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Evaluation of polymeric adjuvants associated with cadidate vaccine strain Brucella ovis ΔabcBA in a murine model of Brucella ovis infection

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Abstract

Brucellosis is an infectious disease caused by facultative intracellular Gram-negative bacteria, of great importance in animal and human health. An ideal vaccine against brucellosis should induce protection, not cause disease in animals or humans, and not interfere with serological diagnosis. Vaccine adjuvants can improve the immune response, leading to a more intense and prolonged protection, improving its effectiveness. The *Brucella ovis* Δ*abcBA* strain encapsulated in alginate provides an experimental vaccine formulation that protects against *Brucella* spp. infection. However, the investigation of other polymers such as adjuvants is important for optimizing the efficiency of the candidate vaccine *B. ovis ΔabcBA*. Therefore, this study aimed to evaluate the vaccine potential of the *B. ovis ΔabcBA* associated with different polymeric adjuvants in mice challenged with *B. ovis* . We observed that *B. ovis* Δ*abcBA* encapsulated by alginate with chitosan, but not copolymer Poloxamer 407, resulted in the lowest bacterial recovery in both the spleen and liver of challenged animals compared to non-vaccinated mice. While copolymer Poloxamer 407 did not induce significant humoral immune response, the alginate and chitosan vaccine formulation induced higher levels of Immunoglobulin G, with an increase in the IgG2b subclass, indicating a Th1 type of response, which is known to play a critical role in controlling infections by intracellular agents.

Keywords

ABC transporter, alginate, chitosan, immune response, mice model, ovine brucellosis

Introduction

Brucellosis is an infectious disease caused by facultative intracellular Gram-negative bacteria, of great importance in animal and human health. Several species of *Brucella* , such as *B. melitensis* , *B. abortus* , *B. suis* , and *B. canis*, can cause human infections and economic losses in small ruminant, cattle, swine and dogs, respectively (Moreno 2021). Although *B. ovis* does not have zoonotic potential, it causes significant economic impact on the sheep industry due to subfertility mostly secondary to epididymitis in rams (Burgess 1982, Carvalho Júnior *et al.* 2012).

Vaccination of domestic animals is the most important strategy for controlling animal brucellosis, which indirectly prevents human brucellosis. An ideal vaccine against brucellosis should induce good protection, not cause disease in animals or humans, and not interfere with serological diagnosis of the disease (Corbel 2006).

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There are no commercially available vaccines for preventing *B. ovis* infection in sheep (Carvalho *et al*. 2020a). Previous studies from our group demonstrated the potential of a vaccine candidate *B. ovis* lacking a species-specific ABC deficient mutant strain (*B. ovis* Δ*abc BA*). This candidate vaccine strain is strongly attenuated *in vitro* and *in vivo* in mice (Silva *et al*. 2011, Silva *et al*. 2014) or sheep (Silva *et al*. 2013). When *B. ovis* Δ*abc BA* is encapsulated in alginate there is an increase in immunogenicity supposedly due to a prolonged release of the vaccine strain (Silva *et al.* 2015b). This formulation induced protection in mice against various *B. ovis* isolates (Carvalho *et al.* 2020b). Importantly, this strain promoted sterile immunity in experimentally challenged rams (Silva *et al.* 2015a). *B. ovis* Δ*abc BA* encapsulated with alginate also protected mice against *B. melitensis* (Costa *et al.* 2020) and *B. canis* (Eckstein *et al.* 2020) associated with a Th1-directed immune response. Thus, this vaccine strain has the potential for development of a polyvalent *Brucella* spp. vaccine.

Adjuvants are used in vaccine preparations to improve the immune response, leading to a more intense and prolonged protection (Brunner *et al.* 2010). Natural polymers derived from microorganisms or plants or synthetic polymers such as polyesters, polyanhydrides, or copolymers demonstrated adjuvant potential in association with various antigens. They favor the activation of humoral and cellular immune responses and promote a slow release of the antigen. Solubility, molecular weight, degree of branching and the conformation of the polymeric backbone are factors that can influence the adjuvant potential of a polymer. Furthermore, biocompatibility, biodegradability, easy production and purification, and absence of toxicity are important characteristics for a polymer to be a good candidate for vaccine adjuvant (Shakya and Nandakumar 2013).

Synthetic copolymers as Poloxamer 407 have been investigated as a controlled delivery system for drugs and antigens (Dumortier *et al.* 2006, Kojarunchitt *et al.* 2015, Bobbala *et al.* 2016). Poloxamer 407 is a non-ionic sulfating triblock copolymer composed of polyethylene and polypropylene with thermo-responsive properties. Its polymerization at body temperature forms a hydrogel that promotes prolonged release of pharmacological agents, while at room temperature, this polymer has a fluid state that facilitates the administration of the formulation (Dumortier *et al.* 2006).

Alginate is a natural polymer extracted from algae that has been used for microencapsulation of vaccines (Arenas-Gamboa *et al.* 2009, Silva *et al.* 2015a, Costa *et al.* 2020). Alginate is a biopolymer that incites a more effective immune response against intracellular pathogens, eliciting production of cytokines such as IFN-γ and TNF, in in the absence of allergic reaction (Kesarwani *et al.* 2021). In this study we will associate alginate with chitosan, which is a polysaccharide from chitin found in crustaceans and cell wall of fungi. Chitosan is biodegradable, biocompatible, and non-toxic compound that has immunomodulatory activity with increasing application in vaccine formulations (Heffernan *et al.* 2011, Abkar *et al.* 2015, Dumkliang *et al.* 2021). Polyonic alginate-chitosan complexes improve the adjuvant properties of its individual components by reducing the microcapsule pores, which results in better retention and optimization of antigen release and better results in experimental vaccines (George and Abraham 2006, Rahaiee *et al.* 2015, Rocha *et al.* 2021).

The biochemical characteristics of biopolymers favor the vaccine response by stimulating the immune system, mainly through the slow release of antigen to antigen-presenting cells, allowing the adaptive immune response to be adequately developed. *B. ovis* Δ*abc BA* vaccine is characterized by its rapid elimination from the host's organism, which may insufficiently induce the adaptive immune system. Thus, the use of an adjuvant with biopolymers that prolongs the time the antigen is exposed to the immune system is desirable and justifies research. Therefore, the goal of this study is to evaluate the adjuvant effect of copolymer Poloxamer 407 and alginate/chitosan complexes on the protective response induced by the candidate vaccine strain *B. ovis* Δ*abc BA* against experimental challenge with wild type *B. ovis* in mice.

