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Effect of Multi-species Probiotic Supplementation on Growth Performance, Antioxidant Status and Incidence of Diarrhea in Neonatal Holstein Dairy Calves

Mandouh MI*, RA Elbanna and HA Abdellatif

Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. 12211
***Corresponding author:** Mandouh.m93@gmail.com

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ABSTRACT

A study was conducted to investigate the effect of probiotic on growth performance and general health status of newborn dairy calves. Twenty-four Holstein dairy calves were used in a 20-day experiment, and were divided into three groups. A control group (CON) was fed on pasteurized whole milk non-supplemented with probiotic for 20 days, the second group was fed on pasteurized whole milk supplemented with 20 grams of probiotic for 5 successive days only, then fed on pasteurized whole milk non-supplemented with probiotic until the end of the experiment (PRO5) and the third group was fed on pasteurized whole milk supplemented with 20 grams of probiotic for 10 successive days, then fed on pasteurized whole milk non-supplemented with probiotic until the end of the experiment (PRO10). Body weight, body conformation, and fecal scoring were recorded on d 0, 10, and 20. Blood samples were collected on d 10 and d 20. Key antioxidant enzymes were assessed beside the liver and kidney function indicators. Data were statistically analyzed using PROC MIX procedures of SAS 9.4 software. Body weight and body weight gain were not affected by probiotic supplementation; but heart girth tended to increase in probiotic fed calves ($P = 0.08$). Incidence of calf diarrhea was 50% in CON and 37.5% in both PRO5 and PRO10 groups. Serum levels of aspartate aminotransferase (AST) and reduced glutathione (GSH) were reduced ($P = 0.05$), while globulin level tended to increase ($P = 0.10$) in probiotic treated calves. Our results suggest that supplementation of suckling calves with multi-species probiotic is beneficial to reduce the incidence of diarrhea.

Key words: Neonatal calf diarrhea, Probiotic, Growth performance, Dairy calves.

INTRODUCTION

Neonatal calf diarrhea is one of the leading health problems that causes productive and economic losses in the dairy industry. It causes high mortality rate, increased medication costs, diminished growth performance and increased culling and replacement rate (Cho and Yoon, 2014). Half of the mortalities among un-weaned calves were attributed to diarrhea, with most cases occurring in calves less than a month old National Animal Health Monitoring System (NAHMS, 2007).

Neonatal calf diarrhea is a multifactorial disease results from the interaction between many variables, including pathogen, calf, immunological and nutritional status, management and environmental factors (Izzo *et al.*, 2011). Colostrum deprivation, abrupt dietary or environmental changes and stressors such as transportation, vaccination, dehorning and weaning increase the incidence of diarrhea in neonatal calves (Mingmongkolchai and Panbangred, 2018). Under such stressful conditions, probiotics may be used to reduce the risk and/or severity of diarrhea caused by disruption of the normal intestinal environment. Probiotic has been defined as "Live microorganisms, which, when supplemented to the host in appropriate amounts, confer a health benefit on the host" (Uyeno *et al.*, 2015). The observable benefits of probiotics on calf growth may be minimal in generally healthy calves, in which the microbial community is relatively stable.

As reported by Riddell *et al.*, (2010) early feeding of probiotics to newborn animals is necessary to establish and keep normal microorganisms and modify intestinal microbial balance, alleviate calf scours and improve general health and performance of the animal. Probiotics generally target the small intestine and act to stabilize the gut microbiota and limit the risk of pathogens by inhibiting their colonization (Chaucheyras-Durand and Durand, 2009). The majority of the probiotics currently used are based on lactic acid bacteria, mainly *Bacillus*, *Enterococcus*, *Lactobacillus* and *Streptococcus* (Song *et al.*, 2014 ;

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Mingmongkolchai and Panbangred, 2018). Many trials have reported that the supplementation of probiotics including *Bacillus* and *Enterococcus* species can effectively reduce the incidence of neonatal calf diarrhea, decrease mortalities and improve feed efficiency and average daily gain (Jatkauskas and Vrotniakiene, 2010; Renaud *et al*., 2019).

