Arch Med Vet **47, 237-244 (2015)**

COMMUNICATION

Faecal culturable microbiota, growth and clinical parameters of calves supplemented with lactic acid bacteria and lactose prior and during experimental infection with *Salmonella* Dublin DSPV 595T

Microbiota cultivable fecal, crecimiento y parámetros clínicos de terneros suplementados con bacterias ácido lácticas y lactosa previo y durante una infección experimental con *Salmonella* Dublin DSPV 595T

LP Soto[a,b], LS Frizzo[a,b], ML Signorini[b,d*], MV Zbrun[a,b], L Lavari[c], E Bertozzi[b], GJ Sequeira[b,c], MR Rosmini[b,c]

[a]Laboratorio de Análisis de Alimentos, Instituto de Ciencias Veterinarias del Litoral, Consejo Nacional de Investigaciones Científicas y Técnicas (ICIVET-CONICET), Santa Fe, Argentina.

[b]Departamento de Salud Pública, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Esperanza, Provincia de Santa Fe, Argentina.

[c]Universidad Católica de Córdoba, Facultad de Ciencias Agropecuarias, Córdoba, Provincia de Córdoba, Argentina.

[d]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto Nacional de Tecnología Agropecuaria, EEA RAFAELA, Santa Fe, Argentina.

RESUMEN

El objetivo de este trabajo fue evaluar el efecto del inóculo probiótico + lactosa sobre el peso, la microbiota intestinal, la morbilidad y la mortalidad de terneros jóvenes desafiados con *Salmonella* Dublin DSPV 595T. Se utilizaron 24 terneros divididos en un grupo control (GC) y un grupo probiótico (GP). Al GP se le administró 100 g lactosa.ternero^{-1}.d^{-1} y 10^{10} UFC.ternero^{-1}.d^{-1} de cada cepa de un inóculo probiótico compuesto por *Lactobacillus casei* DSPV318T, *Lactobacillus salivarius* DSPV315T y *Pediococcus acidilactici* DSPV006T a lo largo de todo el experimento. El patógeno fue administrado el día 11 del experimento, en una dosis oral de 10^9 UFC.animal^{-1}. Las poblaciones de *Lactobacillus* y levaduras fueron modificadas en el GP a causa de la administración del inóculo + lactosa. La severidad de la diarrea fue menor en el GP. No se encontraron diferencias en el resto de los signos clínicos, el peso vivo y la mortalidad entre los dos grupos analizados. En este estudio, la administración periódica de un inóculo probiótico de origen bovino y lactosa, favoreció el establecimiento de una microbiota intestinal más estable y balanceada, aun durante una infección con *Salmonella*. El modelo de infección aguda le dio la oportunidad al probiótico de ejercer su efecto benéfico sobre la severidad de la diarrea. Sin embargo, para futuros estudios se recomienda el uso de dosis menores de *S.* Dublin DSPV 595T para generar un modelo menos severo para evaluar si el inóculo es capaz de ejercer una respuesta diferente en los signos clínicos de terneros jóvenes.

Palabras clave: terneros, probióticos, *Salmonella,* lactosa.

SUMMARY

The aim of this study was to evaluate the probiotic inoculum + lactose effect on weight, intestinal culturable microbiota, morbidity and mortality of young calves challenged with *Salmonella* Dublin DSPV 595T. Twenty eight calves were used, divided in control group (CG) and probiotic group (PG). The PG was provided with 100 g lactose.calf^{-1}.d^{-1} and 10^{10} CFU.calf^{-1}.d^{-1} of each strain of a probiotic inoculum composed of *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T throughout the experiment. The pathogen was administered on day 11 of the experiment, at an oral dose of 10^9 CFU.calf^{-1}. *Lactobacillus* and yeast populations were modified in PG because of inoculum + lactose administration. Severity of diarrhea was lower in PG. No differences were found on the rest of clinical sings, live weight and mortality between the two groups analysed. The periodic administration of a probiotic inoculum of bovine origin and lactose, favoured the establishment of a more stable and balanced intestinal culturable microbiota, even during an infection with *Salmonella*. The generated model of acute infection gave opportunity to the probiotic to exert its beneficial effect on severity of diarrhea. However, the use of lower doses of *S.* Dublin DSPV 595T are recommended for future studies, to generate less severe model in order to evaluate if the inoculum is able to exert a differential response in the clinical symptoms of young calves.

Key words: calves, probiotics, *Salmonella,* lactose.

INTRODUCTION

The balance of the intestinal microbiota is of vital importance in the host nutritional status and is of particular interest in farm animals that are raised in intensive systems (Rosmini *et al* 2004). The balance of the intestinal ecosystem can be altered in intensive farming systems due to separation from their mothers, feeding with milk replacers and the subsequent elimination of the benefits of cow's milk, inadequate colostrum intake, stressful situations, and use of antibiotics (Signorini *et al* 2012, Bayatkouhsar *et al* 2013). This imbalance leaves the animals in a state

Accepted: 20.08.2014.

* P. Kreder 2805. (CP S3080HOF) Esperanza, Santa Fe, Argentina; marcelo.signorini@gmail.com

of increased susceptibility to infections. One of the most common culturable bacterial pathogen found in calves is *Salmonella*, being *Salmonella enterica* serotype Dublin the predominant type (Paulin *et al* 2002).

