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Probiotic supplementation improves seroconversion in cattle vaccinated against rabies

Suplementação de probiótico melhora a soroconversão de bovinos vacinados contra a raiva

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ABSTRACT: Probiotic supplementation has demonstrated beneficial effects on the health of cattle. The aim of this study was to evaluate the effect of probiotic supplementation on the humoral immune response in male cattle with only one dose of rabies vaccine. Ninety male calves were randomly divided into three groups: control, without probiotic supplementation; and groups of animals supplemented with 4 and 8 grams of probiotic in the mineral mixture. On the first day of the experimental period, a blood sample was collected from each animal and vaccinated with a dose of anti-rabies vaccine. After 30, 60, 90 and 120 days, blood samples were collected from all cattle and antibody titers were determined using the serum neutralization technique. The results were compared using the analysis of variance test, followed by the Tukey test. All analyzes were performed using Biostat 5.3 software, with a significance level of 5%. The results revealed a significant increase ($P \leq 0.05$) in anti-rabies antibody titers in groups of animals supplemented with probiotics and this increase demonstrated to be dose-dependent. Furthermore, in animals supplemented with probiotics, levels remained higher during the experimental period than in the group of cattle not supplemented with microorganisms.

Keywords: Antibody titer; Humoral immune response; Neutralizing antibodies.

RESUMO: A suplementação de probióticos demonstrou efeitos benéficos na saúde de bovinos. O objetivo com este estudo foi avaliar o efeito da suplementação de probióticos na resposta imune humoral em bovinos machos com apenas uma dose de vacina antirrábica. Noventa bezerros machos foram divididos aleatoriamente em três grupos: controle, sem suplementação de probiótico; e grupos de animais suplementados com 4 e 8 gramas de probiótico na mistura mineral. No primeiro dia do período experimental foi coletada uma amostra de sangue de cada animal e vacinados com uma dose de vacina antirrábica. Após 30, 60, 90 e 120 dias, foram coletadas amostras de sangue de todos os bovinos e os títulos de anticorpos foram determinados utilizando a técnica de neutralização do soro. Os resultados foram comparados pelo teste de análise de variância, seguido do teste de Tukey. Todas as análises foram realizadas no software Biostat 5.3, com nível de significância de 5%. Os resultados revelaram um aumento significativo ($P \leq 0,05$) nos títulos de anticorpos antirrábicos nos grupos de animais suplementados com probióticos e esse aumento demonstrou ser dose-dependente. Além disso, nos animais suplementados com probiótico os níveis se mantiveram mais elevados do que no grupo de bovinos não suplementados com os micro-organismos.

Palavras-chave: Anticorpos neutralizantes; Resposta imune humoral; Título de anticorpos.

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1 INTRODUCTION

Rabies is a severe viral disease, caused by a virus of the Lyssavirus genus, belonging to the Rhabdoviridae family (Nadin-Davis, 2023). This zoonosis affects all mammals, humans, and animals, and has a wide geographic distribution, with Asia and Africa being the continents with the highest incidence rates of the disease (CDC; NCEZID; DHCPP, 2024).

The occurrence of rabies is frequently reported in farm animals, particularly in cattle, which is the most affected species (Mello *et al.*, 2019), and this disease is considered an important cause of economic losses (Andrade *et al.*, 2020).

As *Desmodus rotundus* is becoming relatively common in regions with large herds of domestic cattle, resulting in increased survival rates and population densities, cattle serve as sentinels for regions where the rabies virus is circulating. This intimate relationship between the density of positive rabies cases and the expansion of cattle herds in the Amazon has been reported in the literature (Andrade *et al.*, 2016).

Vaccination is the best method of rabies control, as it is effective and low cost (Mello *et al.*, 2019). However, in Brazil, immunity inefficiency is common after primo vaccination of anti-rabies vaccines, despite antigenic values being within normal limits, according to the manufacturers (Genaro *et al.*, 2014). Therefore, it is necessary to find alternatives to increase the effectiveness of vaccination against the rabies virus (Ferreira *et al.*, 2009).

The use of probiotics in livestock has been suggested to significantly improve their health, immunity, growth performance, nutritional digestibility, and intestinal microbial balance (Kober *et al.*, 2022; Uyeno; Shigemori; Shimosato, 2015). One of the strategies to increase the immune response of animals to induce immunity to infections caused by viruses or bacteria is the use of probiotics, which has demonstrated improvements in restoring immune responses and potentiation of the vaccine in relation to various pathogens (Kober *et al.*, 2022; Vizzotto-Martino *et al.*, 2016).

