PAPER

Effect of spray application of *Lactobacillus plantarum* on *in vivo* performance, caecal fermentations and haematological traits of suckling rabbits

Fulvia Bovera,[1] Francesco Iannaccone,[1] Vincenzo Mastellone,[2] Sandra Nizza,[3] Antonia Lestingi,[4] Luisa De Martino,[3] Pietro Lombardi,[2] Karina Mallardo,[3] Maria Ferrara,[1] Antonino Nizza[1]

[1]Dipartimento di Scienze Zootecniche e Ispezione degli Alimenti, Università di Napoli Federico II, Italy
[2]Dipartimento di Medicina Sperimentale e Clinica, Università di Catanzaro Magna Grecia, Italy
[3]Dipartimento di Strutture, Funzioni e Tecnologie Biologiche, Università di Napoli Federico II, Italy
[4]Dipartimento di Sanità Pubblica e Zootecnia, Università di Bari, Italy

Abstract

Two days before kindling, 228 New Zealand White rabbit does were homogeneously divided into two groups (114 does per group) and fed the same diet. After delivery, the litters were equalized to 8 pups. From 1 to 35 days of age (weaning), the control group (CONT) did not receive any treatment while in the experimental group (LAC) the nests were sprayed with a commercial product containing lyophilized *Lactobacillus plantarum* dissolved in water (12 g/L). *L. plantarum* was sprayed on the litters (5 mL per rabbit) once a day during seven consecutive days after delivery. After one week of rest, the treatment was repeated for another week according to the same experimental protocol. Mortality rate, recorded on all the litters (912 rabbits per group) was significantly lower in the LAC group (9.9 *vs* 17.2%; P<0.05). There were no significant differences in *in vivo* performance of the 24 litters per group, and rabbits of both groups reached a similar weight at weaning (938 *vs* 932 g for LAC and CONT groups, respectively). Rabbits from the LAC group showed fermentative activity of caecal microflora (total volatile fatty acids 24.8 *vs* 14.5 mmol/L; P<0.01) and higher percentage of lymphocytes (73.7 *vs* 63.9% of total white blood cells; P<0.05). Among the microflora population of rabbit caecal content from the LAC group, it was possible to identify *L. plantarum* (1.25×10^6 CFU/g). It might be supposed that the changes in caecal microflora can affect our results and improve the sanitary status of *Lactobacillus*-sprayed rabbits in the period 1-35 days of age.

Corresponding author: Prof. Antonino Nizza, Dipartimento di Scienze Zootecniche e Ispezione degli Alimenti, Università di Napoli Federico II, via F. Delpino 1, 80137 Napoli, Italy.
Tel. +39.081.2536061 - Fax: +39.081.292981.
E-mail: nizza@unina.it

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Introduction

Probiotic *Lactobacillus* are known to confirm various health promoting activities on their host after either parenteral or oral administration in rats (de Waard *et al.*, 2001; Oyetayo *et al.*, 2003). Some of their beneficial effects include prevention of intestinal infection (Tannock, 1983; Casas and Dobrogosz, 2000), control of serum cholesterol (Bertazzoni *et al.*, 2001), enhancement of immunity (Aattouri *et al.*, 2001) in human and rats, and growth enhancement of poultry and pigs (Baird, 1977; Chang *et al.*, 2001). The mechanisms by which these probiotics affect their host and improve gut barrier can be due to: competition for adhesion site, production of inhibitory compounds, and rebalancing of disturbed gastrointestinal microbial composition and metabolism (de Waard *et al.*, 2001; FAO/WHO, 2001). *Lactobacillus* are not regular inhabitants of the digestive tract in rabbits and, according to some authors (Maertens *et al.*, 2006), poorly adhere to epithelial cells; therefore, their usefulness is doubtful in such species (Yu and Tsen, 1993). Studies on different clinical approaches in pet rabbits (Fann *et al.*, 2001) showed that *Lactobacillus* can be successfully used in therapies instituted for antibiotic-associated enteritis, and suggested two possible mechanisms of action. The first is that *Lactobacillus* has been shown to have an inhibitory effect on pathogenic *E. coli* (Abo-El-Khair *et al.*, 1993) and so it would be useful in the event of *E. coli* overpopulation. The second theory is that, also in rabbit, *Lactobacillus* is a normal gut inhabitant (Das *et al.*, 1997) that may be eradicated with inappropriate antibiotic administration.