Probiotics are "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2001). The periodic administration of a probiotic inoculum of bovine origin seems to favour the establishment of a more stable and balanced intestinal microbiota, thus improving the health of calves (Abe *et al* 1995). Lactic acid bacteria (LAB) with probiotic properties has been considered as possibly responsible for controlling the effects of *Salmonella* spp. (Fayol-Messaoudi *et al* 2007, Rishi *et al* 2009, Castillo *et al* 2012). The use of a probiotic inoculum could control the action of this pathogen by improving the intestinal microbial balance. The aim of this study was to evaluate the effect of administration of a probiotic inoculum and lactose on weight, culturable intestinal microbiota, morbidity and mortality of young calves challenged with *Salmonella* Dublin DSPV 595T.

MATERIAL AND METHODS

ANIMALS

A total of 28 calves Holstein (*Bos taurus*) with a mean age of 5 days of life were used in the experiment, divided into two experimental groups of 15 animals in the CG and 13 animals in the PG. All animals were fed with starter supplied without drugs and water *ad libitum* and with a milk replacer (4 $l.d^{-1}$), rationed directly into the feeder twice a day. Spray-dried lactose was also provided to the PG (50 g in each administration) together with the milk replacer (Frizzo *et al* 2011[a]), and 150 ml of probiotic inoculum in the afternoon delivery of milk replacer. The calves were evaluated for 27 d. Animals care was provided following the Guidelines for the care and use of animals in research and teaching (FASS 1998).

MICROORGANISMS

The inoculum used consisted of: *Lactobacillus casei* DSPV 318T, *Lactobacillus salivarius* DSPV 315T and *Pediococcus acidilactici* DSPV 006T. Their accession numbers in GenBank are: FJ787305, FJ787306 and FJ787307, respectively. The pathogen used was *S.* Dublin DSPV 595T, whose accession number in GenBank is FJ997268.

SELECTION OF ANTIBIOTIC-RESISTANT MUTANTS

In order to monitor the inoculated strains, they were marked by antibiotic resistance for distinguishing them from strains present in the gut (Demecková *et al* 2002). The antibiotic resistance of LAB of the inoculum was obtained by successive cultures in *Lactobacillus* Anaerobic MRS with vancomycin and bromocresol (LAMVAB) medium (Hartemink *et al* 1997) from low concentrations up to a concentration of 10 $\mu g.ml^{-1}$ of rifampicin (Demecková *et al* 2002). A similar procedure was used with the strain *S.* Dublin DSPV 595T using xylose, lysine, desoxicolate (XLD) medium and the antibiotics novobiocin (50 µg/ml) and nalidixic acid (10 µg/ml) ($XLD_{nov/nal}$) (Oyofo *et al* 1989) .

PREPARATION AND ADMINISTRATION OF LAB INOCULUM

The bacteria were multiplied in skim milk (100 $g.L^{-1}$) supplemented with casein hydrolysate (50 $ml.L^{-1}$) for 18-20 h at 37 °C unstirred. The culture was dispersed into containers and frozen at -20 °C until use in artificial rearing of calves, which consisted of a total dose of 10^{10} $CFU.calf^{-1}.d^{-1}$ of each strain (Soto *et al* 2009, Frizzo *et al* 2012). This inoculum was administered to the calves of the probiotic group (PG) during the 27 days of the experiment. The control group (CG) was inoculated in the same way but with 150 ml of skim milk (100 $g.L^{-1}$), which served as a placebo.

PATHOGEN INOCULATION

The *S.* Dublin DSPV 595T strain, cultivated in brain heart infusion (BHI) broth for 18 h at 37 °C, was administered together with the milk replacer, on day 11 of the experiment to all calves of the two experimental groups (CG and PG). To establish the strain concentration, ten-fold dilutions were made from a culture. In this culture and its dilutions, absorbance was determined at 560 nm and, simultaneously, counts on plates were performed. Regression analysis was performed with both parameters. Subsequently, to quantify the amount provided we used the equation $y = 0.4735\ln(x) + 8.2162$, where y corresponds to $\log_{10}$ $CFU.ml^{-1}$ and the variable x measures the absorbance of the culture. The pathogen was given at a dose of 10^9 $CFU.animal^{-1(1)}$.

EXPERIMENTAL DESIGN

Live weight data were recorded at the beginning of the experiment, prior to pathogen inoculation and at the end of the experiment (day 1, 10 and 27).

The health status of the animals was determined daily by the following clinical indicators: body temperature, dehydration level, frequency of diarrhoea by a macroscopic analysis of faeces, severity of diarrhoea, presence or absence of ocular discharge, state of the mucosa, cold nose, cold extremities, weakness in the hindquarters and difficulty in standing up. The temperature was determined as normal up to 40.5 °C. The level of dehydration was determined by skinfold time as suggested by Blood *et al* (1986). Faeces were macroscopically analysed according

[1] Data not published

to the scores proposed by Meyer *et al* (2001) (table 1). The indicators for severity of diarrhoea, eye discharge, state of the mucosa and difficulty in standing up are described in table 1. Given these parameters, morbidity was calculated based on the proportion of animals with abnormal appearance in each group.

