Protection Provided by an Encapsulated Live Attenuated Δ*abcBA* Strain of *Brucella ovis* against Experimental Challenge in a Murine Model

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This study aimed to evaluate the *Brucella ovis* Δ*abcBA* strain as a vaccine candidate in the murine model. BALB/c mice were subcutaneously or intraperitoneally immunized with a single dose or three doses of the *B. ovis* Δ*abcBA* strain and then were challenged with wild-type *B. ovis*. Single or multiple immunizations provided only mild protection, with significantly smaller numbers of wild-type *B. ovis* CFU in the livers of immunized mice but not in the spleens. Encapsulation of *B. ovis* Δ*abcBA* significantly improved protection against experimental challenges in both BALB/c and C57BL/6 mice. Furthermore, immunization with encapsulated *B. ovis* Δ*abcBA* markedly prevented lesions in the spleens and livers of experimentally challenged mice. These results demonstrated that the encapsulated *B. ovis* Δ*abcBA* strain confers protection to mice; therefore, this strain has potential as a vaccine candidate for rams.

Brucella ovis is a facultative, intracellular, Gram-negative coccobacillus (1) that causes economic losses due to its potential to induce reproductive failure in sheep (2, 3). The most important lesions caused by *B. ovis* in rams are epididymitis and seminal vesiculitis (4), and *B. ovis* occasionally causes abortion in ewes (5, 6).

In spite of attempts to develop safe, effective vaccines (7–10), there is no available vaccine specifically against *B. ovis*. The ideal vaccine against *B. ovis* must prevent infection and clinical signs or at least reduce the risk of infection and transmission (11). Rev-1, a live attenuated *Brucella melitensis* strain, is the most commonly used vaccine against *B. melitensis*, and it also protects against *B. ovis* (12). However, the Rev-1 strain retains pathogenic potential, so it is capable of infecting humans. It may cause abortions in ruminants, it may interfere with serological tests, and it is resistant to streptomycin (13, 14).

Mice have been extensively used as a model for brucellosis, which is very important due to the limitations of experimentation with larger animals (15–18). The murine model has allowed studies for the development of safe, effective brucellosis vaccines (15, 19, 20).

Inactivation of a predicted *abcEDCBA*-encoded ATP-binding cassette (ABC) transporter of *B. ovis* (21), which is located in *B. ovis* pathogenicity island 1 (BOPI-1), results in strong attenuation of this pathogen in the murine model (22). Although the substrate of this ABC transporter is not known, it is required for expression of *B. ovis* virulence proteins, including Omp31, Sod, and the *virB*-encoded type IV secretion system (23), which is required for *B. ovis* intracellular survival and *in vivo* persistence (17). Furthermore, a *B. ovis* Δ*abcBA* strain is capable of triggering humoral and cellular immune responses like the wild-type strain in rams; importantly, however, the mutant strain is not excreted in semen and urine (24). Therefore, this mutant strain has great potential as an attenuated vaccine strain. However, considering that the Δ*abcBA* mutant strain is attenuated at very early stages during

infection, resulting in poor persistence *in vivo* (22), methods for prolonging release of the vaccine strain *in vivo* may be highly desirable in this context. Vaccine encapsulation has been used as a useful approach for improving immune responses. Capsules are progressively and slowly eroded, and they function as antigen deposits, extending antigen availability in the organisms (25). Alginate is a widely used compound for encapsulation. It is a natural nontoxic biopolymer that has biocompatibility with live microorganisms (26). The aim of this study was to evaluate the protective potential of an encapsulated live attenuated strain of *B. ovis* (Δ*abcBA* strain) against experimental challenge with wild-type *B. ovis* in mice.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Wild-type *B. ovis* strain ATCC 25840, *B. ovis* Δ*abcBA* (22), and a *B. ovis* Δ*abcBA* isogenic strain expressing *mCherry* fluorescence, with the insertion of pKSoriT-*bla-kan-PsojA-mCherry* (23), were used in this study. Bacteria were grown for 3 days at 37°C on tryptic soy agar (TSA) plates with 1% hemoglobin (Becton Dickinson), in a humidified incubator with 5% CO_2, and then were suspended in sterile phosphate-buffered saline (PBS) (pH 7.4). The mutant strain (*B. ovis* Δ*abcBA*) was grown on TSA with 1% hemoglobin and 100 μg/ml