Another consideration is that, being living microrganisms, the application of probiotics to a large number of animals as under commercial conditions must be efficient, should be administered as early in life as possible (Schneitz *et al.*, 1992), and should minimize uncontrolled variables such as water quality and proportioner/medicator function and consistency. These issues can be addressed and minimized if the probiotic were administered by spray application, as observed by Wolfenden *et al.* (2007) in poultry. According to the authors, the spray application offers several advantages over drinking water or individual administration by gavage.

In this study, a sprayed *Lactobacillus*-based probiotic was used in newborn rabbits up to weaning in order to verify if early administration of lactobacilli can promote their adhesion to the intestinal mucosa and, as a consequence, have a positive impact on growth performance, caecal microflora activity and immune status in rabbits.

Materials and methods

Experimental design

The trial was carried out on a commercial rabbit farm in San Giorgio La Molara (BN, Italy). Two days before kindling, 228 New Zealand White rabbit does (average weight 4.25±0.36 kg, average parity 3±0.5) were homogeneously divided into two groups (114 does per group) and fed the same diet. The does were housed in flat-deck cages measuring 50×70×32 cm high and provided with nesting boxes. Building heating system and forced ventilation allowed the temperature to be maintained at 21±3°C. Rabbit does were kept under 16 h of light and 8 h of darkness. Until Day 20 of lactation, females received *ad libitum* an antibiotic free commercial diet for reproductive rabbit does (177 g CP, 155 g starch and 191 g ADF kg^{-1} DM). The does were

inseminated at Day 11 *post-partum*, and pregnancy was detected by palpation two weeks after insemination. After kindling, the litters were equalized to 8 pups. The control group (CONT) did not receive treatments. In the experimental group (LAC), the nests were sprayed with a commercial product (MIX-AVI® pro, IVS-Wynco LLC, Springdale, AR, USA) containing lyophilized *Lactobacillus plantarum* (Table 1). According to the manufacturer's instructions, *L. plantarum* was dissolved in water to an expected concentration of 5×10^9 CFU/mL (12 g *L. plantarum* /L). Once complete solubilization had been obtained, the mix was used to spray the litters (~40 mL per nest, 5 mL per rabbit) using an ordinary manual garden sprayer. The *L. plantarum* was sprayed on the litters once a day through seven consecutive days immediately after delivery. Then, after one week off, the treatment was repeated for another week according to the same experimental protocol.

In vivo performance

For each group, mortality rate of pups was recorded daily on all the litters (114 nests, 912 pups per group). The *in vivo* performance was recorded on 24 nests (192 pups) randomly chosen per group. Up to 21 days of age, the nests were opened in the morning (~8.30 a.m.) to allow the entrance of the does and closed immediately after suckling, remaining closed for the rest of the day. Thus, milk consumption of the litters was measured by weighing does before and after milking. At 21 days of age, the nests were opened all day and rabbits began to ingest solid feed, represented by the same weaning diet in both groups (Table 2). This weaning diet differed from the lactating diet and was unique to does and litters. The weaning diet was antibiotic free. Milk and food intake of pups were not measured after 21 days of age. Up to 35 days (weaning age), the live weight of litters was recorded weekly in order to calculate the daily weight gain.

Chemical analysis of diet

Diet of does and litter were analyzed for dry matter (method n. 934.01, AOAC, 2004), EE, ash, CP (method n. 945.18, AOAC, 2004), ADF and ADL (method n. 973.18, AOAC, 2004), and amylase-treated NDF (method n. 2002.04, AOAC, 2004).