Material and methods

Ethics statements

The experimental animal procedures in this study strictly followed all applicable laws and regulations. These experimental procedures were approved by the Ethics Committee in the Use of Animals of the Federal University of Minas Gerais, under protocol CEUA/UFMG No. 28/2020. The animals were kept in an environment with controlled temperature and humidity and received water and food *ad libitum*. The animals were euthanized using an anesthetic dose composed of xylazine hydrochloride (2%, 30 mg/Kg, Syntec, Brazil) and ketamine hydrochloride (1%, 210 mg/Kg, Syntec, Brazil), mixed and injected intraperitoneally in a volume of 100 µL followed by cervical dislocation.

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Bacterial strains and culture conditions

Bacterial strains *B. ovis* wild type ATCC 25840 and *B. ovis* Δ*abc BA*, a mutant strain generated from the deletion of open read frame (orf) *abc A* and orf *a bc B* from the *abc EDCBA* locus (Silva *et al.* 2011), and the *B. ovis* Δ*abc BA* mCherry strain which express constitutively red fluorescence (Silva *et al.* 2014), were used in this study. The bacteria were cultured on tryptone soy agar (TSA, Invitrogen, USA) containing 1% hemoglobin (Becton-Dickinson, USA) for three days at a constant temperature of 37°C in a humidified oven and at 5% CO_2, 100 µg/mL of kanamycin (Gibco, Brazil) was added to the plates of the mutant strains. Bacteria were resuspended in phosphate-buffered saline (PBS/pH 7.4, Gibco, Thermo Fisher Scientific, USA) and bacterial concentration was estimated by measuring optical density at 600 nm (OD600) in a spectrophotometer (Bio-Rad, USA).

Evaluation of copolymer stability

The ideal gelation condition of the final formulation at 37°C was determined during the pre-experimental phase using a factorial design with variations in ionic strength, Poloxamer 407 concentration (data not showed). The concentrations 185 µg/mL and 190 µg/mL were selected to mix to bacterial suspension. Poloxamer 407 (PLX, Sigma-Aldrich, USA) is a copolymer that is liquid under refrigeration and polymerizes at 37ºC. The behavior of the PLX associated or not with the bacterial suspension of *B. ovis* Δ*abc BA* at a concentration of 10^{10} CFU/mL was evaluated. Time required for polymerization (solidification) at a temperature of 37°C (in a humidified oven with 5% CO_2) or 21°C (at room temperature) and the adequate refrigeration condition for its maintenance in liquid phase (ideal for inoculation) keeping suspension on 0°C or 4°C were measured. Analyzes were performed in triplicates with 2 mL of the PLX at two different concentrations (185 µg/mL and 190 µg/mL) associated or not with the bacteria.

Vaccine preparation with copolymer.

The bacterial suspension containing $1x10^{10}$ CFU/mL of *B. ovis* Δ*abc BA* was prepared in PBS to define the concentration in OD600, resuspended directly in the solution of Poloxamer 407 (Sigma) at a concentration of 185 µg/mL, remaining under refrigeration until inoculation.

Preparation of vaccines with alginate and chitosan

The encapsulation of the vaccine strain with alginate (Sigma-Aldrich, USA) and chitosan (Sigma-Aldrich, USA) was performed with a bacterial suspension containing a concentration of 1 x 10^{10}CFU/mL of *B. ovis* Δ*abc BA*, resuspended in an alginate solution at 1%, as previously described (Silva *et al.* 2015a), Then the alginate capsules were immersed in a solution composed of chitosan at 1% acetic acid and sodium acetate at pH 5.0 (Rocha *et al.* 2021).

Fluorescence microscopy

To visualize the *B. ovis* Δ*abc BA* associated with the adjuvants used in this study, vaccines were prepared with the*B. ovis* Δ*abc BA* mCherry bacterial strain, under the same conditions and concentration described for the preparation of the vaccines used in the immunization of mice. The vaccine suspensions under glass slides were observed and images captured on a Leica photomicroscope, DM 4000 B (Leica Microsystems, Germany).

Immunization of mice

Thirty 6 -7-week-old C57BL/6 female mice were randomly divided into five groups with six animals each as follows: alginate + chitosan + 1 x 10^9CFU/mL of *B. ovis* Δ*abc BA* (AC+Δ*abc BA*), alginate + chitosan (AC), copolymer Poloxamer 407 + 1 x 10^9 CFU/mL *B. ovis* Δ*abc BA* (PLX+Δ*abcBA*), Copolymer Poloxamer 407 (PLX) and the control group inoculated with PBS (not vaccinated). Mice were inoculated subcutaneously with 100 µL of the vaccine preparations or sterile PBS in a single dose. In animals immunized with preparations containing AC, the vaccine dose was divided into two application sites (dorsal cervical region and pelvic region). Since immunization, the mice were observed daily until the 42nd day after vaccination to measure body weight and thickness at the site of inoculation with caliper, and to observe behavioral changes related to pain or local inflammatory reaction.

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Protection assay

Four weeks after vaccination, all groups were submitted to the infectious challenge with a dose of 1 x 10^6 CFU/animal of the wild type *B. ovis* ATCC 25840 strain. Two weeks after the challenge, to determine the bacterial recovery, the mice were euthanized and fragments of liver and spleen were collected, weighed and macerated using a tissue homogenizer (Ultra Stirrer, Biotech, USA). Serial dilution was performed and plated in TSA with 1% hemoglobin. TSA plates with or addition of 100 µg/mL of kanamycin were intended for the recovery of the vaccine strain. The seeded plates were kept in an oven at a temperature of 37°C and 5% CO_2 and colonies were counted after 3 to 5 days.

Histopathological evaluation

Tissue samples from the vaccine inoculation site, liver, and spleen of mice were fixed in 10% buffered formalin, processed for paraffin embedding, sectioned in a microtome (3-4 µm thick sections), and stained with hematoxylin and eosin. Histopathological changes were scored according to the intensity of the inflammatory infiltrate: 0 (zero) absent, 1 (one) mild, 2 (two) moderate, and 3 (three) severe, and the absence of necrosis 0 (zero) or the presence of necrosis 1 (one), resulting in a total maximum score of 4 (four). The slides were blindly evaluated by two veterinary pathologists.

Evaluation of humoral immune response

The humoral immune response induced in mice after vaccination was evaluated by Indirect Enzyme Linked Immunosorbent Assay (ELISAi) measuring specific titers of immunoglobulins, including IgM, total IgG and its subclasses, IgG1, IgG2a, IgG2b, and IgG3. ELISA plates (Costar, Sigma-Aldrich, USA) were sensitized with 100 µL of sonicate crude total *B. ovis* antigen at a concentration of 0.25 µg per well for 18 hours at 4°C. After antigen adsorption, the plates were washed twice with PBST 0.05% Tween 20 (Sigma-Aldrich, USA) and blocked with 200 µL of PBS plus 5% bovine serum albumin (BSA) for 1 hour at 37°C. After blocking, the solution from the wells was removed and samples of animal sera were diluted (1:100) in PBS solution with 2.5% BSA, added to wells, and incubated for 1 hour at 37°C. Next, the plates were washed three times with 0.05% PBST and 100 µL of the secondary anti-mice antibody (IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3) conjugated with peroxidase (Sigma-Aldrich, USA) diluted 1:2,000 in PBS-BSA 2.5% were added to wells. After incubation at 37°C for 1 hour, the plates were again washed three times with the washing solution, and then 100 µL/well of substrate (0.1 M anhydrous citric acid, 0.2 M sodium phosphate, 0.05% OPD and 0.1% H_2O_2) was added. The plates were protected from light for 5 minutes with the developer solution, and the reaction was stopped by the addition of 50 µL of H_2SO_4. The resulting absorbance was analyzed in an ELISA reader at 492 nm (MR-96A Microplate reader, Mindray, China). All assays were performed in duplicates.

