\log_2$.

Statistical analysis

Data generated in the present experiment were analyzed by using the Statistical Package for Social Sciences (SPSS, version 20.0 Chicago, USA) and presented as mean ± standard error.

Data from digestibility trials (intake and digestibility) were analyzed as a randomized complete design using the General linear model of the SPSS based on the statistical model:

$Y_{ij} = \mu + Ti + eij,$ where,

Y_{ij} = dependent variable of the *j*th calf on the *i*th treatment

μ = overall mean

Ti = the fixed effect of *i*th treatment effect (*i*= 100, 200, 300 mL/calf/day of PFM)

e_{ij} = random residual (error) associated with the dependent variable from the *j*th calf on the *i*th treatment. Means were tested using Duncan's multiple range tests.

Before statistical analysis, fecal microbial counts were transformed into $\log_{10}$. Continuous data collected over time (i.e., monthly structural growth measurements, forthnightly average fecal score, monthly faecal pH, faecal ammonia N, lactate, VFAs and humoral and cell mediated immunity) were analyzed using the linear model:

$Y_{ijk} = \mu + T_i + P_j + (TP)_{ij} + e_{ijk}$ where

μ = general mean

T_i= effect of *i*th treatment (*i*= 100, 200, 300 mL/calf/day of PFM)

P_j= effect of *j*th 90 days period

$(TP)_{ij}$= effect of interaction between treatment and 90 days period

e_{ijk}= random error. Treatment differences with ($P < 0.05$) were considered as a significant statistic.

RESULTS

Body structural Measurements and apparent nutrient digestibility coefficient

Data concerning to structural growth measurements (Table 2) implied that initial hip height (cm), heart girth (cm), wither height (cm) and body length (cm) were similar ($P > 0.05$) across the groups. Final hip height was improved ($P < 0.05$) by the inclusion of probiotics in all the supplemented groups. In a similar line, heart girth was increased in probiotics fed groups with the trend of PFM300~ PFM200> PFM100>CT. On the other hand, wither height and body length was higher ($P < 0.05$) in PFM200 and PFM300 compared to CT, whereas, PFM100 had a characteristic that was comparable to that of other groups. The values of apparent

digestibility coefficient of various nutrients [Dry matter (DM), Organic matter (OM), Crude protein (CP), Neutral detergent fiber (NDF), and Acid detergent fiber (ADF)] are furnished in Table 3. There was no influence ($P > 0.05$) on supplementation of probiotic fermented milk on apparent digestibility coefficient among the groups.

Faecal biomarkers

Faecal score, examined per pen individually, remained unvaried ($P > 0.05$) in all groups but period-wise (Figure 1) significant ($P < 0.05$) impact was also observed. Fortnightly the average faecal consistency scores are given in Table 4. The average faecal pH (Table 4) was decreased ($P < 0.05$) in probiotic fermented milk administered groups as compared to control. The period-wise (Figure 2) comparison also revealed significant decreased ($P < 0.05$) effect on faecal pH.

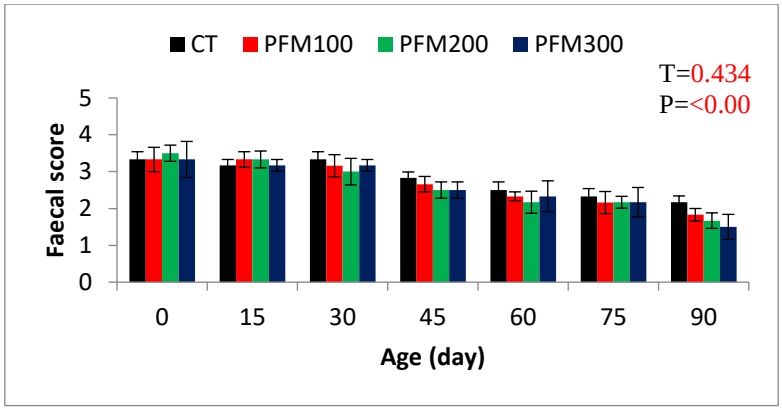

Figure1. Fortnightly faecal score in Murrah buffalo calves supplemented with probiotic enriched fermented milk