Microbiological assay

At weaning (35 days of age), 8 rabbits per group were slaughtered in a specialized slaughterhouse after 12 h fasting. The caecum was tied at both ends, separated by sterile instruments from the rest of the gastrointestinal tract, placed in tightly closed plastic bags and put in a pre-warmed thermos. After sampling, the material was transported as soon as possible (approx. 1 h) to the laboratory. Once in the laboratory, samples of caecal content were aseptically collected and immediately diluted in distilled water, using the following serial dilutions: 0, -4, -7, -9. From each dilution, 0.1 mL were spread on plates of *Lactobacillus* selection agar (BD Diagnostic Systems, Heidelberg, Germany) and incubated under microaerobic conditions for 48 h. *Lactobacillus* colonies were identified using API System 50 CH carbohydrate fermentation strips (bioMérieux, Inc., Marcy l'Etoile, France).

Caecal fermentations

Two quotes of caecal content (each approx. 5 mL) were used for volatile fatty acid (VFAs) determination. After dilution of the samples with 0.03 M oxalic acid (1:4, v/v), the VFAs were analyzed by a gas chromatography system mod. GC 8000top (CE Instruments Rodano, Milano, Italy) with Fused Silica Capillary Column NUKOLTM 30 m×0.25 mm×0.25 µm film thickness (Supelco Analytical, Bellefonte, PA, USA); analysis temperature 135°C, flame ionization detector temperature 180°C, carrier gas, helium, constant flow 1.0 mL/min, pressure 133 kPa. Elution time of standards ranged from 3.9 and 8.4 min, and the total time of the race was 14 min. No internal standards were used. Branched chain proportion (BCP) was determined as the sum of isobutyrate and isovaleriate divided by the total VFA production.

Haematological traits

Before slaughtering, immediately after stunning, samples of blood were collected from each rabbit by heart puncture and put into two different tubes, one of them containing K3EDTA. Serum was obtained by centrifugation at 2000×g for 15 min and was used for the determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and cholesterol. All samples were assayed specrophotometrically (BIOMATE6, Thermo Fisher Scientific Inc., Waltham, MA, USA) using commercial kits (Spinreact, Sant Esteve de Bas, Spain), serum proteins were assayed using the Electrophoresis SELVET24 system (Seleo Engineering S.r.l., Melito, NA, Italy). K3EDTA samples were used to assay hemochrome parameters by an ADVIA 120 hematology system (Siemens, München, Germany).

Statistical analysis

Data were analyzed by ANOVA using the General Linear Model procedure of SAS (2000) to test the effect of treatment with *L. plantarum*. Mortality rate was analyzed using the χ^2-test.

Table 1. Microbiological composition of the *Lactobacillus*-based probiotic° as reported by the producer.

Lactobacillus plantarum	10×10^9 CFU/g
Enterobacteria	100 CFU/g
Sporulated *bacteria*	200 CFU/g
Yeasts	<10 CFU/g
Escherichia coli	Absent
Salmonella spp.	Absent
Staphylococcus aureus	Absent

°MIX-AVI® pro, IVS-Wynco LLC, Springdale, AR, USA.

Table 2. Chemical composition of the diet° administered to the does and the litters from 22 to 35 days of age.

Dry matter, %	89.7
Ash, % DM	8.09
Ether extract, % DM	2.75
Crude protein, % DM	14.9
Crude fibre, % DM	20.2
Neutral detergent fibre, % DM	38.9
Acid detergent fibre, % DM	26.3
Acid detergent lignin, % DM	7.46

°Ingredients: alfalfa meal, barley, wheat bran, wheat middling, sunflower meal, beet pulp, calcium carbonate, sodium chloride. Supplied per kg of diet: vitamin A, 8375 U; vitamin D_3, 750 U; vitamin E, 20 mg; vitamin K_3, 1 mg; vitamin B_1, 1 mg; vitamin B_2, 2 mg; vitamin B_6, 1 mg; nicotinic acid, 20 mg; choline chloride, 250 mg; magnesium, 290 mg; manganese, 20 mg; zinc, 60 mg; iodine, 1.25 mg; iron, 26 mg; copper, 10 mg; cobalt, 0.7 mg.

Table 3. Mortality rate (percentage and number of died/total number of rabbits per week) recorded along the lactating period (1-35 days of age).

	LAC N=912	CONT N=912	P
1-7 d	1.0 (9/912)	3.1 (28/912)	0.15
8-14 d	1.5 (14/903)	3.2 (28/884)	0.29
15-21 d	2.0 (18/890)	5.0 (43/856)	0.14
22-28 d	4.4 (38/872)	4.7 (38/813)	0.80
29-35 d	1.0 (9/834)	1.3 (10/775)	0.96
1-35 d	9.6 (88/912)	16.2 (147/912)	0.04

LAC, *lactobacilli* treated group; CONT, control group.

