EFFECTS OF ENCAPSULATED PROBIOTIC *ENTEROCOCCUS FAECIUM* STRAIN ON DIARRHOEA PATTERNS AND PERFORMANCE OF EARLY WEANED CALVES

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Abstract. The effects of probiotic (*Enterococcus faecium* M74 NCIMB 11181 strain) administration were studied at the Lithuanian University of Health Science in an experiment using 30 Lithuanian Black-and-White calves from 4 to 67 days of age after weaning. Calves were randomly assigned to two groups (control group -T1 and experimental group - T2) with 15 calves for each group and weighing on average of 39.7 (36–43) kg and 39.8 (37–41) kg respectively. The probiotic supplement was added to daily milk intake in experimental group: from day 4 to day 25 of age at a dose of 1.8 g (9.0×10^{10} CFU) per calf per day and from day 26 to day 67 of age at a dose of 0.3 g (1.5×10^{10} CFU (colony forming units)) per calf per day. Feed intake, body weight gain, feces microbial composition and general health condition of all calves were observed. Also condition of feces was examined daily and the occurrence of diarrhea was recorded throughout the experiment.

Probiotic supplementation reduced the number of diarrheic days per animal, severity and actual percentage of calves having diarrhea from 67 to 47%. Herewith, probiotic supplementation at day 67 of age reduced the faecal count of clostridia by 28.4 % ($P<0.05$), when the population of enterococci was increased by 35.6 % ($P<0.05$). Mean values of body weight at the end of experiment and weight gain during 63 days of experiment for treatments probiotic (T2) group and control (T1) group were 78.4 and 74.7 and 38.6 and 35.1 kg ($P<0.01$) respectively. Dry matter intake illustrated that calves fed probiotic (T_2) from day 4 to day 46 of age had numerically greater (by 3.8% $P=0.059$) dry matter intake than control (T1) calves. For the entire study period (63 days), calves fed probiotic (T_2) had greater by 10.1% ($P<0.01$) daily weight gain ($P<0.01$), also calves fed probiotic (T2) had numerically better feed conversion ratio than control (T1) calves ($P=0.299$). The results of this study indicated that present probiotic (*Enterococcus faecium* M74 strain) have beneficial effects in rearing calves.

Keywords: calves, probiotic, growth performance, diarrhea, fecal flora.

Introduction

The gastrointestinal tract is inhabited by a diverse population of bacteria that perform a variety of functions such as acting as a barrier to pathogens and macromolecules of the digestive epithelium. When the homeostatic control is disturbed, chronic inflammation and diarrhea and other diseases may occur (Fuller, 1989; Simon, 2010). A normal intestinal bacterial flora is critical to „out-compete“ the pathogenic bacteria, keep them from becoming established in the gastro intestinal tract and to maintain health (Donohue et al., 2002). When animals are exposed to significant stress, it is possible for the growth of these normal enteric bacteria to become impaired. This allows the growth of potential pathogens, thereby increasing the risk of diseases (Nabuurs et al., 2001; Soderholm and Perdue, 2001). Probiotics can manipulate bacterial flora of the gastrointestinal tract positively, potentially reducing the incidence of disease and improving performance. Beneficial effects include prevention of pathogenic bacterial growth, production of antimicrobial agents, stimulation of the mucosal barrier function, and alteration of immunoregulation (Novak and Katz, 2006). Many papers have addressed the antidiarrheal capacities of different probiotic strains in calves (Abe et al., 1995; Vahjen et al., 2002; Fleige et al., 2007, Samli et al., 2007). The use of probiotics is expected to improve body weight gain, feed efficiency, and health of livestock, because probiotics promote the establishment of a beneficial gut flora and inhibit the growth of pathogenic bacteria in the intestine (Hou et al., 2007). Among *Enterococcus* species, *E. faecium* is the most widely used in commercial probiotics. *E. faecium* is a lactic acid bacterium that is a normal inhabitant in the gut that shows effects against enteropathogens (Willard et al., 2000; Benyacoub et al., 2005). According to Vahjen et al. (2002) the probiotic *E. faecium* initiates a positive feedback mechanism which stimulates metabolic activity of lactobacilli by lactate formation. However, how much a probiotic needs to be consumed to achieve the probiotic effects is crucial information. Therefore, food regulatory/advisory bodies generally stipulate that foods containing probiotic organisms need to have $>10^6$ - 10^7 cfu g^{-1} at the time of consumption (Doleyres et al., 2005). The consumption of probiotic organisms at high doses is safe (Fuller, 1989). However, in practice the addition of considerable overage can be an expensive proposition given the relatively high cost per weight of probiotic cultures.

