



Use of Postbiotics as an Immunomodulatory of the Immune Response against Brucellosis in Nellore Calves Immunized with S 19 Vaccine

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Authors' contributions

This work was carried out in collaboration among all authors. Authors PFL, HMH and CEO conceptualized the writing. Data collection and analysis were performed by authors HMH, AART, FMS, JBVC, CPS, TFS, ERAG, CSPCT and CEO. Data validation by authors FMS, CANR and CEO; Formal analysis by author FMS; Investigation performed by authors PFL, HMH, and CEO; Resources, by authors CEO and HMH; and data curation performed by authors CANR, FMS and CEO. Original draft preparation writing by author CPS; review, editing and visualization by authors CEO and HMH. Supervision and funding acquisition by authors CEO and HMH; project administration by authors PFL, FMS and HMH. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2021/v31i430312

Editor(s):

(1) Dr. Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.

Reviewers:

(1) Mohamed Abd El-Aziem Hashem, Zagazig University, Egypt.

(2) Sherein S. Abdelgayed, Cairo University, Egypt.

Complete Peer review History: <http://www.sdiarticle4.com/review-history/70182>

Original Research Article

Received 20 April 2021

Accepted 25 June 2021

Published 30 June 2021

ABSTRACT

Background: Brucellosis is an important public health disease and a great problem in the cattle production.

Objectives: The aim of this study was to evaluate the immunomodulatory efficiency of a commercial

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postbiotic in Nellore calves immunized with the *Brucella abortus* S19 vaccine.

Methods: We used 40 calves negative for *Brucella* spp. organized into four treatments during 15 days: InRum (*Ingulbal Ruminant*®); InPro (*Ingulbal Protein*®); RumPro (*Ingulbal Ruminant*® and *Ingulbal Protein*®); and Cont: control. Collections of whole blood and serum samples were performed at the beginning of the administration of postbiotic and at 15, 45, 75 and 105 days after start the experiment. At 15 days, all animals received the mandatory vaccine S19. In order to assess immunological parameters, the means of total white cells counts, total lymphocytes, monocytes and neutrophils, and total IgG antibodies were determined.

Results: It found a significative increase ($P<0.05$) of white cells counts and serum IgG levels in the three treatment groups throughout the experiment. Additionally, we observe a tendency not significative in a greater number of lymphocytes, monocytes and neutrophils counts in the treat calves.

Conclusion: Our results suggest that the supplementation with postbiotic is a promising alternative to modulate both the cellular and humoral immune response of S19 vaccine against bovine brucellosis.

Keywords: *Brucellosis, cellular immunity; I-ELISA; humoral immunity; S19 vaccine; postbiotics; calves.*

1. INTRODUCTION

Brucellosis is a zoonosis caused by Gram-negative bacteria belonging to the genus *Brucella*, that infects humans and a huge variety of domestic and wildlife mammals species [1]. This disease is responsible for large economic losses in livestock production due to abortion and, in spite of it is controlled in some countries, it is enzootic in Mediterranean Europe, Northern and Eastern Africa, Central Asia and Central and South America, ranking 3rd among the most neglected zoonotic diseases [2].

Vaccination is largely used to control and prevent bovine brucellosis, as well as avoid risks of *Brucella* spp. transmission to humans through contamination of dairy products. The vaccines recommended by the International Organization for Animal Health are S19, used in calves and RB51 in cows [3,4]. In Brazil, the Ministry of Agriculture, Livestock and Supply, created in 2001 the National Program for the Control and Eradication of Brucellosis and Tuberculosis with the main objective of reducing the incidence and prevalence of animal and human brucellosis. Among its goals, it established the massive vaccination of buffaloes and calves between 03 and 08 months of age with the attenuated vaccine *B. abortus* S19 [5].

Since no developed vaccine has achieved a better efficacy than S19, even though it has about 70% efficacy, probably attributable to the lack of adequate adjuvants to produce the desired protective immune response, S19 vaccine is still widely used [6,7]. A potential

approach to improve vaccine effectiveness involves modulating the immune system through the use of postbiotics. Postbiotics have mechanisms that bring benefits, such as modification of the gut microbiota, competitive adherence to mucosa and epithelium, improvement of epithelial lining barrier function and modulation of the immune system [8,9].