Due to the importance of rabies as one of the main zoonoses in the world, the current study aimed to evaluate the effects of different concentrations of a combination of probiotic microorganism supplementation added to the mineral mixture on the humoral immune response of cattle primo vaccinated with the rabies vaccine.

2 MATERIAL AND METHODS

Ninety uncastrated male cattle of pure origin (PO), of the Nelore breed, and 12 months of age were used, randomly divided into three groups (n=30). Randomization was performed using a computer-generated list to prepare sealed, sequentially numbered envelopes. In the first seven days, the adaptation period, the animals were randomly allocated in three areas of 15,000m^2, with similar topography and soil (Latossolo Roxo), fertilized, irrigated, and containing Tanzania grass (*Panicum maximum* Jacq. Cv. Tanzania). Each of the three areas was divided into 15 paddocks of 1,000m^2, that contained drinking fountains and shade, designed for a rotational grazing system. During this period, the average consumption of 70 grams of mineral mixture/animal/day was established (Arenas *et al.*, 2007).

The proposed groups/treatments were: GC group (control) - cattle vaccinated with one dose of rabies vaccine on the first day of the experimental period and supplemented with 70 grams of mineral mixture/animal/day; and groups G4P and G8P - cattle vaccinated with one dose of rabies vaccine on the first day of the experimental period and supplemented with 4 and 8 grams, respectively, of a probiotic microorganism combination in 70 grams of mineral mixture/animal/day. The treatments were packed in dark bags and administered blindly during the experimental period.

The combined probiotic microorganisms included: *Lactobacillus acidophilus* (~2.2x10^9 UFC/Kg); *Estreptococus faecium* (~2.2x10^9 UFC/Kg); *Bifidobacterium thermophilum* (~2.2x10^9 UFC/Kg); and *Bifidobacterium longum* (~2.2x10^9 UFC/Kg) (Brazilian Enterprise to Increase Livestock Productivity – Embraupec, Paranavaí – PR, Brazil) and the mineral mixture used was Fosbovi Seca® (Tortuga Companhia Zootécnica Agrária, São Paulo, Brazil). The rabies vaccine (Rabmune®, Ceva Saúde Animal Ltda, Paulínia, SP, Brazil) contained a suspension of inactivated PV rabies virus. The application of the vaccine was performed on day zero (primo vaccination) of the experiment, subcutaneously in all cattle, at a dose of 2 mL, following the guidelines of the Ministry of Agriculture, Livestock and Food Supply (MAPA, 2023).

On the morning of days 0, 30, 60, 90, and 120, the cattle were taken to the corral and professionals collected blood samples blindly by puncturing the jugular vein of each cow/group. The samples were centrifuged, and serum aliquots were withdrawn for Eppendorf tubes and stored at -20°C. The samples were individually identified for the determination of individual titers of neutralizing antibodies. Blinding of the evaluations of the anti-rabies titers result was carried out using the seroneutralization technique of the BHK21 clone 13 cells, this test being based on the Test of Fast Fluorescent Focus Inhibition (RFFIT) (Genaro *et al.*, 2014; Smith; Lange; Marks, 1996).

The Shapiro-Wilk test was used to verify the assumption of data normality, and showed that the measurements of serum antibody titers presented parametric distribution. Therefore, a one-way analysis of variance (one-way ANOVA) was performed with contrasts through the Tukey method to determine whether the experimental groups differed within each moment, and the paired t-test was used to determine any differences between moments within each group. All analyses were conducted using Biostat 5.3 software and the significance level was set at $P \leq 0.05$ (Ayres, 2007).

The Ethics and Research Committee of the Western Sao Paulo University, Presidente Prudente, SP, Brazil, approved the experiment under no. 4935, developed between February 2023 and May 2023, with an experimental period of 120 days.