Results

Table 3 shows the mortality rate recorded during the study. Considering the entire experimental period, the LAC group had a lower (P<0.05) mortality rate than the CONT group. No significant differences were recorded in the period 1-21 days between the groups for average daily milk intake of litters, and in the period 1-35 days for average body weight and body weight gain (Table 4). The total volatile fatty acid production (Table 5) was 71% higher in the LAC than in the CONT group (P<0.01) but the molar proportion of the main VFAs was unaffected by *Lactobacillus* treatment. The C3 to C4 ratio was 35% less (P<0.05) in rabbits from LAC group. There was no significant difference in BCP value between the groups. White cell counts and their fractions are reported in Table 6. No differences were recorded in total count of white blood cells but the cell profile showed some differences between the groups. In particular, the LAC group had the highest percentage of lymphocytes (P<0.05), and the lowest of neutrophils (P<0.05) and eosinophils (P<0.01). No differences were recorded for monocytes and basophils. Rabbits from the CONT group had higher (P<0.01) levels of red blood cell, haemoglobin and haematocrit (Table 7) with respect to the rabbits from the LAC group. No significant differences were recorded for all the other parameters reported in the table even if the high individual variability in platelet counts did not allow statistical significance to be reached. However, it is interesting to note that the CONT group had average platelet counts that were almost double those of the LAC group. Finally, cholesterol was higher (P<0.01) in the CONT group.

When evaluating lactobacilli population in rabbit caecum, as expected, *L. plantarum* (the same strain contained in the probiotic product) was isolated only in the rabbits from LAC group and an average was caluclated 1.25×10^6 UFC/g. No lactobacilli were detected in the control group.

Discussion

The use of *Lactobacillus plantarum* in newborn rabbits up to weaning (1-35 days of age) seemed to give interesting results. *In vivo* performance of rabbits was unaffected by the treatment and, as a consequence, the live weight at weaning was similar in both groups. In spite of this, the characteristics of the digestive system and the immunitary status of rabbits sprayed with *L. plantarum* seem to better prepare the animals for the post weaning period. This is a delicate period in which the mortality rate is often very high. In our trial, the digestive system of *Lactobacilli*-treated rabbits seems to have some different fermentative characteristics from untreated rabbits. In fact, the higher production of total VFA in LAC rabbits suggests a higher fermentative activity of caecal microbial population even if there was no difference in the molar proportion among acetate, butyrate and propionate between the groups. The C3 to C4 ratio for both groups was

Table 4. *In vivo* performance of lactating rabbits.

	LAC N=24	CONT N=24	RMSE	P
Individual milk intake, g/d				
1-7 d	132	132	27.2	0.92
8-14 d	202	195	38.4	0.55
15-21 d	229	242	36.2	0.22
Average body weight, g				
1 d	82.6	81.0	10.0	0.60
7 d	182	181	21.0	0.89
14 d	285	290	34.6	0.59
21 d	423	441	57.4	0.29
28 d	698	755	111	0.09
35 d	938	932	116	0.34
Average daily weight gain, g/d				
1-7 d	14.2	14.3	2.10	0.87
8-14 d	14.7	15.6	2.61	0.24
15-21 d	19.7	21.7	4.49	0.15
22-28 d	37.9	41.8	11.4	0.05
29-35 d	34.3	24.9	9.85	0.04
1-35 d	24.2	23.7	5.11	0.68

LAC, *lactobacilli* treated group; CONT, control group; RMSE, root mean square error.

Table 5. Volatile fatty acid production (molar percentage of total volatile fatty acid) in the caecal content of the rabbits.