Statistical analysis

The statistical analysis of the data obtained in the CFU count and antibody measurement were normalized by logarithmic transformation and submitted to the analysis of variance (ANOVA). Then the means were compared using the Tukey test. The score of the lesions in the tissues evaluated were analyzed using the non-parametric Kruskal-Wallis test. All these analyses were performed with GraphPad Prism software version 8.0.1 (GraphPad Prism software 8.0.1, Inc, USA). Values were considered statistically different when P value <0.05.

Results

Characteristics of copolymer Poloxamer 407 in vaccine formulation

The copolymer PLX is liquid at low temperatures and solid at body temperature, which are highly desirable features for a vaccine adjuvant. Since it is liquid during preparation and inoculation, and it polymerizes at body temperature at the site of inoculation, which favors a slow release of the antigen. Therefore, we evaluated physical properties of PLX *in vitro* under conditions similar to those found in the field or usual conditions for veterinary vaccine preparations. The vaccine formulation at the concentration of 185 µg/mL of PLX polymerization at 21°C (room temperature) began at 133,2 ± 0.004 (n = 3) seconds after removal from the 0°C and it was completed at 679,8± 0.043 (n = 3) seconds. In formulations with 190 µg/mL, there was no significant differences in the initial and final polymerization times at 21ºC (Figure supplementary 1A). Maintenance of the vaccine formulation with PLX in liquid phase was evaluated in usual conservation media, either under 0ºC (crushed ice) or 4°C (reusable ice). The polymerized PLX (after removal from

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the 37°C incubator) liquefied in less than 1 minute and remained in constant liquid form (for up to 3 hours evaluated) at 0°C in both concentrations, 185 µg/mL and 190 µg/mL. However, when stored at 4°C it liquefied only partially (Figure supplementary 1B). This time in liquid phase in flake ice was considered enough for preparation, suspension and inoculation of a vaccine in the field. Due to similar liquid phase stability, and polymerization time at room temperature, the lower concentration, 185 µg/mL, was chosen for the mouse immunization experiment. These results suggest that the PLX, presents characteristics that make its use feasible during vaccine preparation.

Bacterial strain in the vaccine formulations with copolymer PLX or alginate and chitosan microcapsules

Evaluation of the vaccine formulations by fluorescence microscopy using the *B. ovis* Δ*abcBA* mCherry strain associated with the polymeric adjuvants demonstrated bacteria retained inside of polymers. The AC capsules showed irregular ovoid shapes, ranging in size from 500 µm to 900 µm (Figure 1 A). Evaluation of the PLX revealed an amorphous material associated with diffusely distributed bacteria (Figure 1 B). The schematic figure represents interaction of bacteria with potential polymeric adjuvants (Figure 1 C)

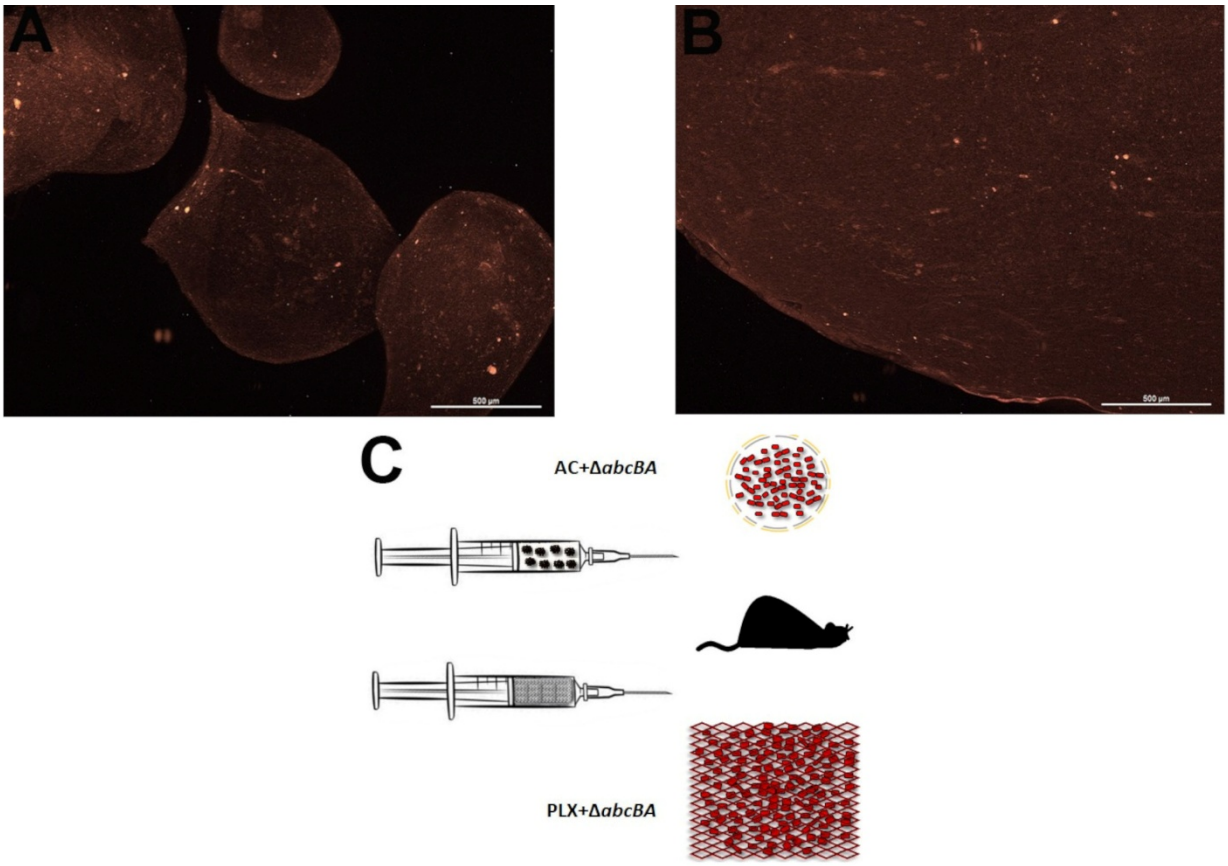

Figure 1. Fluorescence microscopy image demonstrating numerous red bacteria (Brucella ovis ΔabcBA expressing mCherry) associated with alginate capsules with chitosan (A) or the copolymer PLX (B). Schematic figure vaccine formulations - polymers associated to mCherry *B. ovis* (C).