3 RESULTS

On the first day of the experimental period, blood samples were collected from animals in the control group and from animals in the groups supplemented with four and eight grams of probiotic. The results did not reveal anti-rabies antibody titers. Thirty days after administration of the anti-rabies vaccine, the results showed that the GC group had significantly lower anti-rabies antibody titers (1.20 ± 0.44 IU/mL) than the G4P group (2.78 ± 0.67 IU /mL), and the G8P group (3.53 ± 1.10 IU/mL), and all groups differed from each other ($P \leq 0.05$). Sixty days after vaccination, the results demonstrated that the GC, G4P, and G8P groups presented antibody titers of 0.76 ± 0.38, 2.05 ± 0.78, and 2.79 ± 1.13 IU/mL respectively and differed significantly from each other ($P \leq 0.05$). The average results of the GC, G4P, and G8P groups 90 days after vaccination, were respectively: 2.23 ± 0.78, 1.52 ± 1.00, and 0.46 ± 0.14 IU/mL and when statistically compared, they differed significantly from each other ($P \leq 0.05$). At 120 days, a significant difference ($P \leq 0.05$) was also evident between the CG, G4P, and G8P groups (G8P = 1.32 ± 0.78 IU/mL, G4P = 0.91 ± 0.54 IU/mL and GC = 0.35 ± 0.14 IU/mL) (Table 1).

Table 1. Anti-rabies antibody titer[1] in primary vaccinated cattle fed with increasing doses of probiotic added to the mineral mixture

Collection (days)	Experimental groups[2, 3, 4]		
	GC	G4P	G8P
0	0[A]	0[A]	0[A]
30	1.20 ± 0.44[A]	2.78 ± 0.67[B]	3.53 ± 1.10[C]
60	0.76 ± 0.38[A]	2.05 ± 0.78[B]	2.79 ± 1.13[C]
90	0.46 ± 0.07[A]	1.52 ± 1.00[B]	2.23 ± 1.31[C]
120	0.35 ± 0.14[A]	0.91 ± 0.54[B]	1.32 ± 0.78[C]

[1] Titers of anti-rabies antibodies (International unit per milliliter - UI/mL). [2] The cattle were supplemented with 0, 4, and 8 grams of probiotic (groups: control – GC; 4 grams of probiotic - G4P; and 8 grams of probiotic - G8P, respectively) /bovine/day added to 70 grams of mineral mixture. [3] The results are mean ± SD (n=30). [4] Different letters in the same line indicate statistically significant differences (P≤0.05).

Thirty days after vaccination, only 93% of the cattle in the control group and all animals (100%) in the G4P and G8P groups had antibody titers ≥ 0.50 IU/mL. At 60 days, all animals in the G8P group (100%), 93.33% in the G4P, and 76.67% in the GC group had antibody titers ≥ 0.50 IU/mL. Ninety days after the application of the rabies vaccine, 96.67%, 83.33%, and 53.33% of the animals in the G8P, G4P, and GC groups, respectively, had antibody titers ≥ 0.50 IU/mL. At the end of the 120-day experimental period, 86.67%, 67.67%, and 36.67% of the animals in the G8P, G4P, and GC groups, respectively, had antibody titers ≥ 0.50 IU/mL (Table 2).

Table 2. Effects of supplementation with different doses of probiotic added to mineral mixture, on the frequency (%) with which cattle, primo vaccinated against rabies, demonstrate protective levels of anti-rabies antibody titers (titers ≥ 0.5 UI/mL)

Days after primo vaccination	Experimental groups[1]		
	GC	G4P	G8P
0	0	0	0
30	93.33	100	100
60	76.67	93.33	100
90	53.33	83.33	96.67
120	36.67	67.67	86.67

[1] The cattle were supplemented with 0, 4, and 8 grams of probiotic (groups: control – GC; 4 grams of probiotic - G4P; and 8 grams of probiotic - G8P, respectively) /bovine/day added to 70 grams of mineral mixture. Source: The authors

First-degree regression models were fitted of the relationship between rabies antibody titers in cattle vaccinated with one dose and the evaluation period (days). The adjusted models showed reasonable explanatory power for the GC (R^2= 0.50709), G4P (R^2= 0.44609), and G8P (R^2= 0.34826) groups. At 30 days, the regression models showed a lower adjustment to the results obtained, after which, the models presented higher estimates than those observed (Figure 1).

