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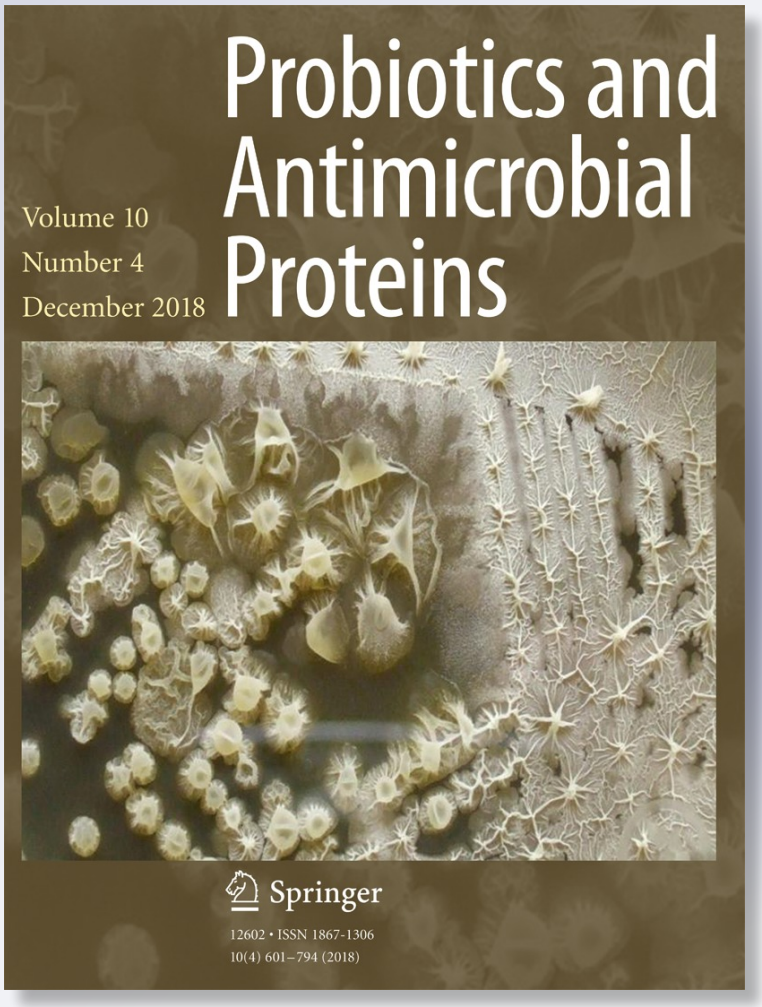

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Effect of Milk Fermented with Lactic Acid Bacteria on Diarrheal Incidence, Growth Performance and Microbiological and Blood Profiles of Newborn Dairy Calves

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Abstract The effect of the administration of milk fermented with lactic acid bacteria to calves was evaluated. The strains included were: *Lactobacillus murinus* CRL1695, *Lact. mucosae* CRL1696, *Lact. johnsonii* CRL1693, and *Lact. salivarius* CRL1702, which were selected for their beneficial and functional properties and isolated from healthy calves in the northwestern region of Argentina. The trial was conducted on a dairy farm located in Tucumán (Holando-Argentino calves). A randomized controlled trial was performed in which 56 new-born animals were divided into two groups: the treated group (T) received the fermented milk for 60 days and the control group (C) only milk. The animals were fed a solid diet ad libitum. The treated group was given a daily dose of 1×10^9CFU of the probiotic fermented milk while the control group was fed milk. Body weight and biometrical parameters were recorded between 15 and 60 days of age, and average daily gain was calculated with three samplings per animal throughout the trial. Rectal swabs and fecal and blood samples were also collected. Results showed the efficacy of the probiotic: lower morbidity and mortality of calves (morbidity was 69.20% in animals without the probiotic, and 46.15% in probiotic-treated animals, with $P = 0.09$; mortality in C was 34.61 and 7.69% in animals fed with ferment milk; $P = 0.02$).The calves fed with probiotic evidenced an improvement in nutritional parameters, body condition and weight gain (health index $P = 0.01$; average daily gain $P = 0.03$).Viable bacterial numbers showed no differences between the two experimental groups. Hematological parameters and serum proteins were not modified by the treatment. The results suggest that the fermented milk containing lactic acid bacteria can be a viable veterinary product for young calves due to its beneficial effects on health and growth.