RECOVERY OF LAB INOCULUM, TOTAL LACTOBACILLUS AND OTHER CULTURABLE POPULATIONS FROM THE INTESTINAL MICROBIOTA

Faecal samples (approximately 5 g) were obtained from three calves of each group by rectal massage on days 1, 5, 10 and 27 of the experiment, and then weighed, diluted 1.100^{-1} in Ringer ¼ solution and homogenised on a magnetic stirrer according to Frizzo *et al* (2011[b]). Serial dilutions of each sample were pour-plated with different media for the count of the population detailed in table 2.

DETECTION OF SALMONELLA DUBLIN DSPV 595T IN FAECES

The faecal samples were collected from all experimental animals at the beginning of the experiment and on days 15 and 27 by rectal massage. Faeces were cultured either in selenite cystine broth for 12 h at 42 °C and in Rappaport Vassiliadis broth for 18 h at 42 °C (1g and 0.1g respectively). After incubation, the culture was inoculated in XLD $_{nov/nal}$ agar plates, which were incubated at 37 °C for 24 h. The finding of typical colonies with positive agglutination test with a polyclonal antibody (OS-A and OS-B, A.N.L.I.S Dr. Carlos G. Malbrán) showed a positive result for *Salmonella* Dublin.

STATISTICAL ANALYSIS

The effect of probiotic administration on weight, temperature, and microbial counts was analysed in a factorial ANOVA (treatment x time) and Duncan`s test. Ordinal variables (clinical indicators: macroscopic analysis of faeces; severity of diarrhoea; eye discharge; state of the mucosa; difficulty in standing up) were analised using the Mann-Whitney test. Dichotomic variables (clinical indicators: cold nose; cold extremities; weakness in hind quarters) were performed using Chi-squared test. A Kaplan-Meier survival analysis (using Breslow test) was performed to evaluate the effect of probiotic supplementation on mortality as consequence of *Salmonella* infection. For these analyses, SPSS 11.0 for Windows software was used with $P < 0.05$ representing a significant difference between means.

Table 1. Severity level for each clinical indicator.
Nivel de severidad para cada indicador clínico.

Clinical indicators	Level			
	1	2	3	4
Macroscopic analysis of faeces	Normal: firm stools, but not hard, their original shape is distorted slightly when it falls and sits on the floor	Soft: shapeless, when they fall they are deposited in mounds and spread slightly	Fluid: they disperse rapidly in sheets of 6 mm deep	Watery: liquid consistency (diarrhoea)
Severity of diarrhoea	mucosa	bloody	egg yolk	fibrinous
Eye discharge	serous	purulent	fibrinous	-
State of the mucosa	normal	pale-hyperemic	with petechiae	icteric
Difficulty in standing up	normal	slow	with help	prostrated

Table 2. Microbial population studied, media and culture conditions used.
Poblaciones microbianas estudiadas, medios y condiciones de cultivo utilizadas.

Microorganisms	Media	Culture conditions
Lactobacillus spp.	LAMVAB	Anaerobiosis, 48 h, 37 °C
Inoculum	$LAMVAB_{rif}$	Anaerobiosis, 48 h, 37 °C
Coliforms	Violet Red Bile Lactose (VRBL)	Aerobiosis, 24 h, 37 °C
Yeast	Fungi and yeasts (F & Y) modified *	Aerobiosis, 48 h, 37 °C
Enterococci	Slanetz and Bartley (S & B)	Aerobiosis, 24 h, 37 °C

* With the addition of 2% dextrose and 0.5% pluripeptone.

RESULTS AND DISCUSSION

At the beginning of the experiment the weight of calves was similar for both groups (41.8 kg PG and 40.9 kg CG, $P > 0.05$) and no differences were found between the groups on any of the next evaluated days. During the first 10 days of the experiment, the calves from both groups lost weight (CG animals lost 1 kg whereas PG ones lost 0.1 kg in average), which is considered normal during the first weeks of life for calves and may be due to transport stress (Adams *et al* 2008). On day 27, animals that survived had a weight gain of 7.3 kg for PG and of 3.8 kg for CG as compared to the start of the experiment.

Prior to the beginning of the experiment the animals were free of the pathogen. On day 15 of the experiment, which corresponds to day 4 post-infection, *Salmonella* was detected in faeces of both groups (60% of animals of GC and 33% of animals of PG) and on day 27, only a small percentage of the PG (16%) was positive to *Salmonella*. There were no differences ($P > 0.05$) in the presence/absence of *Salmonella* between groups for any of the times evaluated. *Salmonella* detection not always is an indicator of the real presence of this pathogen, because bacteria are not excreted continuously. This was evident when all animals manifested typical signs of illness (day 15), but not all of them were positive to *Salmonella* detection. Similarly, it is expected that even after the animals are recovered, the pathogen is found in the stool, because excretion of the pathogen can remain in the surviving animals for months and even years (Klee 2005).