	LAC N=8	CONT N=8	RMSE	P
Acetate	73.0	73.1	2.11	0.42
Propionate	7.10	9.03	0.92	0.138
Butyrate	18.1	15.0	0.95	0.10
Isobutyric	0.56	0.70	0.024	0.052
Isovalerianic	0.65	0.55	0.0364	0.205
Valerianic	0.81	1.59	0.143	0.659
C3:C4	0.39	0.60	0.039	0.0105
Total VFA, mmol/L	24.8	14.5	2.09	0.0002
BCP	0.012	0.012	0.0007	0.199

LAC, *lactobacilli* treated group; CONT, control group; RMSE, root mean square error; VFA, volatile fatty acid; BCP, branched chain proportion [(isobutyric, mmol/L + isovalerianic, mmol/L)/total VFA].

Table 6. Total white blood cells and white cell profile in rabbit from experimental groups.

	LAC N=8	CONT N=8	RMSE	P
WBC, $10^3\times\mu L$	7.96	8.48	0.92	0.4064
Neutrophils, %	19.13	25.95	3.26	0.0118
Lymphocytes, %	73.64	63.88	5.33	0.0211
Monocytes, %	2.31	2.19	0.69	0.8011
Eosinophils, %	1.15	2.35	0.37	0.0024
Basophils, %	2.36	2.32	0.37	0.8769

WBC, white blood cells; LAC, *lactobacilli* treated group; CONT, control group; RMSE, root mean square error.

in the physiological range of weaned rabbits but was significantly lower in LAC group, indicating the prevalence of butyrate in these rabbits. It is well known (Van Soest, 1993) that acetic acid production results from the fermentation of structural carbohydrates by cellulolytic bacteria, while propionate results from that of non-structural carbohydrates by amilolytic bacteria. Butyrate seems to be a preferential source of energy for the hindgut cells (Carabano *et al.*, 1998), and the higher proportion recorded in the LAC rabbits could suggest a better sanitary status of intestinal cells due to a more intense cellular turnover. Considering the specific VFA profile for the rabbit (Gidenne *et al.*, 1988), with a predominance of acetate (60-80% of total VFA), followed by butyrate (8-20%) and then by propionate (3-10%), our results fall within the normal range for both groups. The lack of any difference in molar proportions of isobutyric and isovalerianic acids, used in BCP calculation, or in valerianic acid are the result of, respectively, the degradation of the amino acids valine, leucine and proline (Van Soest, 1994). This suggests a similar microbial activity in protein degradation (Bovera *et al.*, 2007). Neither was there a difference between the groups in the BCP value, considered an index of protein degradation. However, the higher amount of branched chain VFA considered as absolute value seems to confirm the more intense fermentative activity of caecal microbial population of LAC group rabbits. So, our hypothesis is that the higher number of end products from protein degradation in rabbits from the LAC group can be combined to the higher amount of carbon chains from structural and non-structural carbohydrate fermentation in order to produce amino acids for bacteria protein synthesis. This could suggest a higher microflora caecal population and/or a higher bacteria turnover. In growing rabbits, Amber *et al.* (2004), using *Lactobacillus acidophilus*, found a positive effect on average daily gain (+9.6% in respect of the control group) and on feed conversion ratio (-6.5%) while no effect was observed on mortality rate. The same author found improvements in the digestibility of nutrients, in particular crude fibre, due to a modification in caecal microflora resulting from an increase in cellulolytic bacteria counts (CFU/mL).

It was not possible to measure milk and solid feed intake of litters in the last two weeks (22-35 days), but we believe that the increased total VFA production in the caecal contents of rabbits from the LAC group is not attributable to a higher feed intake. Thus, considering the similar milk intake in both groups up to 21 days, and the similar daily weight gain recorded in the period 22-35 days, (an average 36.12 *vs* 33.36 g/d, respectively, for the CONT and the LAC group), we can also assume that there was no difference in solid feed intake between the two groups. The effect on haematological parameters was also very interesting. There was no difference in leukocyte count between the groups. White blood cell is important in defending the body against infections (Schalm *et al.*, 1975). The leukocyte count, however, cannot give specific information since this required differential leukocyte counts. In the differential leukocyte count, neutrophil count was higher in the control group. Neutrophils are responsible for phagocytosis of pathogenic microorganisms during the first few hours after their entry into tissues. No significant differences were detected in the basophil and monocyte counts in the treatment groups. Basophil counts increase upon sensitization to an antigen (or allergen) while monocytes are responsible for defending tissue against microbial agents (Schalm *et al.*, 1975; Cheesbrough, 1991). The lymphocyte counts of rabbits dosed with *Lactobacillus plantarum* was higher in the LAC group compared to control. The primary role of lymphocytes is in humoral antibody formation and cellular immunity (Schalm *et al.*, 1975; Baker and Silver, 1985). In essence, the increase in the lymphocyte count observed in rabbits of the LAC group shows signs of immunostimulatory effect. Aattouri *et al.* (2001) reported that oral ingestion of lactic acid bacteria by rats increases lymphocyte proliferation and interferon A production.