Keywords Beneficial strains · Calves management · Probiotic applications · Fermented milk · Oral administration

Introduction

Diarrhea is one of the most frequent diseases affecting newborn calves in intensive management systems [12, 29]. These systems, increasing in number cause imbalances in the enteric microbiota resulting in inefficient absorption of nutrients and slower adaptation of the transition from liquid to solid feeding [5, 11]. The consequent intestinal diseases and microbiota imbalance cause growth failures and high mortality rates, and economic losses to dairy and beef farms [2, 3, 13, 30]. Moreover, some authors have identified modifications in the immune system of animals exposed to intensive systems as a consequence of high stress levels that result in a decrease in the immune response which leads to unhealthy animals [22].

In recent years, the antibiotic therapy for diarrhea has been applied to specific pathogens and is related only to the severity and duration of the disease [27]. The preventive use of 76 antibiotics as growth promoters been banned in several countries in the European Union mainly because of the acquired

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resistance of the indigenous microbiota and of the residues found in animal products [9, 25]. The use of probiotics has been proposed as a novel alternative for the prevention of intestinal disorders and also as immunomodulators [6, 24, 32]. Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [10]. Different scientists have reported the efficacy of probiotics in calves, pigs and poultry. The beneficial effect of probiotics is not limited to the improvement in nutritional parameters, but also the enhancement of the immune response of animals, thus contributing to a decrease in multiple infections [6, 14, 20, 28, 32].

The aim of this work was to determine the effect of multi-strain fermented milk with autochthonous lactic acid bacteria (LAB) on calf growth and performance along with the incidence and duration of diarrhea. Nutritional parameters, microorganisms in feces and blood parameters on calves were assessed as well. The strains used were previously isolated from calves from the northwestern region of Argentina and selected on the basis of their beneficial, functional, compatible and safety characteristics [16, 17]. Technological studies such as the production of the fermented milk and the resistance of LAB to dairy farm conditions were also conducted [19] .

Materials and Methods

Microorganisms, Growth Conditions and Fermentation Process

Lactobacillus johnsonii CRL1693, *Lact. murinus* CRL1695, *Lact. mucosae* CRL1696, and *Lact. salivarius* CRL1702 were used to prepare fermented milk [18]. Lactobacillus strains were isolated from calf˝ feces and previously selected on the bases of their beneficial properties [16]. Microorganism maintenance, elaboration, and conservation of the fermented milk were published in a previous work [18].

Animals and Treatments

Animal experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The study protocol was approved by the CERELA-CONICET Bioethics Committee (Centro de Referencia para Lactobacilos–Consejo Nacional de Ciencia y Tecnología).

The experiment was conducted on a dairy farm located in Trancas (Tucumán) in Holando-Argentino calves. Fifty-two newborn calves were divided into two groups: control (C) and treated group (T), (controls: 13 males, 13 females; treated animals: 13 males, 13 females, initial body weight 25.1 ± 4.5 at 48 h after birth). They were fed the same diet *ad libitum* except for the administration of fermented milk to the T (controls received non-fermented milk). Colostrum feeding of the calves was provided by the dams following calving. After birth, calves were randomly assigned to one of the two groups and ear marked. Twenty-four hours after birth, the animals received the probiotic and the trial period started. Doses of 1×10^9 CFU were administered daily to each calf in the treated group for 60 days. Production of the fermented milk is described in Maldonado and Nader-Macías [18]. No modifications in either breeding scheme or calf management were performed. Newborn animals were kept with their dams for 5–7 days. Then, the animals were housed individually, and fed saleable unpasteurized whole milk up to the beginning of solid feeding (they had free access to hay), and later moved to the pens at 6 months of age. No antibiotics were added to either feed or water. Each calf received two liters of warn milk twice a day (8:00 and 17:00) in individual buckets. The standard treatments for calves with diarrhea and respiratory symptoms or other pathologies were applied as usual on the farm during the experiment, and evaluation of the severity of symptoms was performed by the veterinarian. For diarrhea therapy, a single dose of oxytetracycline was used.

Administration of Fermented Milk to Animals

In newborn calves and older animals, 10 mL of fermented milk was administered with a syringe (Bremen, Seisema, Sanhekou, China) directly in the oral cavity.