Differences ($P = 0.009$) in the *Lactobacillus* count in faeces between the two groups were found at day 10 of the experiment. At this moment, the CG counts decreased showing 2.19 Log CFU.g^{-1} less than those of the PG. On day 5 of the start of the administration of the probiotic, most of the faecal *Lactobacillus* recovered were the strains belonging to the inoculum. This relationship was kept during the following sampling points (figure 3). In the pre infection period *Lactobacillus* population stabilised in the PG at normal concentration reaching the level between 10^7 and 10^8 CFU.g-1 suggested by Anadón *et al* (2006). This population decreases in calves in an artificial rearing system, fed with milk replacer containing antibiotics (Ozawa *et al* 1983). In this work, *Lactobacillus* population decreased in CG even without antibiotic supplementation, showing the effects caused on this bacterial population by artificial rearing system. On day 27 (16 days after infection with *S.* Dublin DSPV 595T) counts of *Lactobacillus* of both groups decreased, being more pronounced ($P = 0.042$) in the CG, which reached 1 Log CFU.g^{-1}, i.e. 5 Log CFU.g^{-1} less than the PG. Both Coliforms as Enterococci were not affected by the probiotic treatment neither before nor after the infection. Coliforms population remained constant over time, except on day 5, when it was increased ($P = 0.01$) for both groups (figure 3), which may have been due to the stress experienced by the relocation of calves, which was reflected in the decrease in weight. This condition was reversed on day 10, when animals were already adapted to such conditions. Using the values of *Lactobacillus* and coliforms populations the relationship between them was analysed. This *Lactobacillus*/coliforms relationship was higher for the PG ($P = 0.011$) from day 10 of the experiment and a relationship of more than 1 for the PG and less than 1 for the CG, both before and after the infection (figure 4). This index *Lactobacillus*/coliforms has been related to the presence/absence of diarrhea by Abu-Tarboush *et al* (1996) propose that animals with diarrhoea have a rate lower than 1, while healthy ones have an index greater than 1. In this study the group that showed an index lower than 1 had

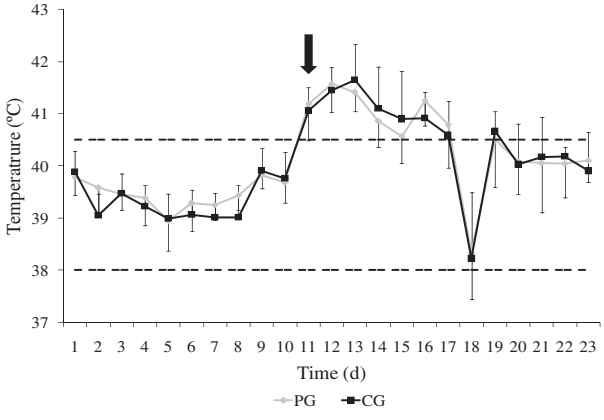

Figure 1. Average daily rectal temperature in control group (CG) and probiotic group (PG). The arrow indicates the time of inoculation with *Salmonella* Dublin DSPV 595T. The dotted lines indicate the range of normal values of rectal temperature in cattle.

Promedio diario de temperatura rectal en el grupo control (CG) y el grupo probiótico (PG). La flecha indica el momento de la inoculación con *Salmonella* Dublin DSPV 595T. La línea de puntos indica el rango de valores normales de la temperatura rectal en terneros.

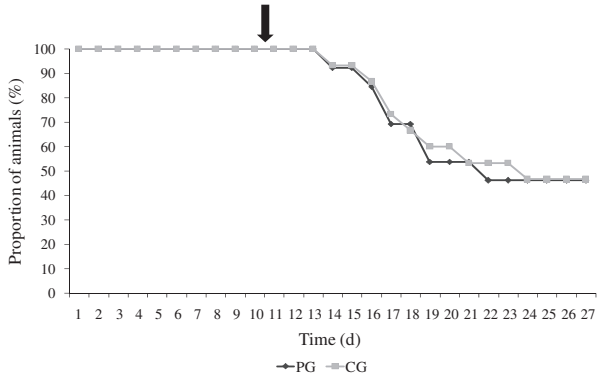

Figure 2. Percentage of live animals of control group (CG) and probiotic group (PG) during the experiment. The arrow indicates the time of inoculation with *Salmonella* Dublin DSPV 595T.

Porcentaje de animales vivos del grupo control (CG) y grupo probiótico (PG) durante el experimento. La flecha indica el momento de la inoculación con *Salmonella* Dublin DSPV 595T.

Table 3. Proportion of animals (%) that showed different severity levels for each clinical sign evaluated: faecal consistency, severity of diarrhoea, state of the mucosa, eye discharge and difficulty in standing up during the period after infection with *S.* Dublin DSPV 595T for control group (CG) and probiotic group (PG).

Proporción de animales (%) que presentaron diferentes niveles de severidad para cada signo clínico evaluado: consistencia fecal, severidad de la diarrea, estado de la mucosa, descarga ocular y dificultad en la incorporación durante el periodo post infección con *S.* Dublin DSPV 595T para el grupo control (CG) y el grupo probiótico (PG).