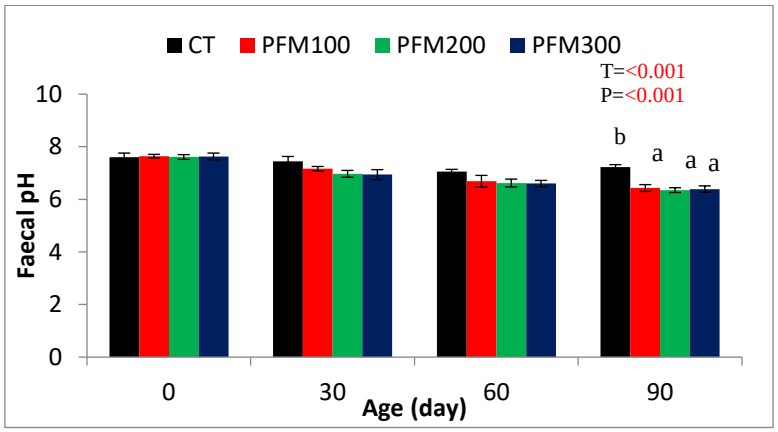

Figure 2. Faecal pH in Murrah buffalo calves supplemented with probiotic enriched fermented milk.

The data apropos of faecal microbiota (Table 5) indicated that *Lactobacillus* population (log_{10} cfu/g in fresh faeces) was improved ($P < 0.05$) in all probiotic supplemented groups as compared to control. In addition, there was also a treatment and time relationship for faecal *Lactobacillus* count (P = 0.048) and this indicated that the lactobacilli count was higher during the experimental period due to probiotic supplementation. The *Bifidobacterium* population in all supplemented groups responded positively ($P < 0.001$) to probiotic supplementation in comparison to control (CT). On the other hand, coliform count (log_{10} cfu/g in fresh faces) was significantly ($P < 0.05$) decreased in all the supplemented group compared to CT, however, no influence ($P > 0.05$) was found for clostridia count (log_{10} cfu/g in fresh faeces) in all the groups.

The data presented in Table 4 illustrates the effect of *L. acidophilus* supplementation on faecal ammonia and lactate. In all probiotic supplemented groups, the concentration of faecal ammonia (µmol/g fresh faeces) was decreased (P < 0.05) relative to the control (CT). While the opposite trend was observed for faecal lactate levels (µmol/g of fresh faeces) which was increased ($P < 0.05$) in all probiotics administered groups as

compared to control. Moreover, there was significant effect of period-wise comparison on faecal ammonia (*P* < 0.001) and lactate (0.005) in Figure 3 and Figure 4 respectively, but period * treatment interaction remained unaffected**.** The total VFAs data illustrated in Table 6 showed that among the faecal VFAs, acetate level was increased (P < 0.05) in PFM200 and PFM300 as compared to the other two groups. However, propionate and butyrate were increased in all the probiotics fed groups as compared to control. On the other hand, there was no effect of period and treatment * period interaction on faecal VFA concentrations with supplementation of probiotics.

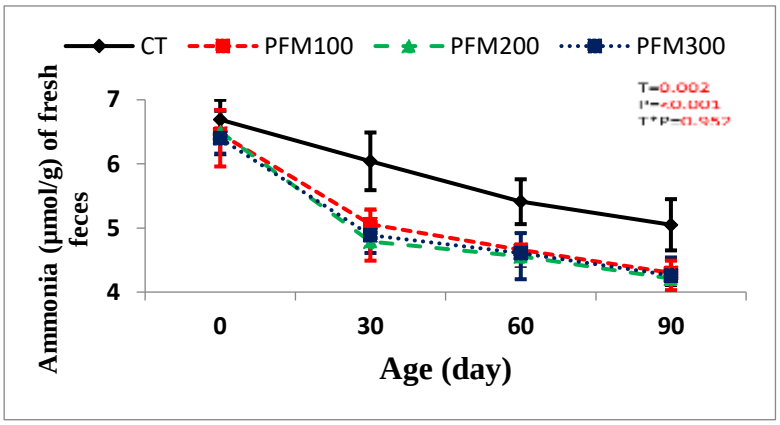

Figure 3. Faecal ammonia in Murrah buffalo calves supplemented with probiotic enriched fermented milk.