Evaluation of Animals

Samples were taken from calves at 15 to 30 days intervals after birth and until they were 60 days old, indicated as follows: sample 1 (5–15 days old) (S1); 2 (15–30 days old) (S2); and 3 (30–60 days old) (S3). Health and nutritional parameters (average daily gain, performance status, stool consistency, body temperature), cultivable bacteria in stools (total mesophilic, enterobacteria, and lactic acid bacteria), and parasitological studies were performed. Biochemical and hematological profiles (red and white blood cell counts, protein electrophoresis, and total proteins) were also carried out. Rectal temperature was determined and digestive and/or respiratory symptoms were registered. Body temperature higher than 40 °C was considered as fever. Intestinal symptoms such as liquid stool and respiratory symptoms like coughing and nasal secretion were registered. Body weight (BW) (kg), chest diameter (cm), and height were determined. Average daily gain (ADG) was calculated as the rate of weight gain per day over the time period evaluated.

Health Index

The health index of the animals was scored during the experiment on the basis of the fecal consistency or diarrhea, body condition, hair coat appearance, chest diameter, height, and ADG. The index was calculated from different parameters. A score of 1 (for each parameter) represents: solid stools, healthy body condition, and healthy (glossy) hair coat; chest diameter, height, and ADG increase. A score of 0 indicates: diarrhea, low body condition, opaque hair coat; no increase in chest diameter, height, or ADG. The index was calculated from the scores obtained divided by the maximum score for each parameter. The indexes were determined in surviving animals (Table 1).

Sample Collection

Stool samples were obtained directly by stimulation of the anal sphincter (8–10 g) and received in sterile containers (Deltalab, Barcelona, Spain) to determine cultivable microbial population. The samples were kept in ice boxes and stored at 4 °C until analysis. For parasitological studies, the samples were collected in 3.5% formaldehyde (Cicarrelli, San Lorenzo, Argentina) (3–5 g). Stool consistency was recorded as normal or scours, and blood and mucus were indicated. Rectal swabs were collected and preserved in Stuart transport medium (Deltalab, Barcelona, Spain) for the investigation of *Salmonella* sp., inoculated on selective media (Selenite broth-Britania, CABA, Argentina) and/or streaked on SS (Salmonella Shigella Agar; Britania, CABA, Argentina). No lactose fermenting (colorless colonies) or SH_2 producer isolates (colonies with a black center) were analyzed by phenotypic characteristics (glucose anaerobic fermentation, urea hydrolysis, indole production from tryptophan, decarboxylation of lysine, and citrate utilization) for further identification. The quantification of cultivable bacteria from stools was performed by the successive dilutions method on peptone water and plating on different selective media. The populations studied were: aerobic bacteria (Plate count agar; Britania, CABA, Argentina); enterobacteria (Mac Conkey Agar; Britania, CABA, Argentina), both later incubated for 24 h at 37 °C, and lactic acid bacteria (in MRS agar incubated for 48 h at 37 °C in microaerophilic conditions). Parasitological studies were performed using the flotation technique with saturated NaCl solution and Barber Koffoyd [4] for identification of protozoa, nematodes, and cestodes. Furthermore, direct microscopic observation of the samples (Olympus, Tokyo, Japan) was performed for cysts or eggs identification.

Blood samples were obtained by jugular puncture. Blood was collected in two plastic tubes (K3 EDTA, Eurotubo K3, Deltalab, Barcelona, Spain; BD vacutainer, BD Franklin Lakes, New Jersey, USA) and a smear was performed on a slide (Deltalab, Barcelona, Spain) for staining. Then, samples were transported under refrigeration to the laboratory for further assays. The hematological profile was performed in blood collected with EDTA as anticoagulant using a hematologic counter. Also, May-Grünwald-Giemsa stain was applied to determine red blood cells morphology and white cells differential counts. Hemoglobin was determined using the cyanmethemoglobin colorimetric method (Wiener Lab, Rosario, Argentina). The different serum proteins were evaluated by electrophoresis techniques quantifying albumin, alpha 1, alpha 2, beta, and gamma proteins (g/L) and albumin/globulin ratio. The Biuret reaction was used to determine protein concentration in blood (g/L).