Therefore, the study was designed to test the efficacy of the probiotic *E. faecium* M74 and to verify the hypothesis that an early (from 4 to 25 days of age) realized supply of defined cell counts of probiotic and further (from 26 to 67 days of age) continuously realized supply of reduced cell counts of probiotic to weaned calves create good conditions for the probiotic strain to act effectively against diarrhea and thus help young animals to improve performance.

Materials and methods

The experiment was conducted at experimental farm of the Institute of Animal Sciences, Lithuanian University of Health Sciences. As recommended by the Scientific Committee on Animal Nutrition (SCAN), the efficacy of the probiotic product was assessed according to the Directive No. 87/153/EEC (EC 1831, 2003).

Experimental animals, feeds and feeding. The investigation was performed in one breeding herd of 160 Lithuanian Black-and-White dairy cows (average milk yield – 6019 kg, milk fat – 265 kg and milk protein - 180 kg per cow per year) from 31 July to 1 October, 2012. After weaning at the day 4 of age calves were moved to a calf house in which they were housed in pens equipped with feeding trough and watering trough as required for calves. Thirty Lithuanian Black-and-White calves were taken from their dams at 4 days of age and divided into two groups: control group (T1) and probiotic group (T2) of which 10 males and 5 females. Both groups were fed whole milk (6 litre per calf per day), calf starter, hay and water. Calf starter, hay and water were offered *ad libitum* intake throughout the trial. The following composition was supplemented (Table 1).

Table 1. **Nutrient content in the diet fed to the calves over 63days from four days of age**

Nutrient content:	Calf starter*	Whole milk	Hay
Dry matter (%)	86.04	12.82	80.03
Crude fibre (g/kg)	47.5	-	246.3
Crude protein (g/kg)	180.7	34.5	86.0
Crude fat (g/kg)	41.9	37.8	23.4
Calcium (g/kg)	21.0	11.6	6.4
Phosphorus (g/kg)	10.4	9.7	3.4
Metabolizable energy (MJ/kg)	11.8	2.51	7.4
*Contains: Co, .004%; I, .025%; Fe, 2.0%; Mg, 5.0%; Mn, 3.0%; K, 7.5%; Se, .015%; S, 10%; Vitamin A, 2200 IU/g; Vitamin D, 660 IU/g; and Vitamin E, 8 IU/g.			

A formulated calf starter contained cereals as the major source of energy and was manufactured by joint-stock company Kėdainių grūdai. The calf starter contained no growth promoters or other zootechnical additives. Hay was made at the experimental farm of the Institute of Animal Science from first cut grass-legume sward. Feeds (whole milk, calf starter and hay), chemical composition and nutrient content were analysed at the Chemical Laboratory of the Institute of Animal Sciences. Samples of each feed were taken regularly during the experiment. The dry matter of feed was determined by oven-drying for 4 h based on the AOAC (1990) method no.930.15. Nitrogen was measured using a Kjeldahl method (AOAC, 1990; method no. 988.05). Crude protein was calculated by multiplying nitrogen by 6.25. Ether extract was determined by the Soxhlet method with petroleum ether as a solvent following AOAC (1990) method no.963.15. Crude fibre was measured With Fibercap (Foss Tecator) using sulphuric acid and Na hydroxide treatment. Crude ash was determined using method 942.05 (AOAC, 2000). Ca - using method 968.08 (AOAC, 2000) dry ashing, atomic-absorption spectrophotometric method, P - spectrophotometric molybdovanadophosphate method.

A probiotic compound containing microencapsulated *Enterococcus faecium* M74, with the total viable count of 50×10^9 CFU (colony forming units) g^{-1}, was supplemented in the daily whole milk intake of experimental (T2) group. From day 4 to day 24 of age experimental group (T2), calves were given probiotic dosage of 1.8 g (9.0×10^{10} CFU) per calf per day and from day 25 to day 67 of age 0.3 g (1.5×10^{10} CFU) per calf per day. The control group (T1) was given no probiotics or other additives. The calves were fed twice a day with whole milk presented in plastic buckets.