	Experimental groups								P
	PG				CG				
	Severity level				Severity level				
	1	2	3	4	1	2	3	4	
Faecal consistency	7.6	21.7	8.3	62.4	6.7	19.0	12.0	62.4	0.677
Severity of diarrhoea	37.1	7.3	12.4	43.3	24.8	14.2	8.7	50.5	0.049
State of mucosa	50.3	47.6	2.1	0.0	47.1	52.6	0.3	0.0	0.568
Eye discharge	85.9	9.3	4.8	-	90.2	7.8	2.0	-	0.103
Difficulty in standing up	80.3	10.7	6.2	2.8	84.7	5.5	6.1	3.8	0.214

Table 4. Proportion of animals (%) that showed presence of clinical signs (cold extremities, cold nose and weakness in hind quarters) after inoculation with the pathogen.

Proporción de animales (%) que presentaron signos clínicos (extremidades frías, morro frío, debilidad en el tren posterior) después de la inoculación con el patógeno.

	Experimental groups		P
	PG*	CG*	
Cold nose	47.4	47.6	0.962
Cold extremities	46.8	46.9	0.812
Weakness in hind quarters	18.2	23.1	0.153

* PG: probiotic group; CG: control group.

higher severity of diarrhoea than the group with a rate greater than 1, suggesting that it might be a correlation between these parameters. Yeast population was affected by the inoculum's administration presenting differences of more than 1.5 Log CFU.ml^{-1} compared to the CG on days 10 (P = 0.039) and 27 (P = 0.014). The PG counts remained stable throughout the experiment, whereas the CG decreased (P = 0.003) from day 10 (figure 3). It had been reported an antagonistic effect of the probiotic on the yeast population by Ozawa *et al* (1983). Instead, our results indicate that the administration of inoculum + lactose remained constant yeast counts in faeces of PG, as opposed to the CG in which this population was diminished over time. For the above might suppose that the administration of probiotics have a synergistic or antagonistic effect depending on the composition of the inoculums. On the other hand, should be clarified that in this work the effect of administration of LAB was studied on some populations and can not excluded that other unstudied populations have been modified by the addition of inoculum. Cultural methods used in this work are useful for the quantification of specific populations. To determine a change in any of the predominant populations in the complex intestinal microbiota is suggested to use rRNA-based analysis (Mayer *et al* 2012, Uyeno *et al* 2010).

All the clinical signs evaluated showed normal values during the first 10 days of the experiment in the pre-infection stage (data not shown), showing the good health of the calves before the *Salmonella*'s inoculation. In the post-infection period, all the animals showed at least one sign of disease, although with different frequencies of occurrence, being more frequent during the first 10 days after inoculation of *Salmonella*. The faecal consistency was one of the clinical signs that clearly showed infection. During the pre-infection stage, most of the calves had a faecal consistency of 2. After the administration of *S.* Dublin DSPV 595T most of the calves had a faecal consistency of 4 (diarrhoea). There were no significant differences between both groups in this parameter. For the indicator severity of diarrhoea was found that the number of animals with more severe diarrhoea was higher in the CG than in the PG (table 3). In the post-infection phase, half of the calves were normal and the other half had a level 2 in the state of the mucosa (pale or hyperemic) (table 3). These characteristic symptoms were seen during the first 10 days of infection. From day 10 post-infection, the number of calves that expressed symptoms decreased gradually and there were no differences between CG and PG. Very few animals showed abnormal levels as regards eye discharge and difficulty in standing up. These two parameters showed no differences between the two groups (table 3). From day 12 of the experiment and during the first 10 days post-infection, presence of cold extremities, cold nose and weakness in the hind quarters in high frequency were observed. After this period, the frequency of occurrence of such signs began to decrease. None of these three signs showed differences between both groups throughout the experiment (table 4). The degree of dehydration was assessed by measuring the skinfold