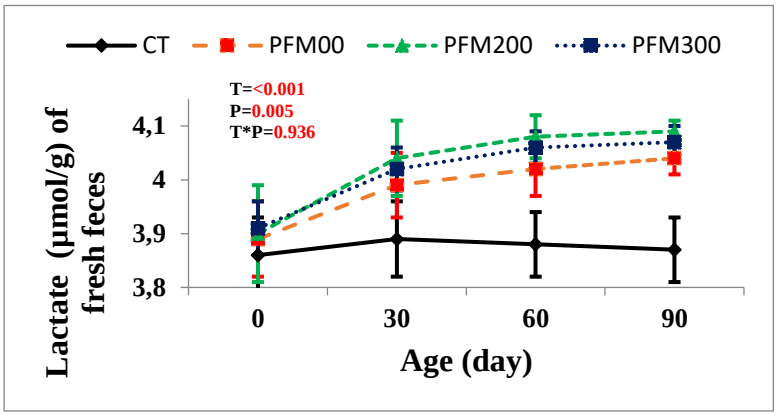

Figure 4. Faecal lactate in Murrah buffalo calves supplemented with probiotic enriched fermented milk.

Immune response

The DTH response results to PHA-p in the form of an absolute increase in skin induration (Figure 5). There was significant (*P* < 0.001) increases in absolute skin thickness (mm) in PFM200 and PFM300 when compared to CT. The HI response data (Figure 6) assessed as antibody responses to chicken-erythrocytes (CRBC) by HA test indicated that the antibody (HA) titre $\log_2$ was significantly higher (P < 0.05) in PFM200 and PFM300 groups as compared to CT and that of PFM100 was comparable to rest of groups. Additionally, the HI response data also showed that titre of antibody in all groups displayed a continuous increase up to 14-days post-inoculation followed by a 21-days decrease.

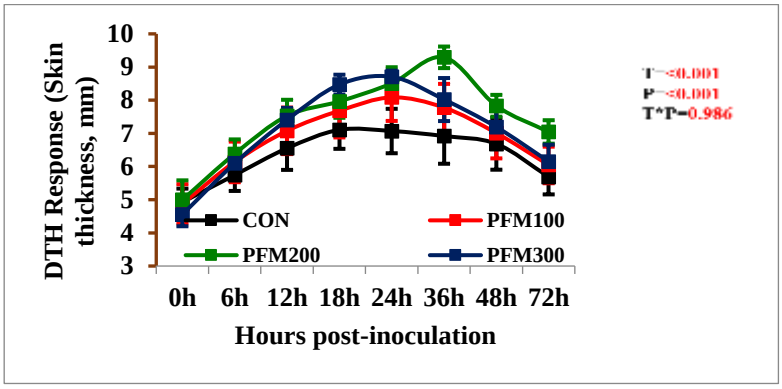

Figure 5. DTH response to intradermal PHA-P in in Murrah buffalo calves supplemented with probiotic enriched fermented milk.

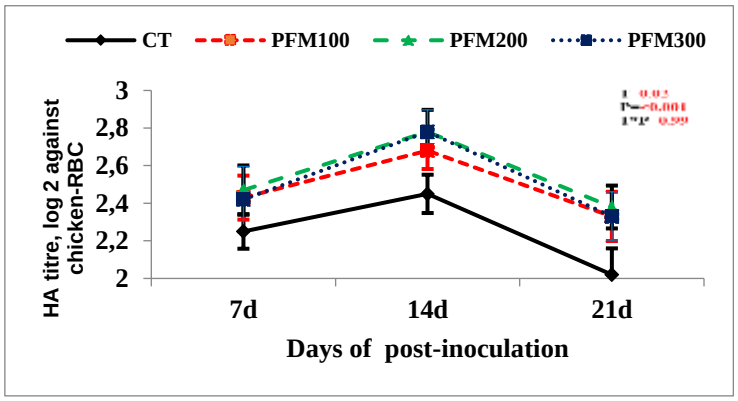

Figure 6. Antibody titre against C-RBC in Murrah buffalo calves supplemented with probiotic enriched fermented milk.