Probiotics have different modes of action including competitive exclusion, some probiotics are able to colonize the gut epithelium and compete for the adhesion site with the pathogenic microbes (La Ragione *et al*., 2004), stimulating the immune system of the animal, production of antimicrobial compounds and enhancing proliferation of beneficial microflora (Wehnes *et al*., 2009; Song *et al*., 2014). *Bacillus* species have been reported to inhibit many pathogens including *Escherichia coli, Clostridium sp., Salmonella typhimurium, Staphylococcus aureus and Streptococcus sp.* (Song *et al*., 2014). *Enterococcus faecium* produces lactic acid which stimulates metabolic activity of *Lactobacilli* and inhibits pathogenic microbes (Jatkauskas and Vrotniakienė, 2014).

Several probiotic products have been introduced to the market; however, research studies on other new multi-species probiotic products were not formerly investigated. Therefore, the aim of the current study was to investigate the effect of multi-species probiotic supplement containing *Bacillus subtilis*, *Bacillus licheniformis* and *Enterococcus faecium* on incidence and severity of diarrhea in neonatal dairy calves.

MATERIALS AND METHODS

Experimental design, animals, and housing

Twenty four newborn dairy calves, 12 males and 12 females, were used in a 20 day experiment and randomly distributed into three dietary treatments as follow: 1) CON; Control group (*n*=8; 4 males and 4 females), was fed on pasteurized whole milk only, 2) PRO5 group (*n* = 8; 4 males and 4 females), was fed on pasteurized whole milk supplemented with 20 grams of the probiotic, Prolyt Pack ® purchased from Biochem, Germany, that contains *Bacillus subtilis* (DSMZ 5750) 3.2×10^{10} CFU, *Bacillus licheniformis* (DSMZ 5749) 3.2×10^{10} CFU and *Enterococcus faecium* 5×10^{10} CFU per kilogram of the probiotic product with a daily dose of 20 grams per calf, for 5 successive days, then fed on pasteurized whole milk non-supplemented with the probiotic for the rest of the experiment; 3) PRO10 group was fed on pasteurized whole milk supplemented with 20 grams of the probiotic for 10 successive days, then fed on pasteurized whole milk non-supplemented with the probiotic for the rest of the experiment. From d 11 to the end of experiment d 20, all calves were receiving pasteurized whole milk non-supplemented with the probiotic.

The trial was conducted at private dairy farm (Cairo-Alexandria Desert Road, Egypt). Calves were housed individually. They were fed on pasteurized whole milk three times a day (total 6 litres of milk / day). All calves were fed from one pooled milk tank via buckets, which were cleaned and sanitized after each feeding. The probiotic was supplemented daily in the morning milk meal for each individual calf in its milk bucket.

Sampling and analyses

Growth performance

Body weight and body conformation (withers height, heart girth and body length) were recorded on d 0, d 10, and d 20. Body conformation parameters were recorded, and body weight gain was calculated according to Kolkman *et al*., (2010). All measurements were performed by the same individual to avoid variations and were done before the afternoon feeding.

Fecal scoring

A 4-point scale was used based on consistency of feces (Table 1), according to Osorio *et al*., (2012). Fecal score ≥ 3 was considered as diarrhea. Calves suffering from diarrhea were recorded and incidence of diarrhea was calculated.

Blood sampling

Blood samples were collected through jugular venipuncture on d 10 and d 20 and left to clot on bench for 20 minutes. Serum was then separated by centrifugation at 3,000 rpm for 10 minutes and stored in -20ºC till time of analysis according to Quezada-Tristán *et al*., (2014).

Antioxidant status

Serum samples were used for analysis of antioxidant indices including superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) using Spinreact kit (Girona, Spain). Samples were analyzed by spectrophotometry using PD-303S spectrophotometer (Apel, Japan).

General health status

Serum samples were used for analysis of total protein (TP), albumin, globulin, albumin:globulin ratio (A:G) was calculated , alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine using Spinreact kit (Girona, Spain). Samples were analyzed by spectrophotometry using PD-303S spectrophotometer (Apel, Japan).

Statistical analysis

The MIXED procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) was used for repeated measures analysis at d 0, 10 and 20 of age. Treatment group, sex, and time (day) were considered as fixed factors in the model, and the random effect was the calf. Significance was determined at $P \leq 0.05$. Because sex had no effect on any of the parameters measured ($P>0.05$), sex was removed from the model.