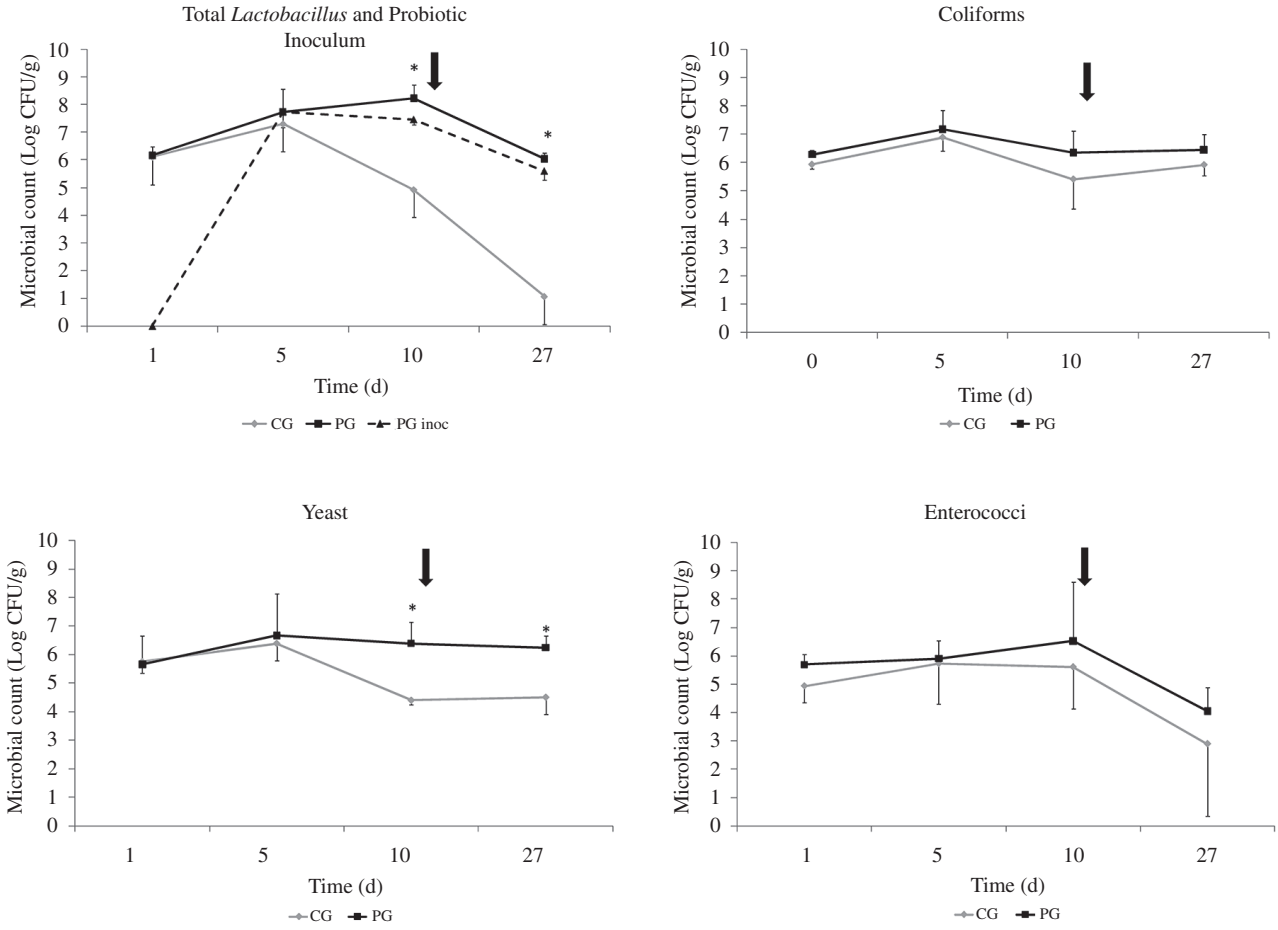

Figure 3. Counting of the populations of *Lactobacillus*, probiotic inoculum, coliforms, enterococci and yeast on days 1, 5, 10 and 27 of the experiment in control group (CG) and probiotic group (PG). * Means significant differences between CG and PG for the same time. PGinoc represents the count of bacteria belonging to the inoculum in the PG. The arrow indicates the time of inoculation with *Salmonella* Dublin DSPV 595T.

Recuento de poblaciones de *Lactobacillus*, inóculo probiótico, coliformes, enterococos y levaduras los días 1, 5, 10 y 27 del experimento en el grupo control (CG) y grupo probiótico (PG). *Significa diferencias significativas entre CG y PG en el mismo momento. PGinoc representa el recuento de bacterias que pertenecen al inóculo en el PG. La flecha indica el momento de la inoculación con *Salmonella* Dublin DSPV 595T.

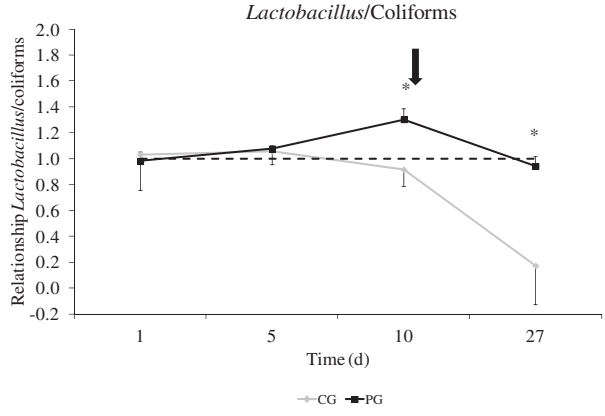

Figure 4. *Lactobacillus* relationship.Coliforms^{-1} in the control group (CG) and probiotic group (PG) during the experiment. *Means significant differences between CG and PG for the same time.

Relación *Lactobacillus*.Coliformes^{-1} en el grupo control (CG) y grupo probiótico (PG) durante el experimento. *Significa que existen diferencias significativas entre el CG y PG en el mismo momento.

time. This feature was normal during the pre-infection period. After inoculation of the pathogen, more than half of the animals showed a skinfold time between 2 and 4 s, thus showing dehydration. No differences between both groups were found for these parameters. The same day that was administered *S.* Dublin DSPV 595T, the rectal temperature began to increase above 40.5 °C, thus showing infection. The peak temperature was observed on day 13 (forty eight h after administration of the pathogen) and then decreased gradually to normal values on day 20 (figure 1). Both groups of calves behaved in the same way. Affected animals with salmonellosis show high fever between 40.5 and 42 °C (Wray and Davies 2000), but the curves of temperature may vary according to the model used, because in general, *Salmonella* infections present a variety of clinical manifestations depending on age of the animal, serotype and pathogen dose (Sarwari *et al* 2001). In this study, high fever became apparent soon after the administration of the pathogen, only 12

h post infection (figure 1) in contrast with other studies that observed a slower response, which led the animals to manifest fever among 24 and 72 h post infection (Paulin *et al* 2002, Silva *et al* 2010).