Postbiotics imply that bacterial viability is not an essential requirement, it refers to inactivated (non-viable) microbial cells, soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis, such as enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, polysaccharides, cell surface proteins, and organic acids [10-12]. They are usually derived from bacteria of the genus *Lactobacillus* or *Bifidobacterium* [13]. Postbiotics present safety advantages over probiotics by reducing the risk of microbial translocation, infection or enhanced inflammatory responses and several attractive properties such as clear chemical structures, safety dose parameters, and longer shelf life [14,15].

Due to the need to adopt alternative strategies that increase immune response effectiveness of S19 vaccine, the aim of this work was to evaluate the immunomodulatory effect of supplementation with commercial postbiotics *Ingulbal Ruminant*® and *Ingulbal Protein*®, as well as the association of these compounds [16]. For this, calves of the Nellore breed had supplemented with postbiotics and subsequent immunization with the S19 vaccine.

2. MATERIALS AND METHODS

2.1 Experimental Design

It was carried out on a Private Farm, located in the central region of the Brazilian Pantanal. A total of 40 Nellore calves with 6 months old and average body weight of 150kg were used in the experiment. The animals were adapted to management for two weeks prior to the beginning of the experiment under pasture conditions with supplementary feed of 500 grams per animal/day and water *ad libidum*. The feed consisted of soybean meal and milled whole corn with conventional probiotics and microelements (calcium, sulfur, phosphorus, potassium, magnesium, zinc, selenium, cobalt, copper and manganese). The postbiotic *Ingulbal Ruminant*[®] and *Ingulbal Protein*[®] used for the treatments were provided by the company Innovación en Gestión y Conservación de Ungulados S.L. (Ingulados)/Spain; University of Extremadura/Spain (<https://ingulados.com>).

Two weeks prior to the beginning of the experiment all calves were tested negative for brucellosis based on serological (acidified buffered antigen – AAT) and molecular tests (PCR), [17]. The screening test (ATT) was performed over an interval of 15 days so that the seronegativity of the calves could be certified, according to the Ministry of Agriculture. After verifying the negativity for *Brucella* spp., the animals were allocated into four groups with ten calves per group: InRum (*Ingulbal Ruminant*[®]); InPro (*Ingulbal Protein*[®]); RumPro (*Ingulbal Ruminant*[®] and *Ingulbal Protein*[®]) and Cont (control).

2.2 Sampling

The sample collections were carried out at the beginning of treatment (T0) and at 15 (T1), 45 (T2), 75 (T3) and 105 (T4) days after T0. The animals belonging to InRum, InPro and RumPro received the defined postbiotics for 15 days (T0 to T1). In T1, all animals were vaccinated with the mandatory vaccine S19. For blood collection, the animals were immobilized individually on beretes. Prior to blood collection, asepsis was performed with the aid of sterile gauze, degerming, iodized alcohol and 70% alcohol. The blood collection for obtaining the serum occurred in a tube without anticoagulant by means of a jugular vein venipuncture with a vacuum needle 38 mm long and 0.9 mm in diameter.

2.3 Evaluation of Cellular Immune Response

The hematological parameters were measured up to eight hours after blood collection in the Neubauer chambers, as described by Voigt [18]. Lymphocytes, monocytes and neutrophils were performed using blood smears fixed with methanol and stained with Giemsa.