Observations and calculations. The consistency of feces from each calf was visually assigned a score from 0 to 2 as follows: 0 = firm, no signs of diarrhea, 1 = soft, slightly loose faecal consistency and 2 = liquid, very loose faecal consistency (diarrhea). Faeces samples were collected from 6 calves from each group at weaning (4 days of age), at day 26 of age and day 67 of age, and microbial groups (clostridia, enterococci and enterobacteria) were counted at the National Food and Veterinary Risk Assessment Institute (Enumeration of clostridia – according LST EN ISO 7937:2004 and ISO 7937:2004, enumeration of enterococci - with Slanetz-Bartley agar and enumeration of enterobacteria – according LST EN ISO 21528-2:2009 and ISO 21528-2:2004). Feed consumption and feed conversion ratio (feed efficiency) are presented per pen for the trial periods of 4 - 24, 25 - 46, 47 - 67 days of age and for the entire trial period (4 - 67 days of age). The chemical composition and nutrient content of the feeds (whole milk, calf starter and hay) were analysed according to the AOAC (1990; 2000). All calves were weighed individually at day 4, at day 25, at day 46 and at day 67 of age. Feed conversion (feed: gain ratio) was calculated as the ratio between the feed intake and body weight gain for each period.

Statistical evaluation. A one-way generalized linear model (GLM) analysis was used in a randomized complete block design with feed-additive treatment as the main factor. When the feed intake and feed conversion rates were statistically analyzed, one pen containing five calves was used as the experimental unit. This was done because feed intake was determined only for a pen of calves. For weight, weight gain and diarrhea respectively, each calf within a pen was used as the experimental unit. This was done because weights could be determined for each individual calf. Due to the limited number of observations in the study, no adjustments for differences in initial weights of calves were made. The Fisher's least significant difference (LSD) procedure at 1% and 5% significance level was used to determine the differences in treatment means. The results were analyzed using GLM of SAS.

Results

Diarrhea incidence and duration. No mortality occurred during the experiment. Diarrhea was observed during the first three weeks after weaning for calves fed the control (T1) diet and only during the first two weeks after weaning for calves fed the experimental (T2) diet. Figs 1, 2 and 3 show diarrhea incidence and the mean fecal scores. Diet supplemented with probiotic lowered the percentage of animals suffering from diarrhea by 20% (40% *vs* 60%). An average of 66.7% of the calves in group T1 had diarrhea while an average of 46.7% had diarrhea in group T2. The percentage of calves having diarrhoea for 1 day, 2 days and 3 days and more was reduced by 20%, 20% and 6.7% respectively. The severity of diarrhea shows that the calves in T2 group had very loose fecal consistency numerically lower (by 20%) than the calves in T1 group. The fecal score calf/day was numerically lower for the probiotic supplemented calves (T2) as compared with that of the control calves (T1).

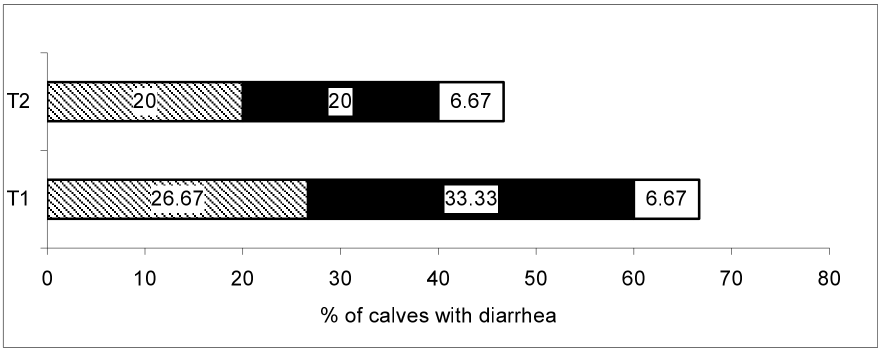

Fig. 1. **Incidence of diarrhea during days 4 – 67 of age in calves fed T1 an T2 diet. The percentage of calves with diarrhea for one day** (hatched plus shaded plus blank column)**, for two days** (hatched plus shaded column) **and for three and more days** (hatched column)