Table 2. Body measurements in Murrah buffalo calves supplemented with probiotic enriched fermented milk.

Attributes	Dietary groups†				Period mean	‡Significance		
	CT	PFM100	PFM200	PFM300		T	P	T×P
				Hip height (cm)				
Initial	79.38±0.84	80.13±1.35	84.23±1.36	83.88±0.66	81.90^{p}±1.37	<0.001	<0.001	0.773
30 days	83.83±0.98	86.16±1.22	89.13±1.53	88.23±0.26	86.84^{q}±1.33			
60 days	89.25±0.96	92.75±1.28	93.75±1.67	92.83±0.40	92.14^{r}±1.30			
Final	96.18±0.72	99.21±0.86	99.50±1.58	99.16±0.69	98.51^{s}±1.11			
Average	87.16^{a}±2.74	89.56^{b}±3.17	91.65^{bc}±2.75	91.03^{c}±2.41				
				Heart girth (cm)				
Initial	76.91±0.89	77.00±1.06	81.75±1.23	81.66±1.11	79.33^{p}±1.41	<0.001	<0.001	0.865
30 days	81.21±1.23	82.33±1.20	86.50±1.23	86.41±0.88	84.11^{q}±1.45			
60 days	85.83±1.01	88.00±1.21	92.5±1.08	92.66±0.98	89.75^{r}±1.58			
Final	89.51±0.82	93.41±1.28	97.66±0.95	97.33±1.05	94.48^{s}±1.69			
Average	83.37^{a}±2.18	85.18^{a}±2.79	89.60^{b}±2.72	89.52^{b}±2.66				
				Wither height (cm)				
Initial	76.98±1.04	78.50±0.44	83.00±1.06	82.21±1.01	80.17^{p}±1.35	<0.001	<0.001	0.997
30 days	81.26±0.93	83.76±0.62	88.00±1.03	87.31±1.17	85.08^{q}±1.44			
60 days	85.66±0.95	88.83±0.61	93.06±1.03	91.73±1.28	89.82^{r}±1.51			
Final	91.96±0.96	94.95±0.51	98.78±1.03	97.13±1.43	95.70^{s}±1.44			
Average	83.97^{a}±2.84	86.61^{b}±2.59	90.71^{c}±2.63	89.60^{c}±2.56				
				Body length (cm)				
Initial	52.16±0.44	53.05±0.66	56.16±1.28	58.06±0.99	54.86^{p}±1.29	<0.001	<0.001	0.933
30 days	56.88±0.34	57.58±0.72	61.31±1.37	62.58±1.16	59.59^{q}±1.36			
60 days	61.58±0.58	63.10±0.71	66.45±1.18	66.66±0.87	64.45^{r}±1.21			
Final	67.68±0.53	69.65±0.79	72.65±1.06	72.06±0.94	70.51^{s}±1.15			
Average	59.57^{a}±2.43	60.84^{a}±2.67	64.14^{b}±2.79	64.84^{b}±2.34				

†Basal diet with no supplementation (CT) or supplemented as *Lactobacillus acidophilus* PFM100 (100 mL/calf/d), PFM200 (200 mL/calf/d), PFM300 (300 mL/calf/d).$^{a,b,c/p,q,r,s}$ Means bearing different superscripts in a row (a,b,c) or column (p,q,r,s) differ significantly (P<0.05) ‡Significant effects of dietary treatment (T), period (P) or their interaction (T**×**P)

Table 3. Nutrient utilization in Murrah buffalo calves supplemented with probiotic enriched fermented milk