Statistical Analysis

Diarrheal incidence, parasitosis, mortality, and association with the treatment applied to control and treated animals were determined using the chi-square test. Health index, ADG, microbial population, hematological formula, hematocrit, total serum proteins, and album-globulin ratio were evaluated using the non-parametric Kruskal Wallis test.

Results

Fifty-six calves were randomly assigned to two groups: 26 to C and 26 to T; calf sex and number of dead and surviving specimens are shown in Fig. 1. Three samplings were performed. Almost all the animals completed the treatment and drank the fermented milk in the T in contrast with the C. In the control group, some of the animals died during the experiments and at the end 17 animals remained. Diarrhea incidence, mortality, antibiotics treatment, respiratory symptoms, and fever were calculated with all animals as described in Fig. 2.

Diarrhea Incidence and Mortality in Animals

Diarrhea prevalence was 69.2% in C and 46.15% in the group fed the fermented milk ($P = 0.09$). Mortality in C was 34.61 and 7.69% in animals fed fermented milk ($P = 0.02$) (Fig. 3); most deaths in control animals occurred during the first month of age with diarrhea symptoms: two of them in week 1, five in week 3, and two later on. Among treated animals, one death occurred during the first week without diarrhea. Only one animal suffering from diarrhea and treated with fermented milk died at 60 days of age.

According to the severity of the diarrhea symptoms, control animals were treated with antibiotics; however, some of them died. The animals in the C that received antibiotics (standard treatment) were 46.30% as against 11.53% ($P = 0.01$) in the T. One of the T animals was treated with antibiotics to prevent infection postmechanical injury.

Table 1 Parameters, categories, and scoring for health index determinations

Parameters evaluated	Categories	Points (score)	Number of determinations (sampling)	Maximum score[b]
Feces consistency	Solid stools	1	3	3
	Diarrhea	0		
Body condition	Healthy body condition	1	3	3
	Low body condition	0		
Hair coat appearance	Glossy	1	3	3
	Opaque	0		
Chest diameter[a]	Increase	1	3	2
	No increase	0		
Height[a]	Increase	1	3	2
	No increase	0		
ADG[a]	Increase	1	3	2
	No increase	0		
Maximum score				15

[a] The increase in chest diameter, height, and ADG was determined by comparing the result of the previous measurement with the one performed in that sampling

[b] The index was calculated from the scores obtained divided by the maximum score obtained for each parameter. The indexes were determined in surviving animals

Respiratory Symptoms and Body Temperature

Six animals from the C had fever, while only two animals in the T showed higher temperature (one of them with a mechanical injury, which could be the reason for the fever). No respiratory symptoms were observed in either of the two groups.

Growth Performance and Calf Health Index

The health index of each animal considering feces consistency (diarrhea), body condition, chest diameter, height, hair coat condition, and ADG showed higher values in calves with fed-fermented milk ($P = 0.01$) (Fig. 4). Treated animals were healthier, with solid consistency of feces and no diarrhea, and

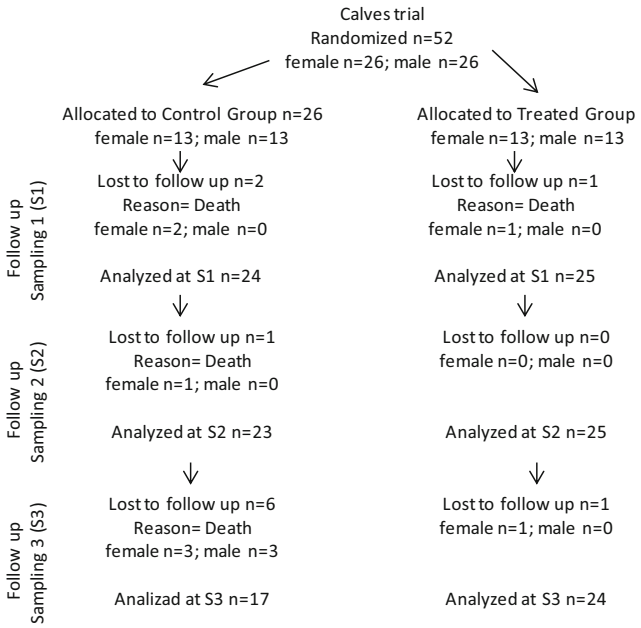

Fig. 1 *Flowchart* of animal trials. Calves were randomly allotted to control and treated groups