2.4 Evaluation of Humoral Immune Response

The antigen was prepared with *Brucella abortus* strain S2308. The sample was plated on trypticase soy agar (TSA) agar, being incubated at 37°C for 72 hours. The isolated colonies were recovered and grown in 10mL of Trypticase soy broth (TSB), kept in a shaker at 37 ° C for 72 hours. The culture was pelleted by centrifuging 14,000 rpm for 15 minutes, the supernatant was discarded and the pellet obtained was washed three times with 1x PBS . After discarding the last supernatant, the pellet was resuspended in 1mL of 1x PBS and then boiled in a dry bath at 95°C for 5 minutes, and then subjected to an ice bath for thermal shock. When cooling, the protease inhibitor was added to prevent protein degradation. The samples were sonicated for 3 pulses of 20 seconds (Branson Sonifier 250) and centrifuged at 14,000 rpm for 15 minutes, in order to separate the soluble and insoluble fractions. The supernatant containing the soluble fraction was transferred to a new eppendorf and stored at -20°C until the plate was prepared. Protein quantification was performed using NanoDrop[®] 2000. For standardization of the ELISA with total antigen stain 2308, optimal dilutions of the antigen, sera, conjugate and buffers were evaluated with four samples of negative and four samples of positive for *Brucella* spp. by the AAT, which were later used as positive and negative controls. After setting the test dilution parameters, the cut-off values were determined using 12 negative sera, establishing the 99% confidence limit according to Frey et al. [19]. The assay was performed in sterile 96-well flat-bottomed microplates (Cralplast[®]), with holes adsorbed with the total antigen. The combination with the best performance was 1: 400 antigen and 1: 200 serum. To prepare the plate, 100µL of the antigen diluted in carbonate buffer (pH 9.6) was added to all wells, and the plate was incubated overnight at 4°C. The blockade was performed with 2% fetal bovine serum (Cultilab[®]) and incubated at 37°C for one hour. After washing the plate, 100 µL of the test sera were applied in duplicate, as well as the

positive and negative controls. The assay blank was made with the addition of 100 μ L of PBST pH 7.2, instead of the serum to be tested. The detection of antibodies was performed with the addition of 100 μ L of the peroxidase-labeled anti-bovine IgG conjugate (Sigma-Aldrich), in the 1:10,000 dilution. The reaction was developed with 50 μ L Sigma Fast® OPD (phenylenediamine o-dihydrochloride) and stopped with 50 μ L of 1M HCl. The result was determined by absorbance at 490nm using Multiskan™ GO UV/Vis microplate spectrophotometer.

2.5 Statistical Analysis

Descriptive statistics (mean \pm standard deviation) was applied to obtain the average of health parameters. The Shapiro-Wilk test was used to establish the normality of the parameters. Subsequently, Analysis of Variance (ANOVA) was applied to determine the differences between treatments and periods according to normality. The Tukey test was used to evaluate the results in pairs of the ANOVA test.

3. RESULTS AND DISCUSSION

3.1 Cellular Immune Response

The hematological parameters showed that the postbiotic were able to induce significant increase in WBC, in all three treatments comparing to control group, throughout the experiment (Table 1). This marked leukocytosis was accompanied by a tendency in increased mean values of monocytes and lymphocytes in InRum, InPro and RumPro during all the experiment, although significant differences have been found only for lymphocytes in InRum at T2, T3 and T4 while in InPro at T2 and T3 (Table 1). We also detected a slight increase for neutrophil averages in the three treatments comparing to control group in T1, T2 and T4, with a significant increase registered in RumPro and InPro at the T1 and T4 respectively (Table 1).

3.2 Humoral Immune Response

Regarding levels of anti-*Brucella* antibodies, we observed an overall increase in IgG concentrations after administration of postbiotics in the three treated groups after the start (T0) until to the end of the experiment (T4) (Fig. 1). The significant increase of IgG serum levels was detected in InRum in T1; in InPro in T2 until to the end of experiment; and in RumPro during T1, T3 and T4 (Table 1).

When comparing the periods, it is possible to observe a typical IgG behavior after

immunization with the S19 strain, with a peak in 30 days after vaccination (T2), returning to initial levels after 60 days (T4). Additionally, we observed significant variations for serum IgG values of all three treated groups: increase between T0 to T1, and T1 to T2 ($P<0.05$); decrease between T2 and T3 ($P<0.05$), remaining the same until the end of experiment (T4) ($P>0.05$).