The animals began to die on day 14 of the experiment, three days after pathogen inoculation. The deaths were "in drip" (1 or 2 calves.d^{-1}.$group^{-1}$). Most deaths occurred during the first 10 days post-infection. Only three calves died after this period. The last calf died on day 24 of the experiment. In total, 53% of the animals in each group died following *Salmonella* challenge (figure 2). Most animals that survived after infection, proved to be in a stage of reversal of the disease. The experiment was closed 72 h after no more deaths were observed. Kaplan-Meier survival test was non-significant (P = 0.904). Mortality accounted for half of the infected animals (figure 2) so we define this as the lethal dose 50 (LD_{50}). This is the first time this dose of strain *S.* Dublin DSPV 595T (10^9 CFU. $animal^{-1}$) was given to generate a model of salmonellosis in calves, which was sufficient to induce the disease in all the animals tested, and the rapid clinical manifestation of the disease observed in this study shows that the model resulted in an acute infection in all animals. Although the dose used proved to be useful for building the model of the disease, the acute manifestation of the illness was not adequate to observe probiotic effects in some clinical signs (Frizzo *et al* 2012). The use of lower doses may be useful for development of symptomatic disease and evaluate the action of probiotic in this context. Therefore, for future studies are suggested to decrease the dose of the pathogen to levels that ensure the development of the disease in all individuals, and also would allow measure probiotic beneficial effects.

In this paper we propose a model of acute infection of *Salmonella* that has been useful to observe probiotics beneficial effects in the intestine. The daily administration of LAB inoculum + lactose supplementation favoured the establishment a stable amount of *Lactobacillus* and yeasts in faeces also before and during infection with *S.* Dublin DSPV 595T. The generated model of acute infection gave opportunity to the probiotic to exert its beneficial effect on severity of diarrhoea. However, for future studies use of lower doses of *S.* Dublin DSPV 595T are recommended, to generate less severe model to evaluate if the inoculum is able to exert a differential response in the clinical symptoms of young calves.

ACKNOWLEDGEMENTS

This study was financed by Universidad Nacional del Litoral, Universidad Católica de Córdoba and Asociación Cooperadoras Argentinas (ACA). L.S. Frizzo, L.P. Soto, M.V. Zbrum and M.L. Signorini are Researchers Career Members from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

REFERENCES

Abe F, N Ishibashi, S Shimamura. 1995. Effect of administration of Bifidobacteria and lactic acid bacteria to newborn calves and piglets. *J Dairy Sci* 78, 2838-2846.

Abu-Tarboush H, M Al-Saiady, A Keir El-Din. 1996. Evaluation of diet containing Lactobacilli on performance, fecal coliform, and lactobacilli of dairy calves. *Anim Feed Sci Technol* 57, 39-49.

Adams MC, J Luo, D Rayward, S. King, R Gibson, GH Moghaddam. 2008. Selection of a novel direct-fed microbial to enhance weight gain in intensively reared calves. *Anim Feed Sci Technol* 145, 41-52.

Anadón A, MR Martínez-Larrañaga, M Aranzazu Martínez. 2006. Probiotics for animal nutrition in the European Union. Regulation and safety assessment. *Regul Toxicol Pharmacol* 45, 91-95.

Bayatkouhsar J, A Tahmasebi, A Naserian, R Mokarram, R Valizadeh. 2013. Effects of supplementation of lactic acid bacteria on growth performance, blood metabolites and fecal coliform and lactobacilli of young dairy calves. *Anim. Feed Sci Technol* 186, 1-11.

Blood DC, SA Henderson, OM Radostits. 1986. *Medicina Veterinaria*. 6ª ed. Ed. Interamericana, México DF, México.

Castillo NA, A de Moreno de LeBlanc, CM Galdeano, G Perdigón. 2012. Comparative study of the protective capacity against *Salmonella* infection between probiotic and nonprobiotic lactobacilli. *J Appl Microbiol* 114, 861-876.

Demecková V, D Kelly, AGP Coutts, PH Brooks, A Campbell. 2002. The effect of fermented liquid feeding on the faecal microbiology and colostrums quality of farrowing sows. *Int J Food Microbiol* 79, 85-97.

FAO/WHO, Food and Agriculture Organization of the United Nations and World Health Organization. 2001. *Evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.* Expert consultation report: Córdoba, Argentina, 1-4 October.

Fayol-Messaoudi D, MH Coconnier-Polter, V Lievin-Le Moal, F Atassi, CN Berger, AL Servin. 2007. The *Lactobacillus plantarum* strain ACA-DC287 isolated from a Greek cheese demonstrates antagonistic activity *in vitro* and *in vivo* against *Salmonella* enterica serovar Typhimurium. *J Appl Microbiol* 103, 657-665.

FASS, Federation of Animal Science Societies. 1998 *Guide for the care and use of agricultural animals in agricultural research and teaching.* 1st rev. ed. Federation of Animal Science Societies, Savoy, IL, USA, Pp 80-84.