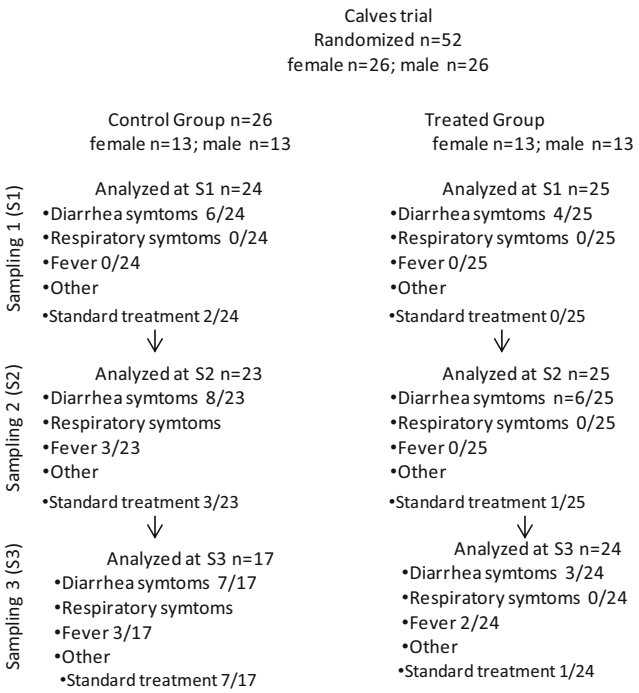

Fig. 2 *Flowchart* of diarrhea incidence, respiratory symptoms and fever in control and treated groups

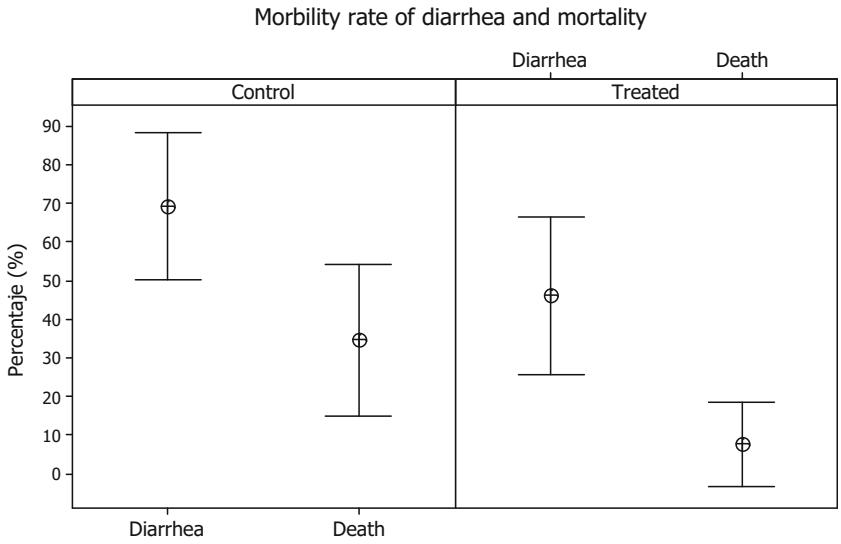

Fig. 3 Mobility rate of calf diarrhea and mortality in control and treated groups fed fermented milk

glossy hair coat; also, average daily gain, chest diameter, and height increased during the experiment. Animals in the T with some diarrhea episodes showed a lower severity of the symptoms and no fever. The individual weight of the animals is plotted in Fig. 5. Statistical analysis of ADG results indicates that there was a significant difference ($P = 0.03$) between C and T, with 0.276 and 0.102 kg/day in treated and control animals, respectively.

Salmonella Identification and Microbial Population

No positive *Salmonella* samples were isolated with the technique applied. The number of cultivable total aerobic bacteria, enterobacteria, and LAB were similar between the two treatments ($P = 0.46$; $P = 0.26$; $P = 0.25$, respectively). However, the number of lactic acid bacteria in the T group was almost similar to the enterobacteria group (samples 1 and 2) in contrast with the control group, where enterobacteria numbers were always higher, as shown in Table 2.

Parasitic Incidence

The prevalence of coccidiosis was similar in treated (47%) and control (50%) animals ($P = 0.87$). Gastrointestinal nematode infection was identified in one animal in the control group.