In general, our results showed that the use of the *Ingulbal Ruminant*® and *Ingulbal Protein*® postbiotics were able to modulate the immune system of calves, before and after vaccination against brucellosis, since the treatment groups have a tendency to increase the numbers lymphocytes and monocytes. In addition, an increase in the levels of immunoglobulin antibodies (total IgG) presented by the treated groups, in relation to the control reinforce the activation of the immune system.

The immunomodulatory effect of postbiotics is related to their ability to induce or suppress the immune system by regulating the production of pro-inflammatory and anti-inflammatory cytokines. Also, postbiotics favor immunity of the intestinal mucosa [20]. The modulation of the immune system can generate an effective adaptive immune response mediated by cells, ideal way to mitigate intracellular infectious agents, such as *Brucella* spp. [21].

In our study, we observed a leukocytosis in the treated groups throughout the experiment due to the activation of the lymphoreticular system for production and transportation of the antibodies in trial to fight the infection. Also the leukocytosis might be due to stimulation of cell mediated immunity [22,23] It is noteworthy that the observed tendency in increase lymphocytes count in the treated calves indicates an adaptive immune response. Indeed, helper T (Th) cells secrete cytokines that assist B cells to build an effective antibody response [24].

Postbiotics are inactivated microbial cells acting as co-stimulate of the induction of the immune response, playing an important role in homeostatic mechanisms due to the presence of peptideoglycans and lipopolysaccharides [13]. These fragments are released in small amounts in the intestines and are fundamental in the activation of macrophages and neutrophils and, consequently, stimulating the production of cytokines, such as IL-1, IL-6, IL-8 and IL-12, in addition to TNF - α . [25]. In addition, there is the hypothesis that the postbiotic can exert

immunomodulation activity by increasing the levels of Th1-associated cytokines and reducing the Th2-associated cytokines [26].

The process of opsonization and phagocytosis of *Brucella* spp. is mediated by Th1 response, that include the production of IFN- γ by CD4⁺ cells, cytotoxic T lymphocytes (CD8⁺ cells), activation of macrophages and production of IgG2a [27]. Although, Th2 response is not effective to intracellular infectious agents, which is associated with the production of IgG1, an intricate interaction between the host and the pathogen requires a balance between the Th1 and Th2 responses. Actually, the dominant IgG2a responses in the early stages of disease can provide protection against brucellosis [28].

Furthermore the occurrence of subacute ruminal acidosis (SARA) in cattle fed a diet rich in grains causes greater acidity, resulting in higher lipopolysaccharide (LPS) activity in the rumen [29]. The increase in ruminal LPS translocates to the bloodstream and can elicit inflammatory and acute-phase protein (APP) responses in cattle and overload liver cells [29-31]. The metabolic consequences of uncontrolled inflammation induced by LPS challenge can be harmful, especially in the early stages of lactation and when there is a marked degree of mobilization of body fat [30,31]. However, studies that administer anti-LPS have observed a decrease in immune-mediated colitis and intestinal inflammation in murine models [32] in addition to mitigating ruminal LPS release and pH depression without following responses in acute phase inflammation or hepatic transcriptomic expression in cattle [31]. Thus, we can suggest that modulate intestinal microbiome can

increasingly serve as a target for regulatory T cell-based immunotherapy [31-33].

The method of evaluating the effectiveness of the vaccine is by measuring levels of antibodies in the serum after vaccination, since they directly correlate with protection and, therefore, these methods can be used as a reference to determine the regulation of probiotics or postbiotics in the immune response system [34]. Regarding the humoral immune response, in our experiment it was possible to observe a typical behavior of total IgG after immunization with the S19 strain, with a peak in 30 days after vaccination and returning to lower levels after this period [35,36].