Inflammatory response of inoculation site to *Brucella ovis* Δ*abcBA* vaccine formulations

Previous studies demonstrated that the use of alginate and chitosan microcapsules elicits an exacerbated inflammatory response at the inoculation site when associated with vaccine antigen (Rocha *et al.* 2021). Therefore, formulations containing alginate and chitosan in this study were applied at two inoculation sites. The vaccine formulations containing PLX were applied at a single inoculation site. When we evaluated the inoculation site of the animals immunized with polymeric adjuvants, only the animals immunized with AC+Δ*abc BA* had significant local changes. Immunization of mice with AC+Δ*abc BA* caused a progressive increase in skin thickness, which peaked at 14 days but remained until 42 days after inoculation (Figure 2A). Three mice immunized with AC+Δ*abcBA* developed a fistula, draining purulent-looking contents. Discomfort during handling for daily weighing was observed in all mice of

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the AC+Δ*abcBA* group. No change in behavior was observed in the animals of the other groups. Despite the changes in the inoculation site, there were no differences in weight gain between the groups throughout the course of the experiment (Figure 2B).

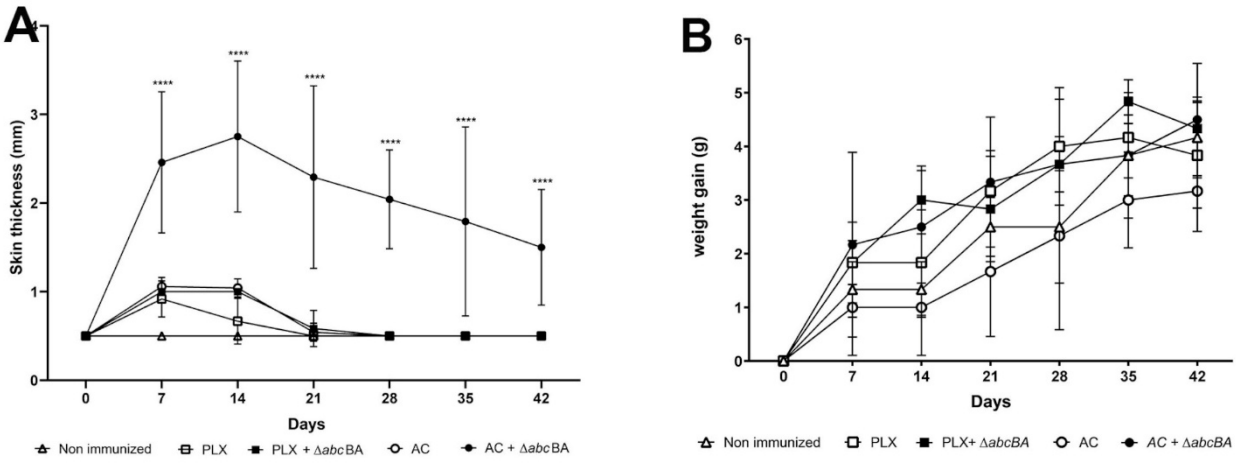

Figure 2. Skin thickness at the vaccine inoculation site (A) and body weight gain (B) of female C57BL/6 mice (n=6) was evaluated for 42 days after subcutaneous immunization with PBS (non-immunized), copolymer (PLX), PLX + B. ovis ΔabcBA (PLX+ΔabcBA), alginate and chitosan capsules (AC), and *B. ovis* Δ*abcBA* encapsulated by alginate and chitosan (AC+Δ*abcBA*). Results were analyzed for normality before being subjected to ANOVA, with mean values compared by Tukey's test. Statistical differences are represented by asterisks (* p < 0.05, ** p

Histology of the skin at inoculation sites sampled at 42 days after immunization, demonstrated a chronic inflammatory lesion in the skin of AC+Δ*abc BA* mice. The dermis presented intense and diffuse inflammatory infiltrate that extended from the superficial dermis to the deep dermis, composed of macrophages with foamy cytoplasm, neutrophils, and some lymphocytes and plasma cells, associated with acanthosis, multifocal areas of moderate necrosis, and marked fibroblastic proliferation. In addition, moderate multifocal deposition of hyaline-looking content was observed associated with the lesion. In contrast, skin from mice in the AC, PLX or PLX+Δ*abcBA* group exhibited mild local inflammatory reaction, characterized by the presence of a discrete number of macrophages, rare lymphocytes and plasma cells, with this inflammatory infiltrate being observed mainly in the deep dermis region, in addition to no histological changes were observed in the skin of unvaccinated mice (Figure 3).

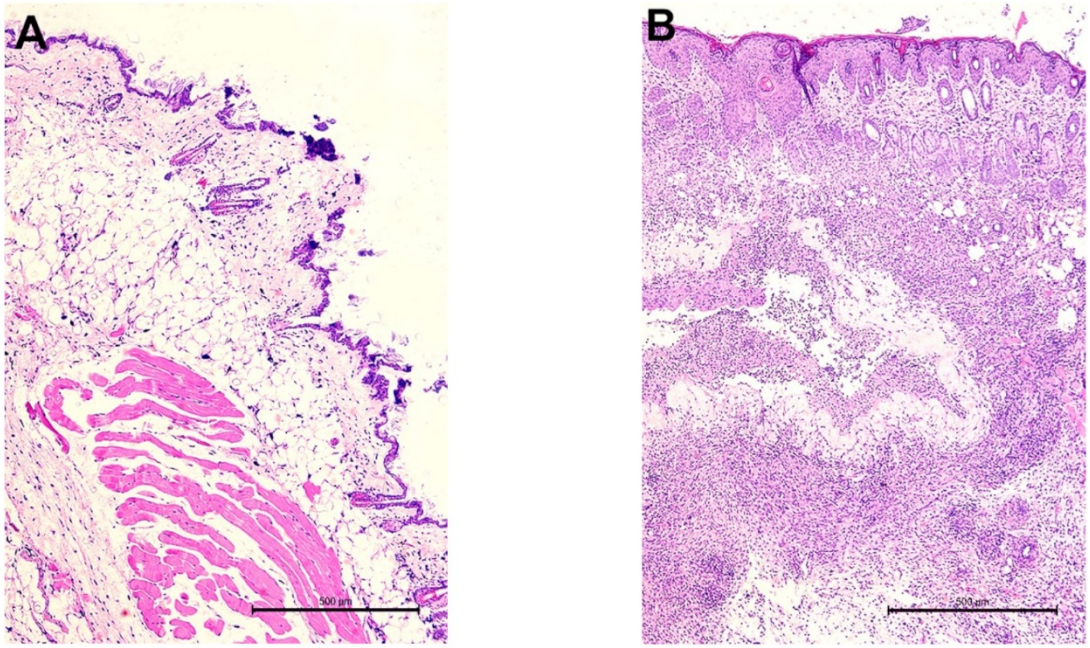

Figure 3a/b. Histopathology of vaccine inoculation skin site of female C57BL/6 mice (n = 6) 42 days after subcutaneous immunization with PBS (non-immunized) (A) and B. ovis ΔabcBA encapsulated by alginate and chitosan (AC+ΔabcBA) (B).