Attributes	Dietary groups†				P value
	CT	PFM100	PFM200	PFM300	
		Dry matter			
Intake (g/day)	1409.5±36.76	1524.3±85.20	1525.7±60.94	1559.6±74.00	0.428
Digested (g/day)	1045.3±36.57	1129.6±73.28	1156.3±46.12	1191.4±44.54	0.262
Digestibility (%)	73.88±1.51	73.61±1.96	75.71±1.31	76.09±1.06	0.560
		Organic matter			
Intake (g/day)	1221.6±43.46	1371.8±76.30	1371.9±55.00	1402.2±66.56	0.189
Digested (g/day)	979.7±78.82	1083.2±67.21	1105.4±48.38	1137.0±45.74	0.327
Digestibility (%)	78.19±3.65	78.49±1.55	80.46±1.29	80.84±0.95	0.755
		Crude protein			
Intake (g/day)	152.41±7.78	170.79±7.21	167.27±7.44	166.29±9.08	0.395
Digested (g/day)	109.08±7.22	123.02±6.88	122.50±5.78	124.13±5.97	0.336
Digestibility (%)	71.26±1.86	71.96±2.44	73.25±1.18	74.83±1.14	0.467
		Neutral detergent fiber			
Intake (g/day)	837.93±32.81	931.43±54.05	958.28±29.16	982.81±41.95	0.095
Digested (g/day)	579.69±28.37	629.04±43.11	670.74±19.90	690.73±18.41	0.064
Digestibility (%)	68.76±1.70	66.69±1.94	69.84±1.38	69.89±1.01	0.442
		Acid detergent fiber			
Intake (g/day)	522.02±18.40	563.21±38.57	572.76±25.89	535.68±32.46	0.606
Digested (g/day)	327.05±18.16	357.02±28.82	367.23±14.38	352.51±33.38	0.709
Digestibility (%)	61.86±2.09	62.40±2.57	64.03±2.64	61.55±3.51	0.922

†Basal diet with no supplementation (CT) or supplemented as Lactobacillus acidophilus PFM100 (100 mL/calf/d), PFM200 (200 mL/calf/d), PFM300 (300 mL/calf/d)

Table 4. Faecal score, pH, ammonia and lactate in Murrah buffalo calves supplemented with probiotic enriched fermented milk.

Attributes	Dietary groups†				Significance‡		
	CT	PFM100	PFM200	PFM300	T	P	T×P
Feacal Score (1-4 point)	2.81±0.10	2.69±0.13	2.62±0.13	2.60±0.15	0.434	<0.001	0.999
Feacal pH	7.33^{b}±0.08	6.98^{a}±0.12	6.89^{a}±0.11	6.89^{a}±0.12	<0.001	<0.001	0.099
Ammonia (µmol/g) of fresh feces	5.80^{a}±0.22	5.12^{b}±0.21	5.01^{b}±0.23	5.04^{b}±0.23	0.002	<0.001	0.952
Lactate (µmol/g) of fresh feces	3.38^{a}±0.03	3.99^{b}±0.03	4.03^{b}±0.03	4.01^{b}±0.02	<0.001	0.005	0.936

†Basal diet with no supplementation (CT) or supplemented as *Lactobacillus acidophilus* PFM100 (100 mL/calf/d), PFM200 (200 mL/calf/d), PFM300 (300 mL/calf/d) ^{a,b}Means bearing different superscripts in a row (a,b) differ significantly (P<0.05) ‡Significant effects of dietary treatment (T), period (P) or their interaction (T×P)

Table 5. Faecal microbiota of Murrah buffalo calves supplemented with probiotic enriched fermented milk.

