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Effects of probiotic bacteria at different concentrations on production of immunomodulatory antibodies against rabies virus in vaccinated cattle

Efeito da concentração de bactérias probióticas como imunomodulador da produção de anticorpos antirrábicos em bovinos vacinados

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Abstract

This study evaluated the effects of supplementation with a combination of probiotic microorganisms, added at different concentrations to the mineral mixture, on the production of serum antibodies against rabies virus in cattle vaccinated with a single dose of rabies vaccine. Forty-two male Nellore cattle, aged 12 months, were randomly divided into three groups (n = 14): the control group (CG) received 70 grams of mineral mixture/animal/day; and the 2 gram probiotic group (G2P) and 8 gram probiotic group (G8P) received 70 grams of mineral mix/animal/day with 2 and 8 grams added, respectively, of a combination of probiotic microorganisms (*Lactobacillus acidophilus, Streptococcus faecium, Bifidobacterium thermophilum and Bifidobacterium longum*). Individual antibody titers were determined using a neutralization in cell-based rapid fluorescent focus inhibition test (RFFIT) technique. One-way analysis of variance (one-way ANOVA) was used with contrasts using the Tukey method to determine whether the experimental groups differed within each time point, and the paired t-test was used to determine whether differences occurred between time points within each group. The level of significance was set at 5%. There were statistically significant differences between the mean serum concentrations of the CG and G8P groups at 30 and 60 days after the first vaccination, and at 60 days, 100% of the animals maintained minimum titers of protective antibodies only in the G8P group. There was also improvement in the production of antibodies in the G2P group compared with the CG after 30 and 60 days, but this difference was not statistically significant. In conclusion, increasing doses of probiotic added to the mineral mix beneficially affected the rabies humoral immune response, as determined by serum antibodies, and enabled the maintenance of minimum protective titers for a longer period in previously vaccinated cattle.

Key words: Antibodies, cattle, probiotic, rabies

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Resumo

Esse estudo avaliou o efeito da suplementação de uma associação de microrganismos probióticos, adicionados à mistura mineral em diferentes doses, na produção de anticorpos séricos antirrábicos em bovinos primovacinados. Quarenta e dois bovinos Nelore machos, com idade de 12 meses, foram divididos em três grupos (n=14): grupo controle (GC) recebeu 70 gramas de mistura mineral/animal/dia; grupos probiótico 2 gramas (G2P) e 8 gramas (G8P) receberam 70 gramas de mistura mineral/animal/dia adicionados respectivamente de 2 e 8 gramas de probióticos (Lactobacillus acidophilus, Streptococcus faecium, Bifidobacterium thermophilum e Bifidobacterium longum). Os títulos individuais de anticorpos antirrábicos foram determinados por meio da técnica de soroneutralização em células baseado no rapid fluorescent focus inhibition test (RFFIT). Os resultados obtidos foram comparados pelo teste t não pareado, com 5% de nível de significância. Houve diferenças estatísticas significativas entre as médias de concentrações séricas entre os grupos GC e G8P, após 30 e 60 dias da primovacinação e após 60 dias, somente o G8P manteve 100% com títulos de anticorpos protetores mínimos. Houve também melhora na produção de anticorpos no grupo G2P em relação ao GC, após 30 e 60 dias, porém não significativa. Conclui-se que as doses crescentes de probiótico adicionadas na mineral interferiram beneficamente na resposta imune humoral antirrábica, determinada pela concentração sérica de anticorpos, assim como permitiu a manutenção por um período maior os títulos protetores mínimos nos bovinos primovacinados.

Palavras-chave: Anticorpos, bovinos, probióticos, raiva

Introduction

Rabies is a viral zoonotic disease with importance to public health characterized by acute and lethal encephalomyelitis (QUEIROZ et al., 2009). In production animals, the disease is 100% lethal, and there is no viable treatment (WILLOUGHBY JÚNIOR et al., 2005). The disease is caused by an RNA virus of the *Rhabdoviridae* family, genus *Lyssavirus* (SCHEFFER et al., 2007; INSTITUTO PASTEUR, 2012; KANITZI et al., 2014).