Table 7. Values of hematological parameters in the two groups of rabbits.

	LAC N=8	CONT N=8	RMSE	P
RBC, $10^6 \times \mu L$	4.34	4.86	0.25	0.006
Hemoglobin, g/dL	9.60	10.84	0.52	0.0021
Hematocrit, %	35.36	40.77	2.76	0.0073
MCV, fL	81.40	83.92	4.61	0.3706
MCH, pg	22.12	22.33	0.60	0.5681
MCHC, g/dL	27.40	26.63	1.17	0.3931
RDW, %	17.68	16.17	2.43	0.3133
HDW, g/dL	2.09	1.91	0.21	0.1840
Platelets, $10^3 \times \mu L$	172	321	239.23	0.3120
MPV, fL	20.84	20.04	4.81	0.7832
PCT, %	0.39	0.75	0.76	0.4333
PDW, %	62.70	62.51	6.56	0.9624
AST, U/L	66.80	61.57	29.36	0.7672
ALT, U/L	42.40	44.14	12.76	0.8205
Cholesterol, mg/dL	53.68	83.43	10.34	<0.001

LAC, *lactobacilli* treated group; CONT, control group; RMSE, root mean square error; RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red cell distribution width; HDV, haemoglobin distribution width; MPV, mean platelet volume; PCT, plateletcrit; PDW, platelet distribution width; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Similar effects on WBC formula were reported by Aboderin *et al.* (2006), who utilized *L. plantarum* in rats. Probably, this improvement in the immune system can explain the lower mortality rate recorded in the LAC group. The CONT group had higher levels of red blood cells, haemoglobin and haematocrit, but it is important to consider that the recorded values of these parameters fall in the physiological ranges for post weaning rabbits (slightly higher for haematocrit) as reported by Archetti *et al.* (2008): red blood cells $3.5\text{-}6.6 \times 10^6 \times \mu L$; haemoglobin 6.7-12.7 g/dL; haematocrit 18.9-34.7%. Studies on chickens (Koenen *et al.*, 2004) showed a positive humoral and cellular immune response using a *Lactobacillus*-based probiotic. In our trial, *L. plantarum* was identified in the caecal content of young rabbits from the LAC group but not in the control group. The number of colonies isolated in the caecal contents (1.25×10^6 CFU/g) is sufficient to have an effect on the animal. In fact, according to Guillot (2001), probiotic organisms must attain concentrations in the order of $10^6\text{-}10^7$ per g in the intestinal content to have any observable effect. Our hypothesis is that in newborn rabbits, the high stomach pH (5-6) of newborn rabbits probably helps the *L. plantarum* to survive the stomachal passage, and enables it to colonize the colon, caecum and large intestine, having a positive effect on animal health. Probably the adequate levels of colonization are also due to the method of *Lactobacillus* application since the spraying method avoids those problems which result from administration by drink water or feed. In fact, in the latter case, the temperatures

reached during the pelletting procedure can deactivate the bacterium. This is particularly critical with non-spore forming bacteria (e.g. *Lactobacillus*, *Pedicoccus* and *Streptococcus*) (Falcao *et al.*, 2007).

The colonization of *L. plantarum* could, of course, induce changes in the relationship among bacteria colonizing the caecum of rabbits.

Conclusions

The application of *Lactobacillus plantarum* by spraying during the pre-weaning period (1-35 days) did not affect rabbit growth but reduced the mortality rate, potentiated the immune system and improved the sanitary status of the animals. This was probably due to changes in caecal bacteria population, characterized by the colonization of *L. plantarum* in the rabbit gut.

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