Attributes	Dietary groups†				Period mean	Significance‡		
	CT	PFM100	PFM200	PFM300		T	P	T×P
Health positive bacteria (log_{10}cfu/g of fresh faeces) *Lactobacillus (log_{10}cfu/g of fresh faeces)*								
0 day	10.00±01	9.96±0.02	10.01±0.01	9.99±0.01	9.99^{p}±0.01	<0.001	0.018	0.048
30 days	9.88^{a}±0.7	10.06^{b}±0.01	10.11^{b}±0.04	10.10^{b}±0.03	10.04^{p}±0.03			
60 days	9.69^{a}±0.4	10.11^{b}±0.02	10.12^{b}±0.04	10.11^{b}±0.03	10.01^{p}+±0.04			
90 days	9.83±0.9	10.13±0.03	10.14±0.04	10.12±0.02	10.05^{p}±0.07			
Average	9.85^{a}±0.07	10.07^{b}±0.02	10.09^{b}±0.02	10.08^{b}±0.02				
Bifidobacterium (log_{10}cfu/g of fresh faeces)								
0day	9.86±0.03	9.86±0.02	9.85±0.03	9.80±0.05	9.84^{p}±0.04	0.019	<0.001	0.091
30 days	9.95^{ab}±0.02	9.94^{a}±0.02	10.01^{b}±0.01	10.00^{ab}±0.02	9.97^{q}±0.02			
60 days	9.99±0.02	10.02±0.02	10.04±0.01	10.04±0.02	10.02^{qr}±0.02			
90 days	9.98^{a}±0.01	10.09b±0.01	10.08^{b}±0.01	10.08^{b}±0.02	10.06^{r}±0.02			
Average	9.94^{a}±0.02	9.98^{b}±0.02	9.99^{ab}±0.02	9.98^{ab} ±0.03				
Health negative bacteria (log_{10}cfu/g of fresh faeces) *Coliform (log_{10}cfu/g of fresh faeces)*								
0day	9.88±0.05	9.86±0.03	9.90±0.02	9.89±0.03	9.89^{q}±0.02	<0.001	0.022	0.413
30 days	9.87±0.04	9.81±0.05	9.83±0.02	9.87±0.03	9.84^{pq}±0.02			
60 days	9.90±0.04	9.79±0.03	9.80±0.03	9.80±0.04	9.82^{pq}±0.02			
90 days	9.90^{b}±0.03	9.76^{a}±0.02	9.77^{a}±0.03	9.78^{ab}±0.04	9.80^{p}±0.02			
Average	9.89^{b}±0.02	9.80^{a}±0.02	9.82^{a}±0.02	9.84^{a}±0.02				
Clostridia (log_{10}cfu/g of fresh faeces)								
0day	9.69±0.05	9.70±0.07	9.70±0.05	9.72±0.06	9.70^{q}±0.05	0.087	0.001	0.396
30 days	9.70±0.08	9.66±0.07	9.65±0.03	9.62±0.09	9.66^{pq}±0.07			
60 days	9.69±0.07	9.59±0.07	9.58±0.08	9.59±0.07	9.61^{p}±0.08			
90 days	9.72±0.06	9.53±0.09	9.52±0.08	9.52±0.09	9.57^{p}±0.11			
Average	9.70±0.01	9.62±0.02	9.61±0.02	9.61±0.02				

†Basal diet with no supplementation (CT) or supplemented as *Lactobacillus acidophilus* PFM100 (100 mL/calf/d), PFM200 (200 mL/calf/d), PFM300 (300 mL/calf/d) $^{a,b/p,q,r}$Means bearing different superscripts in a row (a,b) or column (p,q,r) differ significantly (P<0.05) ‡Significant effects of dietary treatment (T), period (P) or their interaction (T*P)

Table 6. Faecal VFAs of Murrah buffalo calves supplemented with probiotic enriched fermented milk