Variants of the virus have been identified in wild canines and marmosets and in both vampire and non-vampire bats (MOCHIZUKI et al., 2012). Vampire bats, specifically, *Desmodus rotundus*, are the main reservoirs of the virus in Latin America and are principal transmitters of rabies to herbivores (SCHNEIDER et al., 2009).

Ribas (2013) and Lima et al. (2005) reported higher incidence of rabies in cattle in Mato Grosso do Sul and Paraíba, principally occurring in animals under the age of 4 months, which may be related to the lower immunity of young animals, even if they have been previously vaccinated. According to some authors, the use of only one dose of vaccine is insufficient to protect the animals against rabies, with booster doses being required for the vaccination to offer effective protection (ALBAS et al., 2005). In the event of a rabies outbreak, the recommended regimen is two initial doses with an interval of 30 days and annual revaccination of all animals (MAPA, 2009).

It has become necessary to find alternative ways to increase the effectiveness of vaccination against rabies (FERREIRA et al., 2009), one of which, according to Arenas et al. (2007), could be the use of probiotic food components (GENARO et al., 2014).

A probiotic is a live microbial feed supplement that can beneficially affect the host by improving the intestinal microbial balance (NOVAK et al., 2001), thereby increasing the humoral immune response (ARENAS et al., 2009), preventing disease, maintaining intestinal homeostasis (VEMURI et al., 2014) and improving weight gain and feed utilization efficiency (PARDO; REIS, 2008).

The action mechanisms of probiotics include the following: a) production of antimicrobial substances; b) competition for nutritional substrate with pathogenic bacteria; c) competition for adhesion sites with pathogenic bacteria; d) strengthening the intestinal barrier; and e) modulating immune function

(immunomodulation) (THIRABUNYANON, 2011). Probiotics can influence immune function through direct and indirect effects. The direct effects include alterations in the intestinal microbiota and in the profile of pathogen-associated molecular patterns (PAMPs) presented to the associated intestinal lymphoid tissues. Indirect effects may arise from microbial products such as short chain fatty acids (THOMAS; VERSALOVIC, 2010). Evidence from animal models involving rats free of microorganisms indicates that the resident intestinal flora forms defenses and modulates the outcome of viral infections (PANG; IWASAKI, 2011). These microorganisms produce substances (such as lactic acid) that change the pH by acidifying the medium, which leaves it unfavorable for pathogen proliferation and induces the breakdown of proteins with allergenic potential (MORAIS; JACOB, 2006). In addition, some authors have reported that probiotics potentiate vaccines due to a) modulatory influences on the immune response; b) increased production of antibodies; c) activation of macrophages; d) T cell proliferation; e) production of interferon (ROOS et al., 2010); f) ability to interact with Peyer's patches and intestinal cells, stimulating IgA-producing B cells; and g) the migration of intestinal T cells (PERDIGÓN; HOLGADO, 2000; PARDO; REIS, 2008).

Several vaccines are associated with low rates of seroconversion, which results in a low protective effect; however, the oral use of probiotics during the immunization period may act by improving the rates of seroconversion (YOUNGSTER et al., 2011). The efficacy of probiotic supplementation in the diet depends on the concentration added (COPPOLA; TURNES, 2004) because probiotics have only a biological effect in the digestive tract if they are present in a sufficient quantity (MAPA, 2007). In addition, they should be ingested regularly to maintain effective concentrations in the intestinal microbiota (WILLIAMS, 2010). According to Morais and Jacob (2006), the concentration of orally administered probiotics should range from 106 to 109 CFU (colony forming units).

There is convincing evidence that probiotics can influence the immune response to viral infections; however, specific data on responses to the rabies vaccination are lacking. Within this context, this study aimed to evaluate the effects of supplementation with a combination of probiotic microorganisms, added in different doses to the mineral mix, on the production and maintenance of titers of serum antirabies antibodies in previously vaccinated cattle.