RESULTS

Growth performance

Growth performance parameters are presented in Table 2. The dietary supplementation of probiotic did not significantly affect body weight, body weight gain, or body conformation. However, heart girth tended to increase in probiotic-supplemented groups ($P = 0.08$).

Fecal scoring and incidence of diarrhea

Fecal scoring results are shown in Figure 1. Dietary supplementation of probiotic did not affect fecal consistency ($P = 0.14$). However, the incidence of diarrhea was 50% in CON compared to 37.5% in both PRO5 and PRO10 groups as shown in Figure 2.

Table 1: Fecal score classification[1]

Score	Description
1	Firm consistency
2	Soft consistency
3	Runny consistency
4	Liquid consistency

[1]Fecal scoring according to Osorio *et al*., (2012).

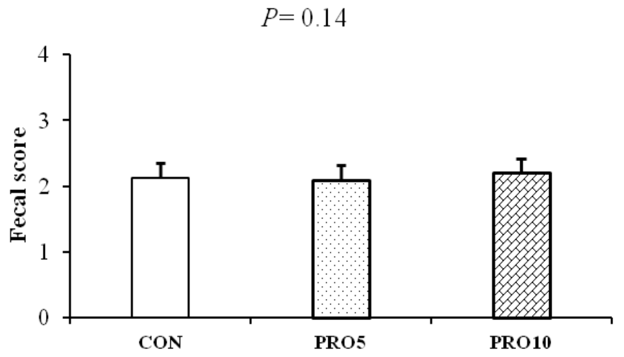

Fig. 1: Effect of dietary probiotic supplementation on fecal scoring of neonatal Holstein dairy calves. Probiotic supplementation did not alter fecal scores of calves (P=0.14).

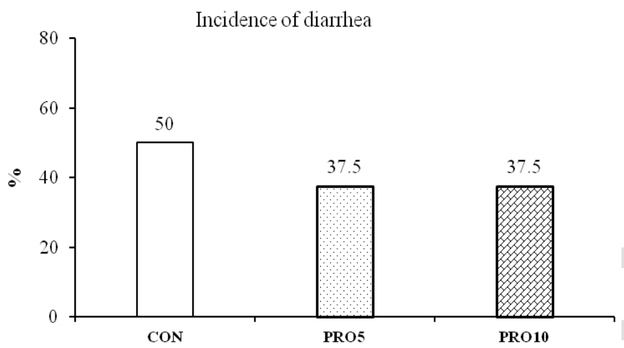

Fig. 2: Effect of dietary probiotic supplementation on incidence of diarrhea in neonatal Holstein dairy calves. Probiotic supplementation reduced incidence of diarrhea regardless of the period of supplementation.

Antioxidant status

The effect of probiotic supplementation on antioxidant status is presented in Table 3. The serum level of GSH decreased (P = 0.05) by 33.33% in both probiotic receiving groups. The serum levels of SOD and CAT were not affected by the treatment (P = 0.24; 0.67 respectively).

General health status

Effect of probiotic supplementation on calves general health status is presented in Table 4. The serum level of AST enzyme reduced (P < 0.01) by 32.86% and 43.09% in PRO5 and PRO10 groups, respectively. Nevertheless, the level of total globulin tended to increase (P = 0.10) in PRO10 group followed by PRO5 group compared to CON group.

DISCUSSION

Using different bacterial strains sharing beneficial properties could have effective results on the calves (Maldonado *et al.*, 2018). Multi-strain probiotic supplements tended to increase the positive impact on the calves rather than using mono-strain inoculum (Signorini *et al.*, 2012).