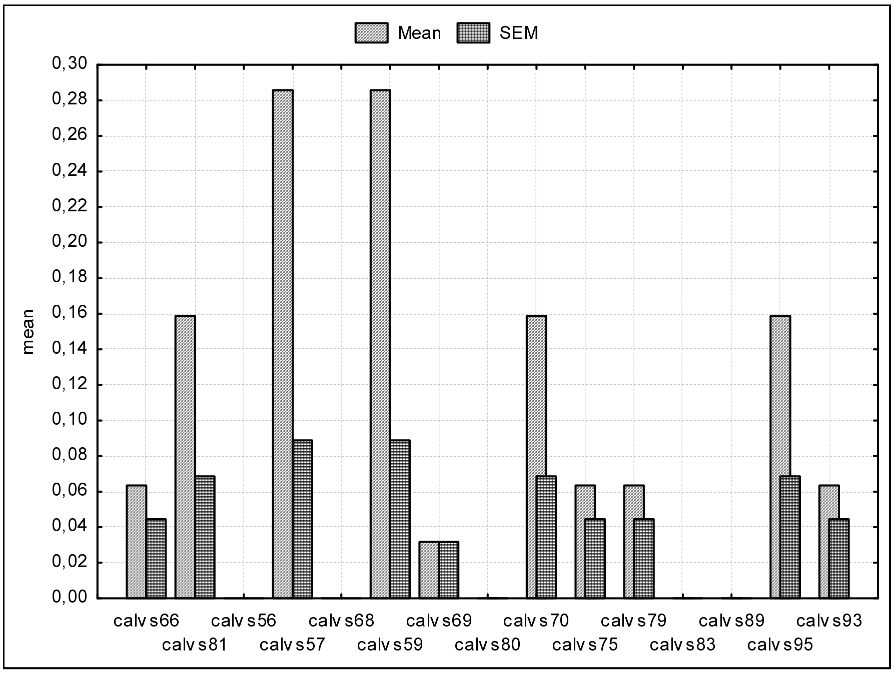

Fig. 2. **Duration of diarrhea** (the diarrhea score per calf per day) **from 4 to 67 days of age in the control group** (T1) **of calves**

The effects of the probiotic *Enterococcus faecium* M74 on the composition of microflora in the feces of the control (T1) and experimental (T2) calves are presented in Table 2.

At day 4 of age enterobacteria were found as the predominant flora and clostridia constituted a minor group, increasing at day 26 of age and then decreasing at 67 day of age in both groups. Enterococci were a subdominant flora and their counts varied from 7.11 to 6.27 log CFU g^{-1} in T2 group and from 7.14 to 8.50 log CFU g^{-1} in T1 group. Probiotic diet (T2 group) reduced the stock of clostridia at day 26 of age by 24.3% (P<0.05), at day 67 of age by 28.4% (P<0.05) and increased enterococci in feces at day 67 of age by 35.6% (P<0.05) compared with control diet (T1).

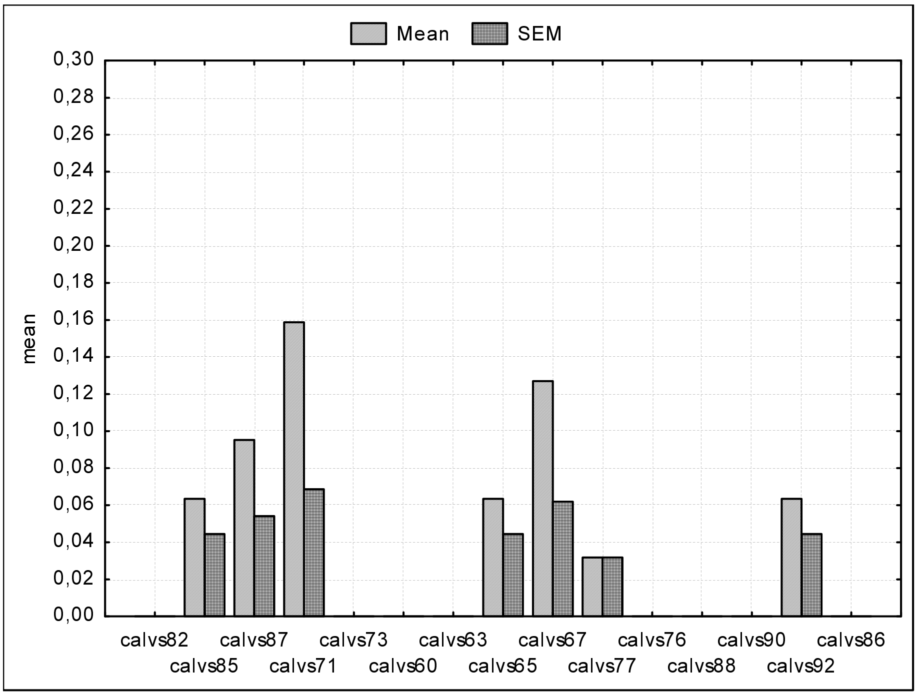

Fig. 3. **Duration of diarrhea** (the diarrhea score per calf per day) **from 4 to 67 days of age in the experimental group** (T2) **of calves**