Attributes	Dietary groups†				Period Mean	Significance‡		
	CT	PFM100	PFM200	PFM300		T	P	T×P
	Acetate (µmol/g) of fresh feces							
0 day	34.03±1.38	32.55±1.47	35.99±0.67	36.78±0.96	34.84±0.71	<0.001	0.472	0.364
30 days	33.10±1.05	35.13±1.35	35.00±1.11	37.08±0.33	35.08±0.61			
60 days	34.02±0.87	35.43±0.79	36.99±0.61	36.91±0.76	35.84±0.49			
90 days	32.16±0.93	35.64±1.17	37.28±0.59	36.98±0.48	35.52±1.42			
Average	33.33^{a}±1.03	34.69^{a}±1.29	36.32^{b}±0.86	36.94^{b}±0.29				
	Propionate (µmol/g) of fresh feces							
0 day	12.55±0.42	16.65±1.14	17.89±0.63	18.43±1.57	16.38±0.82	<0.001	0.452	0.996
30 days	12.45±0.68	17.56±0.55	18.14±0.58	19.39±1.27	16.89±0.87			
60 days	12.87±0.51	17.93±0.45	19.16±1.05	18.90±1.20	17.22±0.85			
90 days	12.69±0.39	17.69±0.72	19.06±0.71	19.80±1.14	17.31±0.90			
Average	12.64^{a}±0.22	17.46^{b}±0.36	18.57^{bc}±0.37	19.13^{c}±0.58				
	Butyrate (µmol/g) of fresh feces							
0 day	4.09±0.19	4.90±0.23	4.67±0.26	4.42±0.27	4.52±0.14	0.013	0.702	0.817
30 days	4.02±0.21	4.53±0.33	4.53±0.31	5.00±0.17	4.52±0.15			
60 days	4.51±0.30	4.71±0.34	4.64±0.28	4.80±0.22	4.66±0.13			
90 days	4.13±0.24	4.83±0.27	4.84±0.33	4.99±0.36	4.70±0.16			
Average	4.19^{a}±0.12	4.74^{b}±0.13	4.6^{b}±0.13	4.80^{b}±0.13				

†Basal diet with no supplementation (CT) or supplemented as *Lactobacillus acidophilus* PFM100 (100 mL/calf/d), PFM200 (200 mL/calf/d), PFM300 (300 mL/calf/d) ^{a,b,c}Means bearing different superscripts in a row (a,b,c) differ significantly (P<0.05) ‡Significant effects of dietary treatment (T), period (P) or their interaction (T*P)

DISCUSSION

Body structural Measurements and apparent nutrient digestibility coefficient

Neonatal Murrah buffalo calves have high metabolizing and fast growth rates, but their growth performance can be limited by several factors [32]. The effect of probiotic can vary with the host's dosages, regimens, bacterial strains, type, age, health and nutritional status [33]. In the present study, increases in structural growth measurements may indicate increased body capacity [34]. Improvement in structural body measurement can result from additional energy in calves receiving probiotic required for skeletal deposition due to increased initial DMI and growth rates in calves supplemented with probiotic [35]. Consistent with our findings, partial replacement of probiotic yogurt for milk in calves significantly increased body length, wither height and hip depth can be attributed to higher DMI and weight gain [36]. Likewise, dietary supplementation of probiotics resulted in an increase in heart girth and wither height relative to control cross-bred calves [37]. Likewise, supplementation of species-specific probiotic in calves significantly improved heart girth measurements in treatment as compared to control [38]. In a recent study carried it was also observed that feeding of probiotic, prebiotic and synbiotic in Murrah buffalo calves significantly improved final hip height and heart girth [11]. However, in contrast to the current results, no significant difference in body measurement parameters with probiotic supplementation was reported [14].

Probiotic supplementation to cattle calves showed no significant difference on apparent digestibility of nutrients between the treatment and control groups which is similar to the observation of the present study [22]. However, contrary to present findings, digestibility coefficient of DM, CP, CF and NDF was significantly ($P < 0.05$) higher in the probiotic fed group [39, 40].

Faecal biomarkers

In the present study, all experimental animals were individually housed and maintained under proper feeding and hygiene program, and diarrhea was rarely recorded, indicating the calves were exposed to good conditions with few environmental stressors that could be the explanation for unchanged faecal score among the treatments. In a similar line, supplementation of live yeast product to young calves did not affect faecal scores [41]. Even administration of *Bacillus* sp. as a probiotic in neonatal calves found no variations in growth performance or risk of diarrhoea [42, 14]. However, contrary to the present findings, supplementation of probiotic significantly reduced ($P < 0.05$) faecal scores and duration of diarrhoea in treatment groups as compared to control groups, possibly due to the the surrounding environment. [43, 44]. Consequently, the benefit from probiotic administration for the health of neonatal calves can depend on the type used, the mode of administration and the environmental status.

Lowering of faecal pH in present study agrees to another study [45] in which supplementation of probiotic caused a significant lowering of faecal pH in calves. It was due to the production of large concentrations of lactic acid during the carbohydrate fermentation by lactic acid bacteria.