Hematological Samples and Serum Proteins

There were no significant differences in WBC counts, RBC percentage, or hemoglobin concentration between controls and calves treated with fermented milk (Table 3). The reference values for total protein concentration were 50–78 g/L, 29–41 g/L for albumin and 20–40 g/L for globulin [31]. No

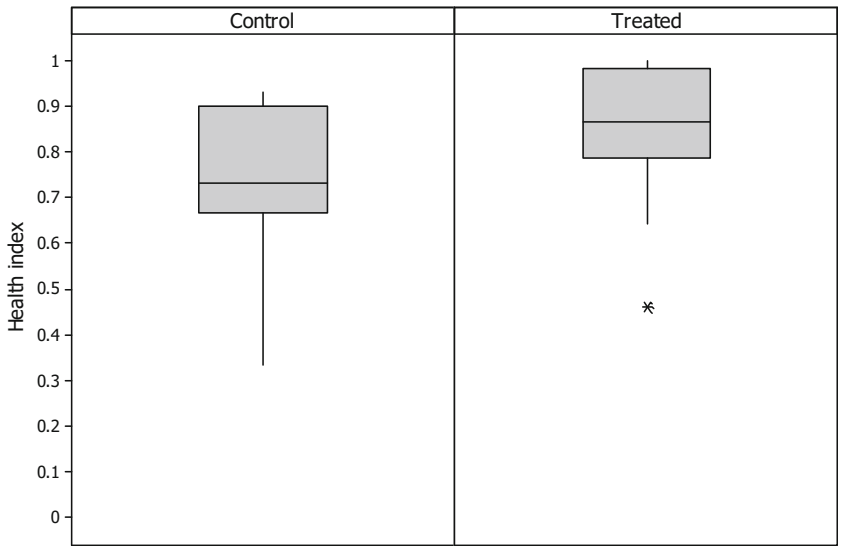

Fig. 4 *Box plot* of the health index calculated for control and treated groups, as indicated in the text. Statistically significant differences were observed between the groups

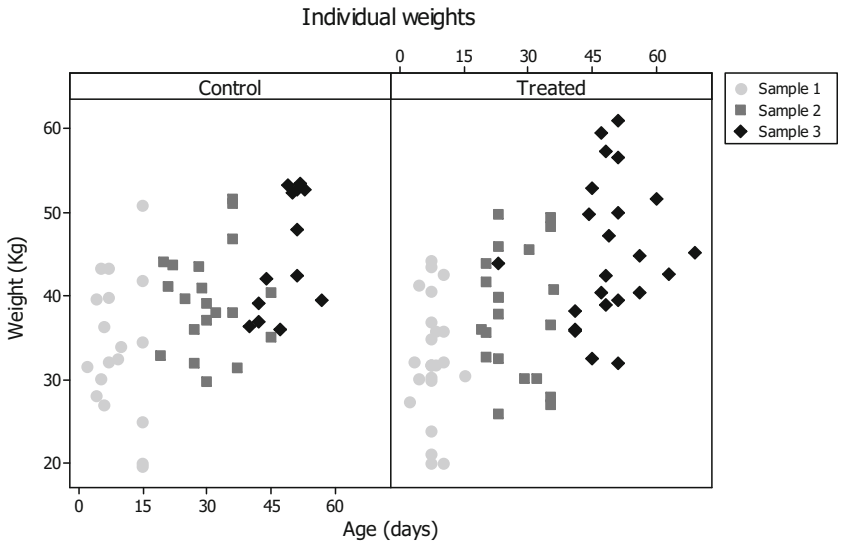

Fig. 5 Individual weight in control and treated group fed-fermented milk

statistically significant differences were found in either albumin/globulin ratio or serum proteins between the two groups (samples 1, 2, and 3).

Discussion

The intensive management systems applied in dairy farms reduce the lactation period in calves, cause numerous modifications in the gastrointestinal tract (GIT) and, in some cases, is responsible for diarrheal episodes [5]. That is why our research group worked on the design of a multi-strain fermented milk for calves to be administered from their birth up to the transition to solid food. The fermented milk was formulated with four different LAB strains, previously selected on the basis of their beneficial properties [16, 18]. The use of different strains sharing beneficial characteristics could have favorable effects on the animals [23, 28]. In this work, administration of the fermented milk reduced mortality and diarrhea incidence in young calves and improved nutritional parameters such as height, weight, and body performance. Similar results of growth performance were obtained by Zang et al. [32]. Also, the supplementation of calf feeding with microorganisms was performed by other scientists with different effects such as a reduction in diarrhea mobility, increase in weight gain and decrease in some bacterial populations such as clostridia [1, 15, 21].