Material and Methods

The study was approved by the Research Ethics Committee, No. 1623, from the University of Oeste Paulista, Presidente Prudente, SP, Brazil.

The research was carried out between September and November 2013 over a period of 90 days. It included 30 days for the animals to adapt to the rotational grazing system and establish a consumption rate of 70 g/animal/day of mineral mixture plus added concentrations of probiotics (ARENAS et al., 2007). This was followed by a 60-day trial period that was conducted on a farm in the district of Pirapozinho, SP, Brazil.

Forty-two uncastrated male cattle of pure origin (PO) of the Nellore breed and aged 12 months were used. They were all vaccinated with a single dose of rabies vaccine and were randomly divided into three groups (n = 14): Control group (CG): mineral mixture; Probiotic group 2 (G2P): two grams of probiotic added to the mineral mixture and Probiotic group 8 (G8P): eight grams of probiotic added to the mineral mixture.

Eight pastures of 2,500 m2 each, similar in topography and soil (Purple Oxisol), were formed with *Panicum maximum* Jacq. cv. Tanzania. The animals were provided with water (water trough) and shade at will, allowing an appropriate rotational grazing system.

The combination of probiotics used was composed of the microorganisms *Lactobacillus acidophilus*

(2.2 10^9 UFC Kg^{-1}), *Streptococcus faecium* (2.2 10^9 UFC Kg^{-1}), *Bifidobacterium thermophilum* (2.2 10^9 UFC Kg^{-1}), and *Bifidobacterium longum* (2.2 10^9 UFC Kg^{-1}), as provided by the Brazilian Company for Livestock Productivity Increase (Embraupec, Paranavaí, PR, Brazil). The mineral mixture used was Fosbovi Seca® (Tortuga Company Agricultural Animal Science, São Paulo, Brazil).

The antirabies vaccine used (Vencofarma of Brazil Ltda., Londrina, Paraná, Brazil) contained Pasteur-inactivated fixed virus adsorbed on aluminum hydroxide gel and produced using cell cultures. The vaccine was administered on day zero of the experimental period in all cattle, subcutaneously at a dose of 2 ml, following the guidelines of the Ministry of Agriculture, Livestock and Food Supply (MAPA, 2013).

On days 0, 30 and 60, all cattle were restrained in a squeeze chute type stall, and blood samples were collected through jugular vein puncture. After centrifugation, aliquots of the serum were placed in plastic tubes and stored in a freezer at –20°C. Individual neutralizing antibody titers were determined from the serum (the Butantan Institute Rabies Laboratory) by means of the seroneutralization in BHK21 clone 13 cells technique, which is based on the rapid fluorescent focus inhibition test (RFFIT) (SMITH et al., 1998).

The Shapiro-Wilk test was used to verify the normality assumption for the data, through which it was found that the measurements of serum antibody titers presented a parametric distribution. Thus, one-way analysis of variance (one-way ANOVA) was used with contrasts using the Tukey method to determine whether the experimental groups differed from each other at each moment, and the paired t-test was used to determine any differences between moments within each group. All analyses were performed using the Biostat 5.0 software with a significance level of 5% (AYRES et al., 2007).

Results and Discussion

On day 0, the animal sera presented no neutralizing antibodies to rabies (Table 1), which demonstrated that there had been no antigenic stimulus prior to the experiment (GENARO et al., 2014). To be considered immune, an individual requires antibody titers ≥ 0.5 IU ml^{-1} (international units per milliliter) (WHO, 1992) and a number of studies have suggested that this dose should also be applied as the minimum for protection in cattle (SIHVONEN et al., 1994; ALBAS et al., 1998; RODRIGUES DA SILVA et al., 2000; ALBAS et al., 2005).

Table 1. Effects of concentrations of supplementation with probiotics added to the mineral mixture, on the frequency with which cattle previously vaccinated against rabies possess titers of antirabies antibodies considered protective.