Table 2. **Population of faecal micro-organisms** (log cfu g^{-1} of feces) **of calves fed control** (T1) **and probiotic diet** (T2) (n = 6 per group)

Item	Age of calves (days)	Diet		P- value
		T1	T2	
Clostridia	4	2.37 ± 0.51	2.33 ± 0.28	0.864
	26	4.41 ± 0.83	3.34 ± 0.76	< 0.05
	67	4.22 ± 0.60	3.02 ± 0.55	< 0.05
Enterococci	4	7.11 ± 0.93	7.14 ± 0.80	0.953
	26	7.06 ± 0.99	7.59 ± 0.53	0.285
	67	6.27 ± 0.98	8.50 ± 0.92	< 0.05
Enterobacteria	4	8.16 ± 0.62	8.12 ± 0.75	0.909
	26	8.59 ± 1.36	8.00 ± 1.16	0.437
	67	8.11 ± 1.22	7.70 ± 1.52	0.610
For each average, the respective standard deviation is added (mean ± S.D.)				

Feed intake, weight gains and conversion rate. All whole milk provided at 6 litre per calf per day was consumed. Therefore, the only components of dry matter intake that could vary were calf starter and hay. During the entire 63-day-study period (from 4 to 67 days of age), the forage and total DM intakes were numerically (by 3.8% P=0.059) higher in the probiotic diet fed group (T2) (Table 3). The average weight of the calves in the control group (T1) and in the experimental group (T2) at day 4 of age was comparable, i.e. 39.7 kg and 39.8 kg respectively. Throughout the whole trial, calves that received probiotic in the whole milk (T2) achieved higher (P<0.01) average live weight when compared to the calves from the control group (T1).

The average weight of T2 calves at days 25, 47 and 67 of age was higher by 1.9% (P<0.05), 3.4% (P<0.01) and by 4.9% (P<0.01), respectively, compared with T1 calves. (Table3). During the entire trial, the average weight gain for the calves fed control diet (T1) and probiotic diet (T2) was 35.0 kg and 38.6 kg respectively. Therefore, average weight gain was by 3.6 kg (10%) higher in probitic (T2) group compared to the control (T1) group and this difference was statistically conclusive (P< 0.01). Table 3 shows that the daily weight gain of the calves fed probiotic diet (T2) was significantly higher than that of calves fed control diet (T1) probiotic free diets in all trial periods: from 4 to 25 days of age (P<0.01), from 26 to 46 days of age (P<0.05) and from 47 to 67 days of age (P<0.01). The average daily weight gain during the entire trial in T2 group was, therefore, higher by 10.1% (P<0.01) if compared to T1 group. For the entire trial period the feed conversion ratio was improved numerically (by 5.3%, P=0.299) in the probiotic treated group.

Table 3. **Body weight gain, average daily gains, feed intake and feed conversion rate of calves fed probiotic diet** (T2) **and control diet** (T1) (for body weight and average daily gain n = 15 per group, for feed intake and feed efficiency n = 3 per group)

Item	Days/periods of calves age	Treatment		P- value
		T1	T2	
Body weight (kg)	4	39.7 ± 1.99	39.8 ± 1.78	0.610
	25	46.9 ± 2.74	47.8 ± 2.48	< 0.05
	46	58.7 ± 4.04	60.7 ± 3.49	< 0.01
	63	74.7 ± 4.70	78.4 ± 4.44	< 0.01
Average daily gain (g)	4-25	346.1 ± 49.22	380.7 ± 43.90	< 0.01
	26-46	561.8 ± 88.68	616.0 ± 66.07	< 0.05
	47-67	761.9 ± 50.98	841.2 ± 56.10	< 0.01
	4-67	556.5 ± 56.13	612.7 ± 50.56	< 0.01
Feed intake (kg DM per calf)	4-25	17.0 ± 0.16	17.1 ± 0.15	< 0.05
	26-46	24.2 ± 0.56	25.3 ± 0.55	< 0.05
	47-67	32.0 ± 1.03	34.3 ± 1.05	0.096
	4-67	73.2 ± 1.63	76.6 ± 1.71	0.059
Feed efficiency (gain/ DMI)	4-25	2.35 ± 0.13	2.14 ± 0.05	0.192
	26-46	2.06 ± 0.13	1.95 ± 0.05	0.408
	47-67	2.00 ± 0.03	1.91 ± 0.03	0.096
	4-67	2.09 ± 0.08	1.98 ± 0.05	0.299
DM- dry matter, DMI- dry matter. For each average, the respective standard deviation is added (mean ± S.D.)				