We observe that the groups fed with postbiotics had a significant increase in the production of IgG in different periods, although it was not possible to distinguish the best treatment with different types of postbiotics. In fact, in the group treated with *Ingulbal Protein*[®], IgG levels increased in all periods after suspension of supplementation with postbiotic and immunization, while the group treated with *Ingulbal Ruminant*[®], the stimulus in the production of IgG was significant, only during supplementation (T1). For the group treated with *Ingulbal Protein*[®] and *Ingulbal Ruminant*[®], the production stimulus was late, and occurred after 75 days of the treatment, remained until the end of the experiment. In addition, although the increase of total IgG levels after S19 vaccination was expected [34], the observed levels of optical density (O.D.) are low, probably due to the I-ELISA test assessing the levels of total IgG while IgG1 and IgG2a antibodies are the ones that confer a protective immune response against *Brucella* spp. [37].

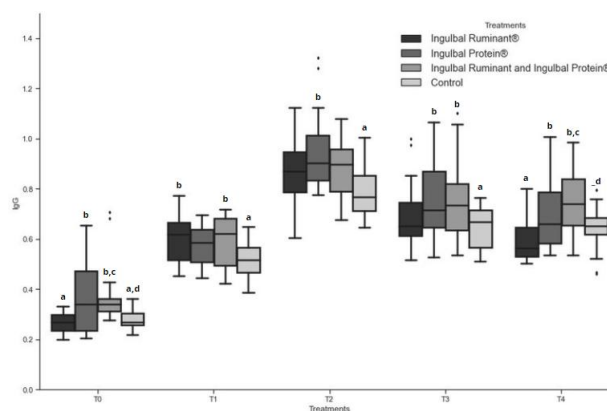


Fig. 1. Values of the optical density of IgG anti-*Brucella* antibodies by the indirect ELISA method at 490nm using Multiskan™ GO UV/Vis microplate spectrophotometer

Table 1. White blood cell counting Nellore calves treated with postbiotics

	Groups	White Blood Cell	Lymphocyte	Monocyte	Neutrophil	IgG anti-<i>Brucella</i>
T0 – 0 days Start of treatment	InRum	15,045 ± 2685	11,184 ± 2,333	460 ± 358	2,874 ± 526	0,2673 ± 0,039623 ^a
	InPro	13,975 ± 3094	9,927 ± 2,360	481 ± 315	3,188 ± 963	0,36146 ± 0,144873 ^b
	RumPro	16,025 ± 1739	1,1178 ± 2,204	1,011 ± 758 ^b	3,559 ± 1,840	0,369045 ± 0,117313 ^{bc}
	Cont	14,600 ± 2864	10,377 ± 2,516	319 ± 361 ^a	3,675 ± 893	0,281095 ± 0,041693 ^{ad}
T1 – 15 days Vaccination	InRum	14,960 ± 3929 ^b	10,999 ± 3,028	329 ± 195	2,951 ± 1314	0,60205 ± 0,099143 ^b
	InPro	15,500 ± 2715 ^b	10,858 ± 3,099	539 ± 325	3,575 ± 1,577	0,57591 ± 0,081023
	RumPro	15,095 ± 2434 ^b	9,568 ± 3,056	505 ± 189	4,464 ± 2,136 ^b	0,602415 ± 0,097993 ^b
	Cont	12,195 ± 2253 ^a	9,238 ± 1,768	302 ± 248	2,221 ± 917 ^a	0,512625 ± 0,069983 ^a
T2 - 45 days	InRum	16,845 ± 2063 ^b	11,273 ± 2,388 ^b	1,237 ± 513	3,358 ± 1,619	0,866515 ± 0,127913
	InPro	16,510 ± 2341 ^b	11,482 ± 3,012 ^b	817 ± 359	3,658 ± 1,169	0,94554 ± 0,156333 ^b
	RumPro	16,040 ± 1922 ^b	10,768 ± 2,927	940 ± 916	3,809 ± 1,408	0,88586 ± 0,113983
	Cont	12,170 ± 1328 ^a	8,181 ± 1,169 ^a	711 ± 431	2,581 ± 629	0,78443 ± 0,091913 ^a
T3 - 75 days	InRum	16,345 ± 1570 ^b	10,597 ± 872 ^b	684 ± 587	4,161 ± 1,438	0,68576 ± 0,135503
	InPro	17,500 ± 2412 ^{b,c}	10,953 ± 2,007 ^b	719 ± 725	4,827 ± 1,344	0,76467 ± 0,170743 ^b
	RumPro	15,435 ± 2560 ^{-d}	9,559 ± 1,467	832 ± 720	4,212 ± 1,442	0,76353 ± 0,163073 ^b
	Cont	13,820 ± 1010 ^a	8,655 ± 1,181 ^a	276 ± 331	4,310 ± 903	0,6466 ± 0,088263 ^a
T4 - 105 days	InRum	16,975 ± 2804 ^b	11,353 ± 2,088 ^b	123 ± 186	4,728 ± 1,536	0,59727 ± 0,084833 ^a
	InPro	17,830 ± 3287 ^{b,c}	9,493 ± 2,512	204 ± 182	7,081 ± 3,235 ^b	0,71265 ± 0,154383 ^b
	RumPro	15,195 ± 1927 ^{-d}	9,214 ± 2,027	223 ± 188	5,287 ± 1,756	0,75672 ± 0,132343 ^{bc}
	Cont	13,555 ± 1225 ^a	8,379 ± 1,648 ^a	126 ± 229	4,509 ± 1,393 ^a	0,642345 ± 0,086923 ^{-d}