Days after first vaccination	Frequency of cattle with titers of antirabies antibodies		
	Experimental groups		
	GC	G2P	G8P
0	0	0	0
30	93	100	100
60	42	64	100

The cattle were considered to have protective titers of antirabies antibodies, when they presented titers ≥0.5 IU mL^{-1}.
The cattle were supplemented with 0 (GC), 2 (G2P) and 8 (G8P) grams of an association of probiotic microorganisms/animal/day added to 70 grams of mineral mixture.

Thirty days after the primary vaccination, 93% of the animals in the CG group presented antibody titers ≥ 0.50 IU mL^{-1} (Table 1). Albas et al. (2005), who also used rabies vaccine, observed a lower percentage than that of the present study, 88.9% of the nine animals evaluated, and Silva et al. (2000) attained superior results, 95.5% of the 22 animals evaluated. According to Montaño et al. (1987), this variation in animal responses may be related to the type of rabies vaccine used or to antigenic differences in the lots. In addition, factors such as the quality of the vaccine, the physiological state of the animals and the individual capacity of the immune system response can all contribute to a reduction in the efficiency and effectiveness of vaccination in cattle herds. It should be highlighted that despite the variation in bovine responses, the vaccines used were all properly released, registered and approved by the Ministry of Agriculture, Livestock and Food Supply (ALBAS et al., 1998; GIOMETTI et al., 2006; MARIA et al., 2009).

In the cattle supplemented with 2 (G2P) or 8 (G8P) grams/day of probiotics in the mineral mixture, 100% of the animals presented minimum protective antibody titers of ≥ 0.50 IU mL^{-1} 30 days after the primary vaccination (Table 1). When comparing the antibody titers among the groups supplemented with probiotics and the groups not supplemented, the G8P group demonstrated a significant difference from the other groups ($P<0.05$). The G2P group presented a higher mean compared to the CG, although this difference was not significant ($P>0.05$) (Table 2). These results indicate that supplementation with probiotics in the mineral mixture had a beneficial effect on the level of antirabies antibodies. According to Macdonald and Bell (2010), the influence of probiotic supplementation on immune response can be determined by the specific antibody levels in serum after vaccination, which correlates directly with the protection of the individual. This increase in antibody titers of groups supplemented with probiotics can be explained by a stimulatory effect on the immune system (ESPARZA; FRAGOSO, 2012; NOGUEIRA; GONÇALVES, 2011) that is attributable to lactic acid bacteria (CROSS, 2002; PARDO; REIS, 2008), which were present in this study (*Lactobacillus* and *Bifidobacterium*). The bacteria in question potentiate vaccines via modulatory influences on the immune response such as increasing the production of antibodies and activating macrophages, T-cell proliferation and interferon production (ROOS, 2006). In addition, these results demonstrate that increases in the concentration of associated probiotic microorganisms in the mineral mixture enhance the beneficial immunomodulatory effects of the probiotic, which was also described by Arenas et al. (2009).