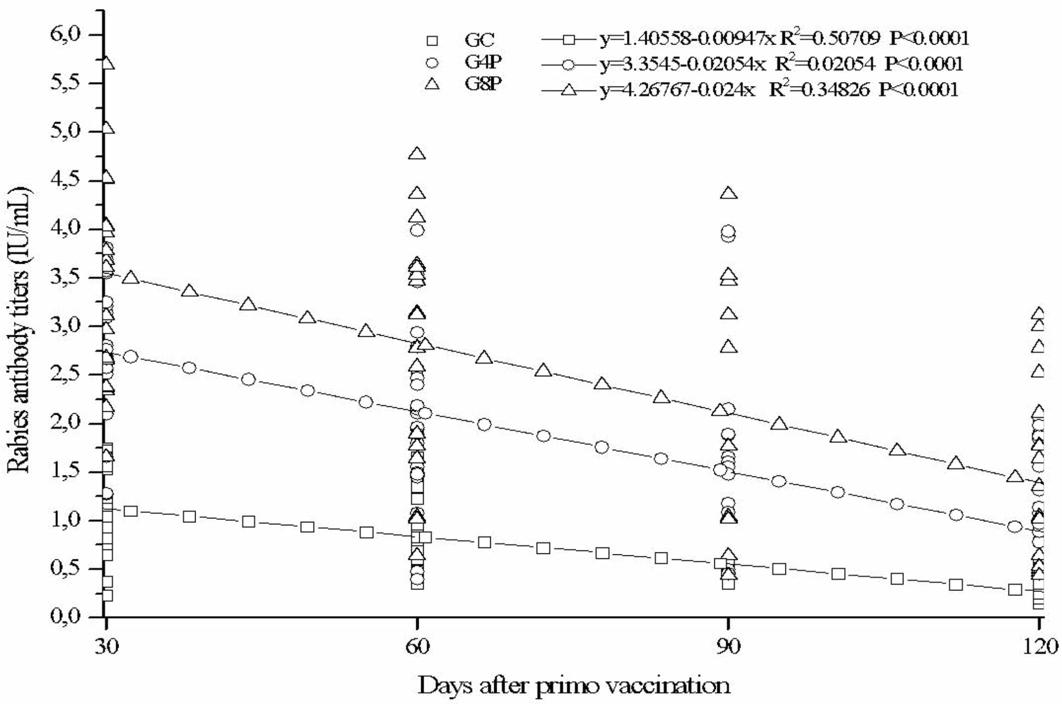

Figure 1. Regression curves adjusted for the titration of anti-rabies antibodies in cattle vaccinated with one dose of anti-rabies vaccine, primo vaccinated, at 30, 60, 90, and 120 days and as a function of the dose of probiotic (0, 4, and 8 g) added to the mineral mixture.

4 DISCUSSION

Rabies is a zoonotic viral disease, preventable by vaccination, that affects the central nervous system of domestic and wild animals, and once clinical symptoms appear, rabies is virtually 100% fatal. Annually, the occurrence of rabies results in a global estimated cost of USD 8.6 billion (Who, 2023) and in Brazil, this neurological disease causes significant economic losses in cattle (Mello *et al.*, 2019).

In Guatemala, a study demonstrated that the brand of rabies vaccine is an important factor, both in the initial response and in the long-term maintenance of an adequate response, especially in areas where there is evidence of the presence of vampire bats and laboratory-confirmed cases of rabies in cattle (Gilbert *et al.*, 2015).

In this study, the results did not reveal anti-rabies titer at the beginning of the experimental period, indicating that the animals had not previously come into contact with the rabies virus or had been vaccinated. After 30 days of subcutaneous vaccination, blood samples were collected and serum analyzes revealed that 93.33% of the animals responded adequately to the primary vaccination. This vaccine response, described as a primary immune response, occurs through the recognition and generation of an immune response to the inoculated antigen (Lynn *et al.*, 2022). A study carried out in Bhutan also did not determine antibody titers at the beginning of the experimental period and after primary vaccination via the subcutaneous route, only 58% of vaccinated cattle showed an adequate immunological response (titers $\geq$ 0.5 IU/mL) (Wangmo *et al.*, 2019), indicating a great variability in vaccine responses. The available information about protection against rabies comes from animal rabies challenge studies. Interestingly, the level of protection determined by challenge studies in dogs, cats, and cattle is the same cut-off of 0.5 IU/mL determined as proof of seroconversion in humans (Albas *et al.*, 2005; Fernandes *et al.*, 2017; Krämer *et al.*, 2010; Mansfield *et al.*, 2004; Moore, 2021; Who, 2023). Therefore, this measurement can be used as a reference to determine the influence of therapeutic interventions on the immune response of cattle vaccinated against rabies (Macdonald; Bell, 2010).