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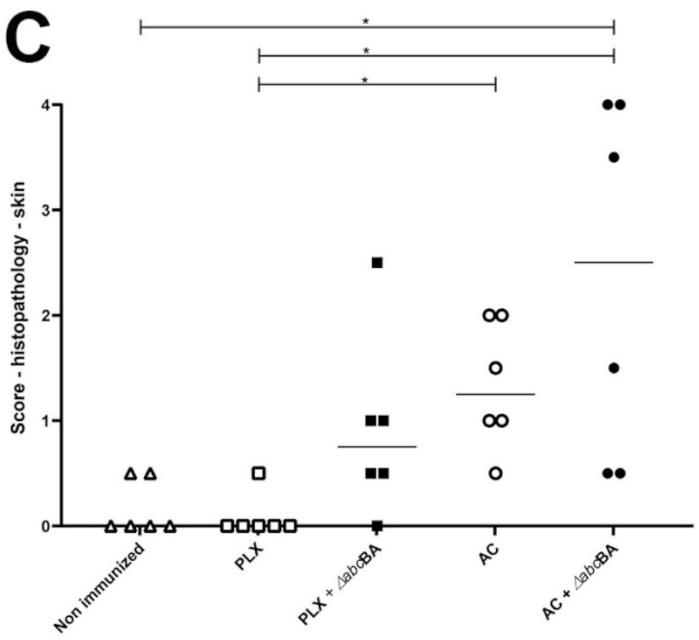

Figure 3c. Histopathological scores are expressed as individual animals (points) and median (line) (C). Results were analyzed using the Kruskal-Wallis non-parametric test. Statistical differences are represented by asterisks (* $p < 0.05$, ** $p < 0.01$).

Vaccine protection of *Brucella ovis* Δ*abcBA* formulations against challenge with *B. ovis*

Mice immunized with alginate capsules with chitosan loaded with the vaccine strain showed a significant ($p < 0.05$) reduction in bacterial load in the liver (Figure 4A) and spleen (Figure 4B) after challenge with the *B. ovis* wild type strain when compared to the unvaccinated group. The AC+Δ*abcBA* vaccine resulted in 0.5 Log protection index in the spleen and 0.6 Log in the liver. Mice vaccinated with PLX+Δ*abcBA* did not show significant CFU reduction in the spleen and liver when compared with unimmunized animals.

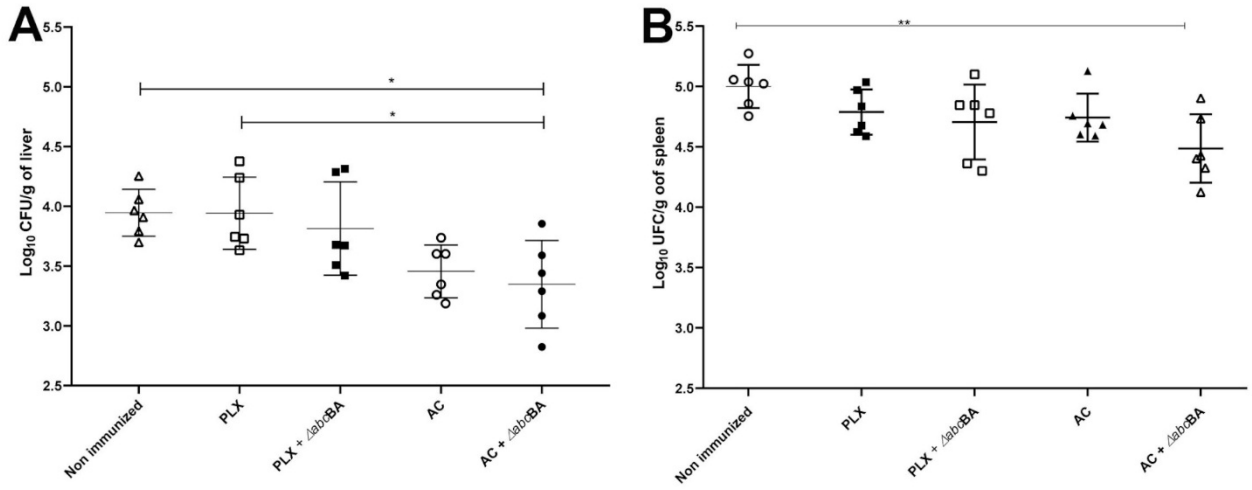

Figure 4. Bacterial recovery from liver (A) and spleen (B) of female C57BL/6 mice (n = 6) immunized subcutaneously with PBS (non-immunized), copolymer (PLX), PLX + B. ovis ΔabcBA (PLX+ΔabcBA), alginate and chitosan capsules (AC), and B. ovis ΔabcBA encapsulated by alginate and chitosan (AC+Δ*abcBA*) and subsequently challenged with the wild strain of *B. ovis*. Data are expressed as individual animals (points) and mean with standard deviation. Results were log-transformed before being subjected to ANOVA and compared by Tukey's test. Statistical differences are represented by asterisks (* $p < 0.05$, ** $p < 0.01$).

Microscopically, liver of unvaccinated mice infected with *B. ovis* exhibited multifocal areas of intense inflammatory infiltrate composed of macrophages and neutrophils (microgranulomas), associated with multifocal random areas of necrosis (Figure 5). Similar lesions were observed in the liver of the non-immunized animals were also observed in mice of the PLX, AC and PLX+Δ*abcBA* groups (Figure 5). However, mice immunized with AC+Δ*abcBA* developed

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less severe lesions in the liver ($p < 0.05$) compared to the other groups (Figure 5).Therefore, vaccination with *B. ovis* Δ*abc BA* encapsulated in alginate and chitosan resulted in reduction of bacterial colonization in liver and spleen with less severe inflammatory lesions in liver after challenge with the wild type *B. ovis* strain .