Frizzo LS, LP Soto, MV Zbrun, E Bertozzi, GJ Sequeira, R Rodriguez Armesto, MR Rosmini. 2011ª. Effect of lactic acid bacteria and lactose on growth performance and intestinal microbial balance of artificially reared calves. *Livestock Sci* 140, 246-252.

Frizzo LS, LP Soto, E Bertozzi, MV Zbrun, ML Signorini, G Sequeira, R Rodríguez Armesto, MR Rosmini. 2011ᵇ. Intestinal populations of *Lactobacilli* and coliforms after *in vivo Salmonella dublin* challenge and their relationship with microbial translocation in calves supplemented with lactic acid bacteria and lactose. *Anim Feed Sci Technol* 170, 12-20.

Frizzo LS, MV Zbrun, LP Soto, E Bertozzi, G Sequeira, LE Marti, ML Signorini, R Rodríguez Armesto, MR Rosmini. 2012. Pathogen translocation and histopathological lesions in an experimental model of *Salmonella* Dublin infection in calves supplemented with lactic acid bacteria and lactose. *J Vet Sci* 13, 261-270.

Hartemink R, VR Domenech, FM Rombouts, 1997. LAMVAB-A new selective medium for the isolation of lactobacilli from faeces. *J Microbiol Meth* 29, 77-84.

Klee W. 2005. Enfermedades de los intestinos. En: Dirksen G, Gründer H, Stöber M. *Medicina interna y cirugía del bovino*. 4ª ed. Inter-Médica, Buenos Aires, Argentina, Pp 468-543.

Mayer M, A Abenthum, JM Matthes, D Kleeberger, MJ Ege, C Hölzel, J Bauer, K Schwaiger. 2012. Development and genetic influence of the rectal bacterial flora of newborn calves. *Vet Microbiol* 161, 179-185.

Meyer PM, A Vaz Pires, AR Vagadlo, JM Correia De Simas, I Susin. 2001. Adição de probiótico ao leite integral ou sucedáneo e

desempenho de bezerros da raça holandesa. *Scientia Agricola* 58, 215-221.

Oyofo BA, JR DeLoach, DE Corrier, JO Norman, RL Ziprin, HH Mollenhauer. 1989. Effects of carbohydrates on *Salmonella* Thyphimorium colonization in broilers chickens. *Avian dis* 33, 531-534.

Ozawa K, K Yabu-Uchi, K Yamanaka, Y Yamashita, S Nomura, I Oku. 1983. Effect of *Streptococcus faecalis* BIO-4R on intestinal flora of weanling piglets and calves. *Int J Food Microbiol* 45, 1513-1518.

Paulin SM, PR Watson, AR Benmore, M.P Stevens, PW Jones, B Villarreal-Ramos, TS Wallis. 2002. Analysis of *Salmonella enterica* Serotype-Host Specificity in calves: avirulence of *S. enterica* Serotype Gallinarum correlates with bacterial dissemination from mesenteric lymph nodes and persistence *in vivo*. *Infect Immun* 70, 6788-6797.

Rishi P, KM Swapandeep, B Sushma, S Geeta, T Rupinder. 2009. Protective efficacy probiotic alone or in conjunction with a prebiotic in *Salmonella*-induced liver damage. *FEMS Microbiol Ecol* 69, 222-230.

Rosmini MR, GJ Sequeira, I Guerrero-Legarreta, LE Martí, R Dalla-Santina, L Frizzo, JC Bonazza. 2004. Producción de probióticos para animales de abasto: importancia del uso de la microbiota intestinal indígena. *Revista Mexicana de Ingeniería Quimica* 3, 181-191.

Sarwari AR, LS Magder, P Levine, AM McNamara, S Knower, GL Armstrong, R Etzel, J Hollingsworth, JG Morris. 2001. Serotype Distribution of *Salmonella* Isolates from food animals after slaughter differs from that of isolates found in humans. *J Infect Dis* 183, 1295-1299.

Signorini ML, LP Soto, MV Zbrun, GJ Sequeira, MR Rosmini, LS Frizzo. 2012. Impact of probiotic administration on health and faecal microbiota in young calves: A meta-analysis of randomized controlled trials of lactic acid bacteria. *Res Vet Sci* 93, 250-258.

Silva DG, PRL Silva, JJ Fagliari. 2010. Hemograma e perfil bioquímico sérico, inclusive hemogasométrico, de bezerros infectados experimentalmente com *Salmonella* Dublin. *Arq Bras Med Vet Zootec* 62, 251-257.

Soto LP, LS Frizzo, E Bertozzi, A Diaz, LE Martí, R Dalla Santina, GJ Sequeira, MR Rosmini. 2009. Milk evaluation as growth and cold preservation medium of a probiotic inoculum for young calves. *J Anim Vet Adv* 8, 1353-1360.

Uyeno Y, Y Sekiguchi, Y Kamagata. 2010. rRNA-based analysis to monitor succession of faecal bacterial communities in Holstein calves. *Lett Appl Microbiol* 5, 570-577.

Wray C, RH Davies. 2000. *Salmonella infections in cattle*. In: Wray C, Wray A (eds). *Salmonella in domestic animals*. CABI Publishing, London, UK, Pp 169-191.