The effectiveness of vaccines in inducing immunity can be influenced by the intestinal microbiota, since cross-talk between intestinal bacteria and the host's immune system beneficially controls immune responses (Kazemifard; Dehkohneh; Baradaran Ghavami, 2022). Studies have shown that regular dietary supplementation of microorganisms, in probiotics, can beneficially modulate the intestinal microbiota and improve vaccine response (Scott, 2018; Wu *et al.*, 2016). In the current study, groups of cattle supplemented daily with 70 grams of mineral mixture containing 4 or 8 grams of probiotic showed dose-dependent improvement in serum antibody titers. A systematic review with meta-analysis of studies in cattle reported that the effect size may also be related to the composition, periodicity and method of probiotic supplementation (Wang *et al.*, 2023). Furthermore, the beneficial results observed may also be due to the beneficial modulation of the host's intestinal microbiota, as in humans, the cross-talk between the intestinal microbiota and the immune system, through the production of various metabolic and antimicrobial peptides, is directly related to innate and adaptive immunity, as well as the production of antibodies (Kazemifard; Dehkohneh; Baradaran Ghavami, 2022; López *et al.*, 2016).

In the groups supplemented with probiotics, the results revealed a greater persistence of the titer. This beneficial result in mature ruminants must be due to the fact that probiotics, when reaching the ruminal compartment, the main site of food digestion, increase performance and immunity (Raabis; Li; Cersosimo, 2019). These results are due to the different modes of action of the supplemented livestock microorganisms, which include: competitive exclusion for binding sites; adhesion to the gastrointestinal tract; strengthening the epithelial barrier; increased digestion and absorption of nutrients; competing with pathogenic bacteria for nutrients in the intestine; production of antimicrobial substances; alteration in gene expression in pathogenic microorganisms; bacterial antagonism; bioconversion; and immunomodulation, due to its impact on the signaling and differentiation of immune cells, as they are involved in driving innate and acquired immunity (Genaro *et al.*, 2014; Kober *et al.*, 2022; Peroni; Morelli, 2021).

This beneficial response of bacterial supplementation is associated with biological effects on the intestinal environment, if they reach a minimum number, which needs to be above 10^7 CFU/g of total lactic acid bacteria, in accordance with the requirements of the Technical Regulation on Identity and Quality of Fermented Milks, Normative Instruction N°.46 (MAPA, 2007). In addition, there is a relationship between the concentration and the effectiveness of the probiotics, so that adequate colonization of beneficial microorganisms occurs in the intestine (Coppola; Gil-Turnes, 2004; Williams, 2010). In the present study, the administration of the mineral mixture containing probiotic supplementation was carried out daily and the concentration of viable bacteria per kg of product was done according to the manufacturer's recommendations.

Unfortunately, vaccination does not appear to provide complete protection, as bovine rabies has been reported even in vaccinated animals (Lima *et al.*, 2005). These results may be related to the type of vaccine, antigenic differences in batches, vaccine quality, physiological condition of the animals, and individual response capacity of the immune system, which are also cited as factors that may contribute to the reduced efficiency and effectiveness of vaccinations in herds (Montaño; Polack; Mora, 1987; Zimmermann *et al.*, 2019). While researchers have noted the potential for probiotics to increase vaccination success, the route of administration of the vaccine and probiotics, as well as the type and dosage of the probiotic strain, play a critical role in increasing the effectiveness of vaccines, as documented in humans, cattle, and sheep (Cross, 2002; Genaro *et al.*, 2014; Vizzotto-Martino *et al.*, 2016; Zimmermann *et al.*, 2019). Furthermore, probiotics are likely to have different effects on antibody production after vaccination and on natural infection, although the mechanisms involved remain largely unknown (Kober *et al.*, 2022).

Despite the beneficial results of probiotic supplementation, these do not eliminate the need to implement a booster dose, as recommended by the Rabies Control Program in Herbivores (Migliavacca *et al.*, 2020). Furthermore, additional studies are needed to determine which combination of probiotic microorganisms best induces seroconversion in ruminants primo vaccinated against rabies, or that received a booster dose. Finally, studies should establish the influence of the nutritional quality of the forage, sex, breed, and age on the vaccine response.

5 FINAL CONSIDERATIONS

The results allow us to conclude that supplementation with probiotics for cattle improves the vaccine response in a dose-dependent manner. Furthermore, it has been shown that supplementation with probiotics induces a greater persistence of the level of humoral immunological protection against rabies.

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