The diarrhea morbidity and mortality rates obtained in the C were in agreement with those reported by González Pereyra *et al*. [7] in Argentinean dairy farms. Probiotic administration reduced the incidence of calf scours, severity, and duration of diarrhea. Similar results were obtained by Mokhber-Dezfouli et al. [21].

Table 2 Cultivable aerobic, lactic acid bacteria, and enterobacteria population in calves' feces in control and treated groups

	Aerobic population (log CFU/g feces)			Lactic acid bacteria (log CFU/g feces)			Enterobacteria (log CFU/g feces)		
	Mean	Max	Min	Mean	Max	Min	Mean	Max	Min
Sample 1[a]									
Control group	10.49 ± 1.33	12.37	6.81	8.32 ± 1.75	10.45	5.32	9.39 ± 1.13	11.59	7.00
Treated Group	10.89 ± 0.60	12.24	9.71	8.48 ± 1.73	11.75	5.94	10.18 ± 0.76	11.28	8.34
Sample 2[a]									
Control group	10.15 ± 0.94	11.16	7.84	8.29 ± 1.64	10.03	4.17	9.19 ± 1.45	11.02	5.30
Treated group	10.37 ± 0.94	12.07	8.37	7.92 ± 1.5	10.82	5.68	9.30 ± 1.35	11.09	6.63
Sample 3[a]									
Control group	10.21 ± 0.99	11.43	7.75	8.09 ± 1.96	10.58	4.83	9.56 ± 1.16	10.82	6.75
Treated group	10.24 ± 0.97	11.99	8.59	8.09 ± 1.33	10.02	5.60	9.38 ± 1.13	10.97	6.70

Results are expressed as mean ± standard error. Max and min correspond to higher and lower values. No significant differences were observed between the two groups at different samplings 1, 2, and 3 ($P < 0.05$)

[a] Samples were collected from calves during the experiment at different ages: Sample 1 (5–15 days old), Sample 2 (15–30 days old) and Sample 3 (30–60 days old)

Table 3 Blood profile and serum proteins in control Group and probiotic treated Group