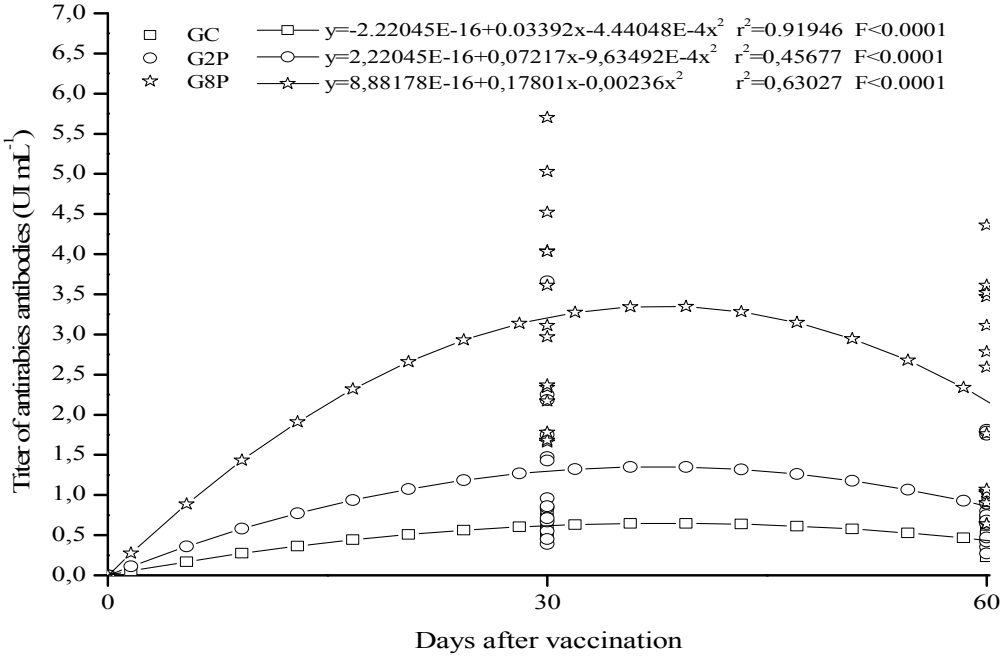
Table 2. Effects of concentrations of supplementation with probiotics added to the mineral mixture, on the titer of antirabies antibodies in cattle previously vaccinated against rabies.

Harvest (days)	Titers of antirabies antibodies (IU mL^{-1})		
	Experimental groups		
	GC	G2P	G8P
0	0^{Aa}	0^{Aa}	0^{Aa}
30	$0,62 \pm 0,10^{Ab}$	$1,30 \pm 0,91^{Ab}$	$3,22 \pm 1,31^{Bb}$
60	$0,44 \pm 0,09^{Ab}$	$0,86 \pm 0,53^{Ab}$	$2,18 \pm 1,30^{Bb}$

The results are presented as mean ± SD, n=14.
Different Uppercase letters in the same line or different lowercase letters in the same column indicate statistically significant differences ($P<0.05$).
The cattle were supplemented with 0 (CG), 2G2P) and 8 (G8P) grams of probiotics/animal/day added to 70 grams of mineral mixture.

Sixty days after the first vaccination, the percentage of animals in the CG with antibody titers ≥ 0.50 IU mL^{-1} had decreased to 42%, whereas the G2P and G8P groups presented 64 and 100%, respectively (Table 1). According to Albas et al. (2005), immunity does not persist for more than 30 days after the first vaccination; however, in the G8P group, the percentage of animals with titers considered protective was sustained (Figure 1). This result indicates that the supplementation caused a persistent immune stimulation effect (MUSA et al., 2009), which was also described by Arenas et al. (2009), who used similar microorganisms and doses, although the probiotics added were enzymes and zinc. According to Maria et al. (2009), zinc has an immunomodulatory role, which may have interfered beneficially in that study. Ferreira et al. (2009) did not find significant differences between their control group and a group supplemented with 4 g of probiotics (*Lactobacillus acidophilus, Streptococcus faecium, Bifidobacterium thermophilum and Bifidobacterium longum*). However, they used 15-month-old (that is, older) stirks, and they performed the study during the winter period, which may have compromised the colonization of microorganisms due to the low nutritional quality of pastures at that time of year.

Figure 1. Effect of different doses of probiotic on the title of anti-rabies antibodies in cattle produced in response to the primary vaccination.



Conclusions

From the results obtained in this study, it can be concluded that the evaluated vaccine demonstrated excellent capacity to induce seroconversion but did not provide minimum protective titer in all of the animals. Probiotic supplementation enhanced the antirabies humoral immune response, but only the group supplemented with eight grams differed significantly from the other groups. All of the cattle supplemented with eight grams of probiotics presented minimum protective titers, and they maintained the titer until the end of the experimental period of 60 days without requiring a booster dose. Other studies should be performed under the same experimental conditions to determine how long the minimum protective titer persists in cattle that are

supplemented with eight grams of probiotics and have previously been vaccinated against rabies.

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