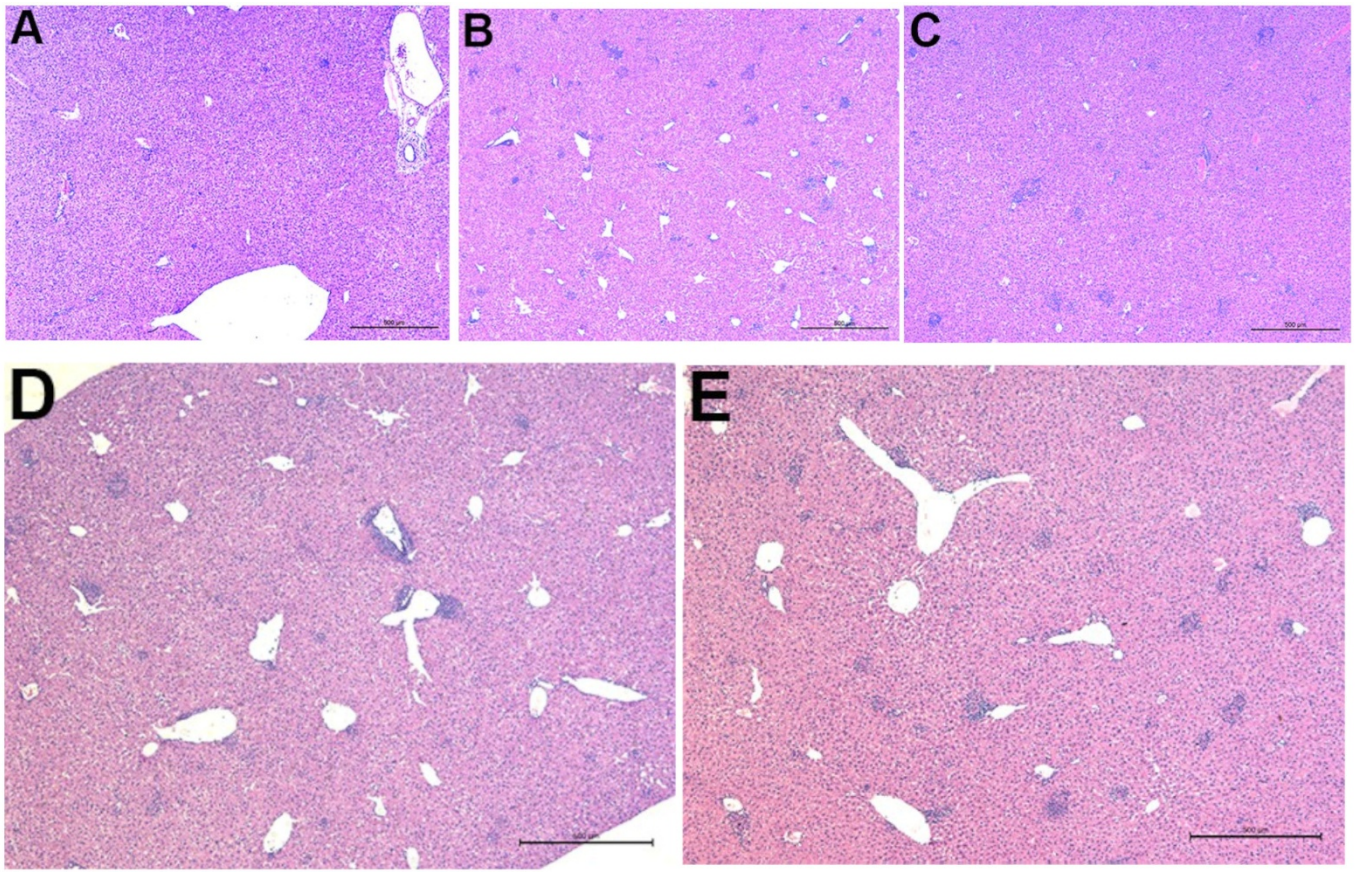

Figure 5a/e. Histopathological evaluation of the liver of female C57BL/6 mice (n = 6) immunized subcutaneously with Brucella ovis ΔabcBA encapsulated by alginate with chitosan (AC+ΔabcBA) represented by black circle (A), AC by empty circle (B), copolymer + *B. ovis* Δ*abcBA* (PLX+Δ*abcBA*) Black square (C), PLX by empty square (D) and PBS (non-immunized) empty triangle (E).

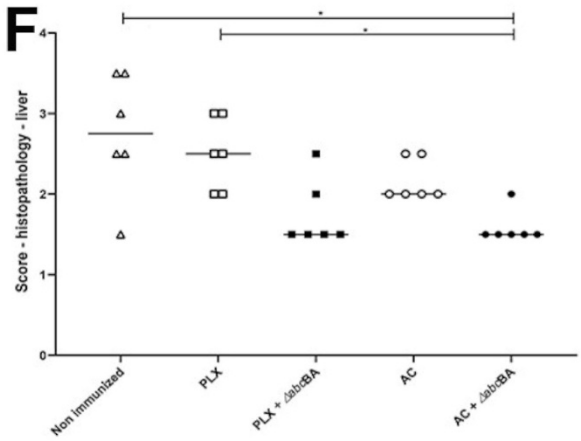

Figure 5f. Histopathological scores are expressed as individual animals (points) and the median (F). Results were analyzed using the Kruskal-Wallis non-parametric test. Statistical differences are represented by asterisks (* $p < 0.05$).

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Humoral immune response in mice Immunized with *Brucella ovis* Δ*abc BA* vaccine formulations