Group	Sample[a] 1		Sample[a] 2		Sample[a] 3	
Blood profiles	Control	Treated	Control	Treated	Control	Treated
RBC (10^6/mm^3)	8.05 ± 0.35	7.74 ± 0.65	8.13 ± 0.39	7.82 ± 0.34	7.92 ± 0.47	7.50 ± 0.41
WBC (10^3/mm^3)	6.68 ± 1.72	7.18 ± 0.69	7.45 ± 0.795	7.46 ± 0.549	7.17 ± 0.84	7.26 ± 0.54
HGB (g/L)	147 ± 42	144 ± 13	146 ± 44	1.42 ± 14	146 ± 43	143 ± 53
HCT (%)	35.2 ± 3.96	37.6 ± 3.87	33.8 ± 4.55	36.8 ± 2.0	33.8 ± 2.79	33.0 ± 3.27
GRA (10^3/mm^3)	1.94 ± 0.81	2.24 ± 0.353	2.34 ± 0.788	2.35 ± 0.90	2.54 ± 0.54	1.35 ± 1.32
GRA(%)	28.6 ± 7.26	31.2 ± 4.08	33.5 ± 4.85	35.2 ± 4.79	35.4 ± 5.73	34.9 ± 4.88
EO (10^3/mm^3)	0.49 ± 0.11	0.18 ± 0.47	0.71 ± 0.88	0.25 ± 0.58	0.83 ± 0.84	0.16 ± 0.58
EO (%)	7.06 ± 1.7	2.73 ± 7.03	1.07 ± 1.22	3.53 ± 0.7.86	1.19 ± 1.17	4.29 ± 1.13
BAS (10^3/mm^3)	–	0.11 ± 0.04	–	–	–	–
BAS (%)	–	1.82 ± 0.59	–	–	–	–
LYM (10^3/mm^3)	4.58 ± 1.14	4.88 ± 0.58	4.38 ± 1.33	4.21 ± 1.57	4.38 ± 0.69	6.91 ± 2.42
LYM (%)	68.9 ± 5.91	67.9 ± 4.28	62.7 ± 4.90	63.0 ± 4.74	61.2 ± 6.39	63.0 ± 5.69
MONO (10^3/mm^3)	0.14 ± 0.15	0.47 ± 111	0.118 ± 142	0.89 ± 124	0.14 ± 0.14	0.14 ± 0.11
MONO (%)	2.90 ± 0.25	6.82 ± 0.16	1.80 ± 0.21	1.41 ± 0.18	1.94 ± 0.18	1.71 ± 1.70
Blood serum proteins						
Total protein (g/L)	59.7 ± 9.99	53.4 ± 7.76	54.0 ± 5.87	56.1 ± 5.35	54.1 ± 6.67	56.0 ± 6.11
A/Gl	0.86 ± 0.36	0.95 ± 0.46	0.76 ± 0.20	0.68 ± 0.40	0.77 ± 0.15	0.98 ± 0.43
Albumin (g/L)	26.7 ± 4.79	25.4 ± 5.03	22.6 ± 1.59	25.0 ± 2.85	23.0 ± 1.53	25.5 ± 3.41
Alpha 1 (g/L)	10.5 ± 4.33	8.18 ± 3.65	10.7 ± 2.66	10.9 ± 1.97	11.3 ± 2.95	8.77 ± 2.90
Alpha 2 (g/L)	7.03 ± 2.14	6.37 ± 2.58	7.73 ± 1.49	9.28 ± 2.70	8.62 ± 1.60	7.56 ± 3.23
Beta (g/L)	1.32 ± 7.75	1.12 ± 4.32	8.66 ± 4.74	1.09 ± 7.58	8.21 ± 3.03	8.91 ± 7.30
Gamma1 (g/L)	2.47 ± 3.96	2.01 ± 4.13	3.79 ± 4.19	3.17 ± 1.23	2.79 ± 4.27	5.19 ± 5.07

Results are expressed as mean ± standard error. No statistical differences were obtained among the blood samples between control and treated animals ($P < 0.05$)

RBC erythrocytes count, *HGB* hemoglobin, *HCT* hematocrit, *WBC* white blood cell count, *GRA* granulocytes count and *GRA (%)* granulocyte percent, *EO* eosinophils count, *EO* eosinophils percent, *BAS* basophils count, *BAS* (%) basophilspercent, *LYM* lymphocyte count, *LYM (%)* lymphocyte percent, *MONO* monocytes count, *MONO (%)* monocyte percent, *A/Gl* albumin/globulin ratio

[a] Samples were collected from calves during the experiments at different ages: Sample 1 (5–15 days old), Sample 2 (15–30 days old), and Sample 3 (30–60 days old).

The techniques applied for the isolation and identification of *Salmonella* were used by different authors [3], with no positive samples in young calves. Although diarrhea can be caused by multiple infectious and non-infectious factors, diagnosis was not performed in this work. With respect to coccidiosis, treated animals showed a lower prevalence than controls, in agreement with the reports of Silva et al. [26] for organic farm dairy herds in southeastern Brazil.

The evaluation of hematological parameters indicated no differences in either WBC counts or serum protein concentration (albumin and globulin) between the probiotic and the control groups, in contrast with the results obtained by Hosseini et al. [8] who found higher WBC counts in broiler chicks and lambs fed with beneficial microorganisms. With respect to serum proteins, the control and treated groups maintained reference values, with no differences between them.

Conclusions

A multi-strain fermented milk was administered to calves. Animal performance, diarrhea incidence, nutritional, microbiological, and hematological parameters were evaluated. The probiotic product proved to be beneficial for young animals as shown by a decrease in diarrhea prevalence and mortality rates. No respiratory symptoms were observed in animals treated with the fermented milk. Also, a health index showed statistical differences between the control and treated groups, indicating that calves fed with probiotic milk were healthier and had a higher weigh gain. Average daily gain was significantly different between T and C in older animals. *Salmonella* was not isolated, and cultivable bacteria numbers in feces were not modified by fermented milk consumption. Parasitosis rate was similar in control and treated animals. Hematological profile and serum proteins were not modified

by the probiotic, despite certain differences observed in the white blood cell (WBC) count.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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