T0 - start of treatment with postbiotics, T1 - end of the administration of postbiotics and immunization with vaccine S19. Hematological data are expressed by mean values ± standard deviations in mm³. Different letters indicate significant difference to $p \leq 0.05$. InRum- Ingulbal Ruminant[®]; InPro - Ingulbal Protein[®]; RumPro - Ruminant[®] + Protein[®] and Cont – control.

The treatment of cattle with functional components has been studied in the interaction with other infectious agents shown effectiveness in potentiating the immune response in immunized animals [38]. Pérez et al. evaluated the performance of supplementation with *Ingulbal Ruminant*® and *Ingulbal Protein*® in cattle infected with *Mycobacterium avium* subsp. paratuberculosis and detected a significant improvement in the health of animals due to increase in red blood cells, and normalization in the levels of total proteins, globulins, creatine and aspartate aminotransferase [16]. In addition, a study conducted by Shenderov revealed that postbiotics have a good capacity for absorption, metabolism, distribution and excretion, indicating satisfactory physiological responses of different organs and tissues of the host [39]. Furthermore, it has been shown that postbiotics can mimic the health effects of probiotics, avoiding the administration of live microorganisms which are not always harmless in addition to having several attractive properties, such as clear chemical structures, safety dose parameters and longer useful life long [13,15].

It is considered that postbiotics should be administered continuously to exert their effects, however, even with the interruption of the postbiotic supply at 15 days, our results show that the modulation of the immune system continued, even 105 days after the start of treatment. This fact may indicate that the immunomodulatory effect is perpetuated by stimulating the population of memory cells, which will respond actively in cases of future contacts with the antigen [40].

4. CONCLUSION

Use of postbiotics *Ingulbal Protein*® and *Ingulbal Ruminant*® may contribute to the improvement the host immune system by stimulating the production of lymphocytes and neutrophils and increase specific IgG antibodies anti-*Brucella abortus* after immunization with compulsory vaccine S19. Supplementation with postbiotics is an accessible, safe, healthy and profitable strategy for optimizing animal production. Additionally, by enhancing the immune system, cattle can cope with different infectious agents more effectively, including *Brucella* spp. This alternative could become an important tool resulting in a favorable cost-benefit ratio, since reduction in the prevalence of brucellosis have positive socioeconomic impacts.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by committee on Ethics in the Use of Animals (CEUA/UCDB) 022/2018.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Carlos Aberto Ramos Nascimento, for the technical support and donation of materials for I-ELISA and to the Research Group InsanaHuna (Campo Grande-MS, Brazil) for assistance in the field work support. This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) by Productivity Grant granted to the Herrera MH (CNPq Process No. 308768/2017-5); Ingulados-Research; Coordination for the Improvement of Higher Education (CAPES) by a scholarship to Santos CP.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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