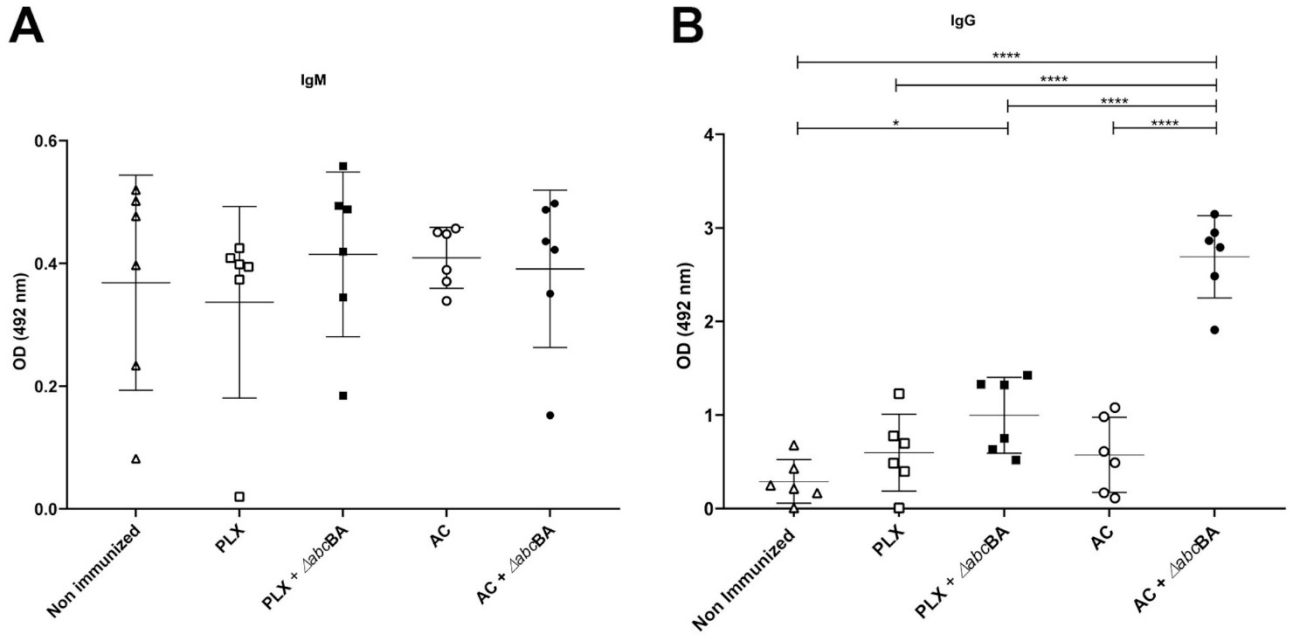
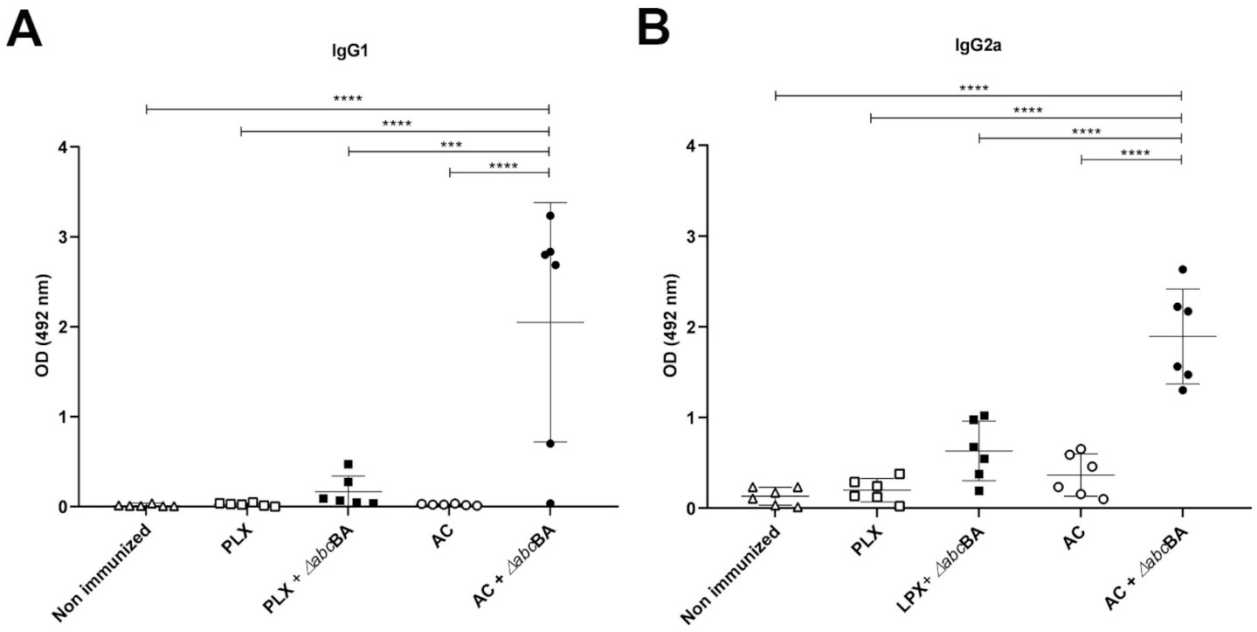


Figure 6. Determination of total IgM (A) and IgG (B) levels by ELISAi of serum from female C57BL/6 mice (n = 6) immunized subcutaneously with PBS (non-immunized), copolymer (PLX), PLX + B. ovis ΔabcBA (PLX+ΔabcBA), alginate and chitosan capsules (AC), *B. ovis* Δ*abcBA* encapsulated by alginate and chitosan (AC+Δ*abcBA*) and subsequently challenged with the wild strain of *B. ovis*. Data are expressed as individual animals (points) and mean with standard deviation. Results were analyzed for normality before being subjected to ANOVA, with the mean values compared by Tukey's test. Statistical differences are represented by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

When evaluating the humoral response of the different groups after challenge with *B. ovis* , we observed that IgM levels were similar between the groups, with no statistically significant differences (Figure 6A). However, the evaluation of IgG levels indicated that the mice immunized with AC+Δ*abcBA* had higher levels of total IgG when compared to the other groups. There was a small but significant increase in total IgG levels in mice immunized with CP+Δ*abcBA* compared to the non-immunized mice (Figure 6B).

Then were evaluating the IgG subclasses, a significant increase in the levels of IgG1, IgG2a and IgG2b subclasses was observed in the mice immunized with AC+Δ*abcBA* compared to the other groups (Figure 7).



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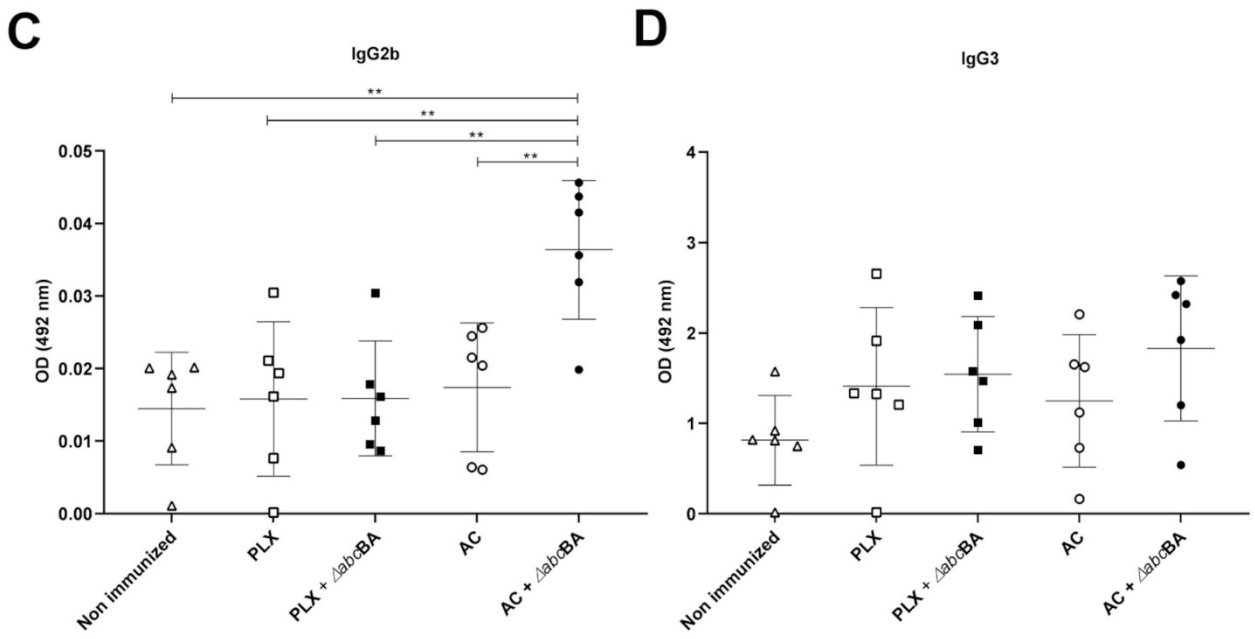