Discussion

The results of this experiment indicated that probiotic treatment systemically reduced the occurrence of diarrhea. *E. faecium* M74 preparation depressed the incidence, severity and duration of diarrhea. This outcome agrees with the results of some other studies with calves (Abe et al., 1995; Khuntia et al., 2002; Frizzo et al., 2010) but in other studies (Cruywagen et al., 1996) no probiotic-induced reduction of the occurrence of diarrhea was observed. Kawakami et al. (2010), Gorgulu et al. (2003) reported that with respect to diarrhoea and faecal scoring calves fed probiotics were superior to control group. The present results showed that supplementation of *E. faecium* M74 had significantly reduced the stock of clostridia at day 26 and 67 of calve age and had significantly increased enterococci in the faeces at 67 days of calves age, suggesting that the bacteria have a probiotic ability to improve intestinal microbial flora. The use of lactic acid bacteria to reduce pathogenic bacteria in the gut has been termed a probiotic strategy, with an overall goal of promoting colonization of protective bacteria in calves during the first weeks of life to compete with pathogenic bacteria. Additionally, lactic acid bacteria can stimulate the development of the immune response against pathogenic bacteria and counteract the negative effects of illnesses (Fleige et al., 2007; Willard et al., 2000; Benyacoub et al., 2005; Frizzo et al., 2010). Young calves are especially susceptible to intestinal infectious diseases during these periods and maintaining or increasing body weight gain could enhance the resistance to disease (Cruywagen et al., 1996). Reports of lactic acid bacteria fed to young calves are inconclusive. Many authors reported beneficial effects of probiotic preparations on animal growth (Abe et al., 1995; Frizzo et al., 2010) and there are those (Cruywagen et al., 1996) who reported no effects. The results of this trial indicate positive effects of the probiotic product *E. faecium* M74 on animal growth and feed efficiency. The increase in both body weight gain and disease resistance places the young calf in a very favourable situation in which it can continue to gain body weight and be better prepared to resist diarrheal pathogens. Different mechanisms of probiotics action have been described (Fuller, 1989; Fleige et al., 2007; Frizzo et al., 2010) and can be summarized as follows: probiotics compete for nutrients and produce antibacterial compounds (e.g., organic acids, hydrogen peroxide, bacteriocins) in the intestinal lumen allowing them to occupy specific niches of the intestinal mucosa and activate the innate immune system of calves. The involvement of each of these mechanisms is directly related to the type of the probiotic strain and the feed consumed by the calves. The improvement in utilization of the feed and consequent improvement in body weight gain is the final consequence of probiotic action.

Conclusions

Addition of probiotic *Enterococcus faecium* M74 reduced the number of calves having diarrhea compared to the control calves, by 20% lowered the percentage of animals suffering from diarrhea and diminished the duration of diarrhea. Besides, probiotic diet reduced the faecal count of clostridia, when the population of enterococci was increased. During the entire 63-day-study period (from 4 to 67 days of age), the forage and total DM intakes were numerically (by 3.8% P=0.059) higher in the probiotic diet fed group compared to control diet.

The average weight of the calves during the first three weeks of the age (when an initial probiotic dose was 1.8 g per calf per day) in the probiotic group was by 1.9% ($P<0.05$) higher compared to the calves from the control group. Reducing the initial probiotic dose to a maintenance level (0.3g per calf per day) from 26 to 67 days of age had no negative effect on the growth of the calves fed probiotic diet. Moreover, the difference in live weight at the end of the trial increased to 4.9% ($P<0.01$) in probiotc diet fed calves compared with control diet fed calves. Adding probiotic to whole milk significantly increased the daily weight gain throughout the whole trial and numerically improved feed conversion ratio.

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