The increased population of *Lactobacillus* and *Bifidobacterium* with a concomitant reduction of Coliforms in the current study might be due to increased lactic acid concentration in GIT of the probiotic supplemented group which in turn led to increase in beneficial gut microbes with a concomitant reduction in the growth of harmful microbes [12]. Furthermore, the rumen of newborn calves were not functional and the microbial population is slowly controlled with age as the animals mature [45,46]. Such causes may be the explanation for the significant treatment and period interaction for *Lactobacillus* count in this trial. Microbiota ferments amino acid to short-chain fatty acid (SCFA) and ammonia to obtain the energy. Probiotic bacteria increase SCFA formation by accelerating carbohydrate breakdown which is resistant to indigenous bacteria [47]. The SCFA acts as a host energy source, supplying 10-30% of the basal metabolic requirement along with energy for hepatic cells, colonocytes and peripheral tissue [48]. Acceleration in net SCFA and lactic acid production by probiotic supplementation likely contributed to lower the net ammonia content. An improved faecal VFA level in probiotic supplemented group is an indicator of better adaptation of probiotic in the gut of calves. Probiotics also aid in the development of rumen in calves by elevating the concentration of VFA in rumen. Supplementation of probiotic as *Bacillus amyloliquefaciens* strain H57 increased the concentration of VFA such as valerate and butyrate in the rumen of dairy calves may have added more energy to the rumen epithelium and assisted in the rumen development [44]. Similarly, an increase in VFA concentration on supplementation of probiotic in calves was observed [49]. However, contrary to this, supplementation of bacteria based probiotic in Holstein calves did not affect ruminal VFA content [50].

Immune response

Proper production of microbiota in the gastrointestinal tract in early weeks of life is critical for developing a healthy immune system as calves are born with a naive immune system [50]. *Lactobacillus* strains augment the integrity of the intestinal barrier function that results in decreased translocation of bacteria across the intestinal mucosa and maintenance of immune tolerance [51]. PHA-p, a lectin from *Phaseolus vulgaris* causes non-specific proliferation of T-cells which are responsible for DTH reactions and used mainly *in vivo* as an indicator of cell-mediated immune response [52]. The proliferative response potential of circulating T-lymphocytes to an injected mitogen such as PHA can be measured by skin thickness test [53]. The reaction depends on specific antigen-dependent T-cell recall response seen as an inflammatory reaction that reaches maximum intensity after antigenic challenge of 24 to 48 h [54] that justifies improved DTH response in PFM200 and PFM300 (Figure 3). The outcome of this study towards the positive impact of probiotics on CMI response aligns with the findings of other researchers who had shown that dietary probiotics enhanced specific immune functions in young dogs [55] and indicated enhanced DTH response on administration of *E. faecalis* in mice [56]. In another study, PHA-P intradermal injection increases the thickness of the skin fold in prebiotic and symbiotic treated calves, but the effect in probiotic treated calves is comparable from treatment and control groups [57]. On the contrary, DTH response to a percutaneous injection of PHA was not affected by the supplementation of probiotics [30]. The HIR is an immunity component that is mediated by secreted antibodies produced in the cells of the B lymphocyte lineage (B cell). Mohamadi [19] have observed increased humoral response after ovalbumin was injected into calves that received synbiotic supplementation against probiotic and prebiotic supplemented groups. Probiotics can prevent intestinal diseases through both humoral and cell-mediated immune modulation [20] as indicated in the present investigation.

CONCLUSIONS

Based on the above findings, it may be concluded that dietary supplementation of probiotic *Lactobacillus acidophilus* as fermented milk improved the body growth indices and neonatal health measured in terms of quality faecal attributes and immunity. Further in-depth analysis is indicated that the observed responses were more evident in 200 and 300 mL probiotic fermented milk-fed groups as compared to 100 mL. So 200 mL PFM is economical for raising Murrah calves. Overall, the findings of this study showed the efficacy of fermented milk enriched with probiotic is the potential feed additive to be used to promote health status and performance of Murrah calves.

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