Figure 7. Determination of IgG1 (A), IgG2a (B), IgG2b (C) and IgG3 (D) levels by ELISAi of serum from female C57BL/6 mice (n = 6), immunized subcutaneously with PBS (Non-immunized), copolymer (PLX), PLX + B. ovis ΔabcBA (PLX+ΔabcBA), alginate and chitosan capsules (AC), and *B. ovis* Δ*abcBA* encapsulated by alginate and chitosan (AC+Δ*abcBA*) and subsequently challenged with the wild strain of *B. ovis* and subsequently challenged with the wild strain of *B. ovis*. Data are expressed as individual animals (points) and mean with standard deviation. Results were analyzed for normality before being subjected to ANOVA, with mean values compared by Tukey's test. Statistical differences are represented by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Discussion

This study evaluated two vaccine formulations with biopolymers: vaccine associated with a diblock copolymer PLX that solidifies at body temperature and vaccine encapsulated in alginate and chitosan microcapsules. We observed that C57BL/6 mice immunized subcutaneously with *B. ovis* Δ*abcBA* encapsulated by alginate and chitosan, showed reduced bacterial colonization in spleen and liver after challenge with wild-type *B. ovis* strain, and induced the production of protective antibodies. Vaccine development for controlling *Brucella* infections have driven a considerable research effort over the past several years seeking novel vaccine formulations that are safe for animals and humans, and effective for controlling infection and clinical manifestation (Carvalho *et al*. 2016, Carvalho *et al.* 2020). Polymers have been increasingly used as vaccine adjuvants because they exhibit stability, safety, and biocompatibility (Golshani *et al.* 2018, Sadeghi *et al*. 2019) and act as an antigen delivery system, protecting them from degradation, leading to slower release by increasing immunogen availability, and improving uptake by antigen-presenting cells, leading to a more organized and durable immune response (Rice-Ficht *et al.* 2010, Kashem *et al.* 2017, Abkar *et al.* 2019).

Thus, this study demonstrates that vaccination with *B. ovis* Δ*abc BA* strain with alginate and chitosan confers protection to *B. ovis* infection in the murine model. Our previous studies demonstrated that *B. ovis* Δ*abcBA* vaccine strain encapsulated only with alginate confers a similar protection (Silva *et al.* 2015b) that was observed in new formulation which it associates chitosan to alginate. Although protection index was not very high, it is very consistent in this mouse model when challenge with reference strain of *B. ovis*. (Silva *et al*. 2015b, Carvalho *et al.* 2020b). The infection with pathogenic field isolates in the murine model, demonstrate that the vaccine formulation maintains its protective ability by reducing bacterial recovery in spleen and liver of the challenged mice (Carvalho *et al.* 2020b). In addition, the vaccine formulation when was used in the preferred host showed to be safe and effective by inducing sterile immunity (Silva *et al.* 2015a).

Previously, Arenas-Gamboa *et al*. (2009) reported better performance of the live attenuated vaccine strain (*Brucella abortus* S19 Δ*vjbR*) when encapsulated by alginate associated to poly-L-lysine/vpB. This formulation demonstrated sustained antigen release resulting in pronounced Th1 cytokine profile in cultured splenocytes, even after eight months of vaccination. The capacity for greater protective induction associated with the use of this combination of adjuvants has also been demonstrated against infection by other pathogens. Vaccination with alginate /chitosan microcapsules containing inactive *Listeria monocytogenes* induced an efficient immune response characterized by a

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decrease in lesions associated with a substantial increase in the CD4+ and CD8+ T lymphocyte and the synthesis of high levels of IFN-γ (Rocha *et al.* 2021). Live vaccine encapsulated by alginate and chitosan administered orally showed protection against *Salmonella enterica* serovar Gallinarum infection in birds associated to higher levels of IFN-γ mRNA expression (Onuigbo *et al*. 2018). Fish orally immunized with bacteria coated with alginate and chitosan against *Streptococcus iniae* and *Lactococcus garvieae* infections demonstrating higher survival rate in immunized animals and elevated levels of IgM and IgG (Halimi *et al.* 2019).

The necessity for encapsulation requires equipment that rise vaccine production costs. The search for polymers that do not require special equipment and can have important adjunct features such as slow release is desirable (Klouda and Mikos 2008). Although PLX copolymer has the behavior of being solid within the body favoring slow antigen release, the formulation used in this study did not induce protection murine model challenged by *B ovis*. The use of PLX induced minimal inoculation site reaction but did not reduce bacterial colonization in spleen and liver, or the inflammation in liver, and did not even induce significant humoral immune response.

Immunization with *Brucella ovis* Δ*abc BA* coated alginate and chitosan capsules induces a strong humoral immune response with high levels of IgG and with higher representation of IgG1 and IgG2b subclasses. According to Abkar *et al*. (2019) *Brucella* spp. infection induces increased IgG production with predominance of IgG1 and IgG2 in mice. IGg2b synthesis favors the Th1-like response, playing an important role in anti-*Brucella* immunity in C57BL/6 mice (Fornefett *et al.* 2018). Th1-like response was linked to IFN production, a cytokine important in controlling intracellular pathogens like *Brucella* (Murphy *et al.* 2001). IFN favors IgG2a, IgG2b, and IgG3 production in mice *Brucella* infected (Fornefett *et al.* 2018).

Vaccine reactogenicity at the application site is tolerable in favor of inducing a protective immune response. However, some adjuvants may lead to undesirable exacerbated inflammatory reaction at the site of application as in the case of Freud's adjuvant (Windsor *et al.* 2005, Windsor and Eppleston 2006). It is important to continually search for new formulations that induce less reactogenicity (Stills Jr 2005, Powers *et al.* 2007) without impairing their immunostimulatory capacity. In this study mice that received AC + *B. ovis* Δ*abc BA*, exhibited a local inflammatory reaction in some cases with fistula formation. The mice showed mild discomfort, but it did not affect weight gain. Preparation of vaccines with these adjuvants combinations should be refined. It is important to emphasize that the reactogenicity in the target host may be different from that observed in mice.

In conclusion, *B. ovis* Δ*abc BA* encapsulated by alginate with chitosan, but not copolymer Poloxamer 407, resulted in reduction of bacterial recovery in challenged animals and induce protective humoral response showing vaccine potential in murine model.

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