In the current study, dietary supplementation of probiotic did not significantly affect body weight, body weight gain or body conformation. The same results were obtained by Riddell *et al*. (2010) who reported that supplementation of calf starter and milk replacer with *B. subtilis* and *B. licheniformis* probiotic did not affect body weight gain and wither height in the calves. Seifzadeh *et al.* (2017) stated that the usage of probiotic containing *Enterococcus faecium*, *Lactobacillus acidophilus* and other bacterial strains in newly born calves had no observed effect on daily weight gain. Also, *Lactobacillus plantarum* and *Bacillus subtilis* based probiotic had no significant effect on average daily gain but improved FCR over the first months of age in calves (Zhang *et al*., 2016). Further, adding a mixture of probiotic containing *E. faecium, Lactobacillus acidophilus, L. casei* and *Bifidobacterium bifidium* did not improve daily weight gain in newly born Holstein female calves (Bayatkouhsar *et al.*, 2013). Bakhshi *et al.*, (2010) reported that milk supplementation with *B. subtilis* and *B. licheniformis* did not enhance body weight gain of Holstein calves in a 7-week trial. Additionally, in a 90-day study on newly born calves the probiotic containing different strains including *E. faecium* only improved growth performance after 90 days of supplementation, and not at day 30 or 60 (Mokhber-Dezfiuli *et al*., 2007).

Surprisingly, the tendency toward increase in heart girth in probiotic-supplemented groups could indicate an improvement in heart health and blood iron, since improved heart girth was formerly reported to be associated with decrease incidence of anemia and improved iron concentration (Ježek *et al*., 2009), which worth further investigation on the effect of probiotic supplementations on heart health and incidence of anemia that is a common condition in newborn calves (Ježek *et al*., 2009).

In this study, the incidence of neonatal calf diarrhea was reduced in newborn calves received the probiotic supplement, indicating the prophylactic role of probiotic. However, there was no significant alteration in fecal consistency between probiotic and control groups. These results were supported by the previous findings by Jatkauskas and Vrotniakiene (2010) and Le *et al.*, (2016) who reported that supplementation of probiotic to newborn calves reduced the incidence of diarrhea by 30 – 40% with increased average daily gain. This improvement in gut health and decrease in diarrhea incidence could be due to the antimicrobial activity of probiotics. Probiotics produce antimicrobial substances and metabolic compounds that suppress the growth of pathogenic microorganisms, *E. faecium* produces certain metabolites such as lactic acid which plays an important role in inhibiting the development of pathogenic bacteria by lowering the pH (Jatkauskas and Vrotniakienė, 2014; Sharma *et al*., 2018). *Bacillus* species have been reported to inhibit many pathogens including *E. coli, Clostridium* sp. *Campylobacter* sp., and *Salmonella typhimurium* in cattle, poultry and pigs and alleviate the risk of diarrhea (Song *et al.*, 2014).

On the other hand Riddell *et al.* (2010) found no difference in the incidence of diarrhea when newborn calves were supplemented with a probiotic containing

Table 2: Effect of probiotic supplementation on growth performance indices of neonatal Holstein dairy calves [1]

Parameter	Treatment[2]				*P-value***		
	CON	PRO5	PRO10	SEM	Trt	Day	Trt*Day
Body weight, Kg	39.14	40.55	39.30	0.69	0.27	<0.01	0.99
Daily BWG, g[3]	271.88	279.17	237.50	37.94	0.66	0.61	0.49
Body length, cm	103.09	102.75	101.97	0.72	0.47	<0.01	0.56
Hip height, cm	78.81	79.91	79.06	0.54	0.31	<0.01	0.65
Heart girth, cm	78.89	80.10	79.06	0.42	0.08	<0.01	0.53

[1] Values are least square mean, *n* = 8 / group: [2] Treatments: CON = no probiotic; PRO5 = probiotic supplementation for 5 days; PRO10 = probiotic supplementation for 10 days: [3] Daily body weight gain: [**] P-value ≤ 0.05 considered significant.

Table 3: Effect of probiotic supplementation on antioxidant status of neonatal Holstein dairy calves at d 10 and d 20[1].

Item	Treatment[2]				P-value**		
	CON	PRO5	PRO10	SEM	Trt	Day	Trt*Day
SOD, u/ml[3]	135.54	116.83	122.24	7.61	0.24	<0.01	0.39
CAT, u/ml[4]	0.61	0.55	0.55	0.06	0.67	0.32	0.39
GSH, mmol/l[5]	0.06[a]	0.04[b]	0.04[b]	0.01	0.05	0.74	0.79

[1] Values are least square mean, *n* = 8 / group: [2] Treatments: CON = no probiotic; PRO5 = probiotic supplementation for 5 days; PRO10 = probiotic supplementation for 10 days: [3] Superoxide dismutase: [4] Catalase: [5] Reduced Glutathione: [**] P-value ≤ 0.05 considered significant

Table 4: Effect of probiotic supplementation on general health status of neonatal Holstein dairy calves at d 10 and d 20[1].

Item	Treatment[2]				*P*-value**		
	CON	PRO5	PRO10	SEM	Trt	Day	Trt*Day
ALT, u/l[3]	31.27	33.40	36.44	6.09	0.80	0.22	0.06
AST, u/l[4]	36.85[a]	24.74[b]	20.97[b]	4.11	0.01	0.87	0.50
TP, g/dl[5]	5.44	5.83	6.26	0.29	0.43	0.25	0.56
Albumin, g/dl	1.94	2.06	2.07	0.45	0.11	<0.01	0.04
Globulin, g/dl	3.50	3.77	4.19	0.23	0.10	0.20	0.03
A: G ratio[6]	0.56	0.56	0.51	0.03	0.61	0.09	0.50
BUN, mg/dl[7]	11.26	10.89	12.57	1.33	0.69	0.19	0.02
Creatinine mg/dl,	0.92	1.01	1.04	0.04	0.25	0.20	0.22

[1] Values are least square mean, *n* = 8 / group: [2] Treatments: CON = no probiotic; PRO5 = probiotic supplementation for 5 days; PRO10 = probiotic supplementation for 10 days: [3] Alanine aminotransferase: [4] Aspartate aminotransferase: [5] Total protein: [6] Albumin: globulin ratio: [7] Blood urea nitrogen: [**] P-value ≤ 0.05 considered significant.

Bacillus subtilis and *Bacillus licheniformis.* Kowalski *et al*. (2009) also found no effect on the incidence of diarrhea, but body weight gain and feed intake were better when used the same probiotic in suckling calves for 8 weeks. Further, no treatment effect was observed due to *Lactobacillus acidophilus* supplementation in calves on diarrhea incidence and there was no relationship between diarrhea, incidence and severity, and body weight changes (Cruywagen *et al.*,1996). In the current study we did not observe a significant difference in fecal score among groups, which could possibly be due to the small number of calves and the short duration of the study. Similar results were reported by (Signorini *et al.*, 2012; Salazar *et al.*, 2019) who pointed out that probiotic supplemented calves did not show any improvement in fecal consistency. In contrast Jatkauskas and Vrotniakienė (2014) reported that fecal score was numerically lower for probiotic supplemented calves compared with control calves.

Compared to the probiotic treated calves, the level of AST was higher in the control group possibly due to the tissue affection and liver affection resulting from diarrhea which is in line with Bartkiene *et al*., (2016). On the other hand, Le *et al.*, (2016) mentioned that there was no difference in AST level and other various serum parameters in response to *Bacillus amyloliquefaciens* supplementation in calves. Globulin concentration in treated calves tended to increase, which could be due to increase in immunoglobulin as formerly confirmed by Al-Saiady (2010), which indicate the positive effect of probiotic on immune status of calves, and hence, reduce incidence of diarrhea. Bayatkouhsar *et al.*, (2013) reported that supplementation of calves with lactic acid bacteria had no effect on serum total protein level during the weaning period. Changes in oxidative biomarkers (GSH, SOD and, CAT) can be used as a good indicator of animal health and physiological status. However, there is no consistent relationship between the changes that occur in serum concentration of antioxidants and those that occur in the gut (Alugongo *et al*., 2017). The observed reduction in level of GSH might be due to its depletion in reactive oxygen species (ROS) scavenging process, as reported earlier by Wang *et al*., (2017), antioxidant potential of probiotics through regulation of multiple antioxidant systems including its ROS scavenging ability. However, level of GSSG and GSH-Px should be measured to understand whether or not the antioxidant effect of probiotic treatment was mediated through GSH system, taking in consideration that none of the other antioxidant enzymes changed between groups.

Conclusions

Probiotics are efficient additives to reduce incidence of diarrhea in neonatal calves and could be used to reduce calf diarrhea medication costs thus decreasing economic losses to the dairy industry. However, its effect on growth performance is minimal.

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