FIG 1 Numbers of CFU of *Brucella ovis* recovered from livers (A) and spleens (B) of BALB/c mice that were not immunized or were immunized with the *B. ovis* Δ*abcBA* strain and were challenged with wild-type *B. ovis*. Vaccination reduced the challenge inoculum by 90% in the liver, compared to controls, when administered by the subcutaneous (SC) or intraperitoneal (IP) route but had no effect in the spleen, a potential site of persistent infection. Bars represent the mean and standard deviation of results for mice ($n = 5$) that were neither immunized nor challenged (NC), mice inoculated with PBS by the subcutaneous or intraperitoneal routes, and mice immunized with *B. ovis* Δ*abcBA* intraperitoneally or subcutaneously. Raw data were logarithmically transformed prior to ANOVA, and means were compared by Tukey's test. Statistical differences between groups are indicated. *, $P < 0.05$; ***, $P < 0.001$.
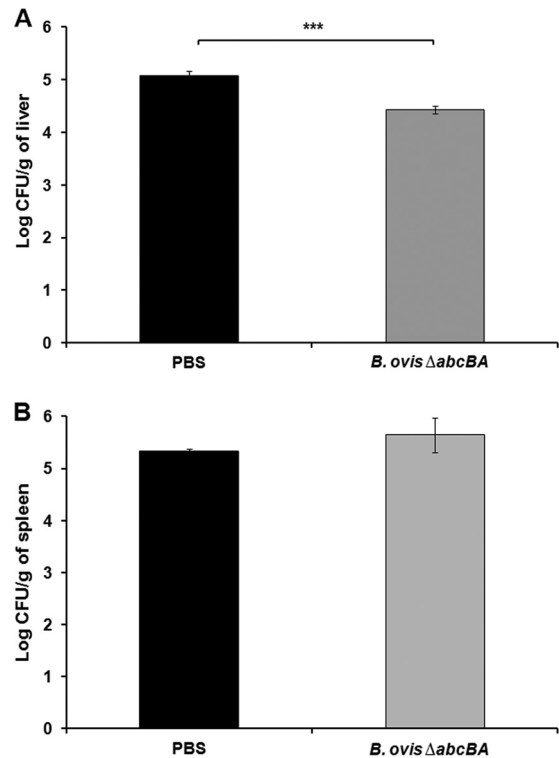

FIG 2 Numbers of CFU of *Brucella ovis* recovered from livers (A) and spleens (B) of BALB/c mice that were not immunized or were immunized three times with *B. ovis* Δ*abcBA* and were challenged with wild-type *B. ovis*. Bars represent the mean and standard deviation of results for 10 mice that were not immunized and 10 mice that were immunized three times. Raw data were logarithmically transformed prior to ANOVA, and means were compared by Tukey's test. Statistical differences between groups are indicated. ***, $P < 0.001$.

kanamycin (Gibco, Invitrogen, Brazil). Inoculum concentrations were estimated using a SmartSpec spectrophotometer (Bio-Rad, Hercules, CA) measuring the optical density at 600 nm (OD$_{600}$). For bacterial cultures, mouse tissue homogenates were plated on TSA with 1% hemoglobin for CFU counting after 5 to 7 days in culture at 37°C in 5% CO$_2$. For quantification of CFU of the mutant strain in tissues, 100 μg/ml of kanamycin (Gibco, Invitrogen, Brazil) was added to TSA with 1% hemoglobin.

B. ovis Δ*abcBA* encapsulation. Encapsulation of the *B. ovis* Δ*abcBA* strain was performed as follows: 100 μl of a suspension containing 1 × 10^{11} CFU of *B. ovis* Δ*abcBA* was added to 2 ml of 1% sodium alginate (Sigma-Aldrich), and 0.2 ml of this mixture was placed in a 1-ml syringe and dripped with a needle (0.23 mm by 4 mm) into 10 ml of a 5% CaCl$_2$ solution. After the solution was dripped, capsules were formed and then homogenized for 15 min. Capsules were washed twice in MOPS (morpholinepropanesulfonic acid) buffer (Sigma-Aldrich), followed by 0.5% poly-L-lysine solution (Sigma-Aldrich), for 15 min under agitation and then were washed in MOPS buffer. Particles were added to a 0.03% alginate solution over 5 min and then were suspended in MOPS buffer. Particles were inoculated subcutaneously at a final dose of 10^8 CFU per mouse. Particle sizes were assessed by light microscopy, and the effectiveness of bacterial encapsulation and density were assessed by encapsulation of *mCherry*-expressing *B. ovis* Δ*abcBA* and fluorescence microscopy (Leica DM4000 B).

Vaccination of mice. Animal experiments were approved by the Institutional Ethics Committee on Animal Experimentation of the Universidade Federal de Minas Gerais (CETEA/UFMG protocols 204/2012 and 395/2013). This study included three independent experiments, which are detailed below.

Subcutaneous and intraperitoneal routes of immunization were compared. Six- to 7-week-old male BALB/c mice ($n = 35$) were used. Ten mice were immunized by the intraperitoneal route and 10 mice were immunized by the subcutaneous route with 100 μl of a suspension containing 10^8 CFU of the *B. ovis* Δ*abcBA* strain in PBS. Ten mice were inoculated with sterile PBS (five by the subcutaneous route and five by the intraperitoneal route). Additionally, five mice were not inoculated or challenged. Six weeks after immunization, the mice ($n = 30$) were challenged with 10^6 CFU of wild-type *B. ovis* (ATCC 25840) by the intraperitoneal route. Two weeks later, the mice were euthanized with an overdose of 2% xylazine hydrochloride (0.1 mg/kg) and 1% ketamine hydrochloride (35 mg/kg) by the intraperitoneal route, followed by cervical dislocation.

In order to evaluate repeated subcutaneous vaccinations, ten 6- to 7-week-old male BALB/c mice were immunized three times, by the subcutaneous route, with 100 μl of a suspension containing 10^8 CFU of *B. ovis* Δ*abcBA* in PBS, with intervals of 1 week between the immunizations. Another ten 6- to 7-week-old male BALB/c mice were inoculated three times with 100 μl of PBS and were used as controls. Six weeks after the last immunization, all 20 mice were challenged with 10^6 CFU of a wild-type *B. ovis* (ATCC 25840) suspension in PBS by the intraperitoneal route. Two weeks later, the mice were euthanized as described above.

The effects of attenuated vaccine strain encapsulation were also investigated. Six- to 7-week-old BALB/c ($n = 20$) and C57BL/6 ($n = 20$) mice
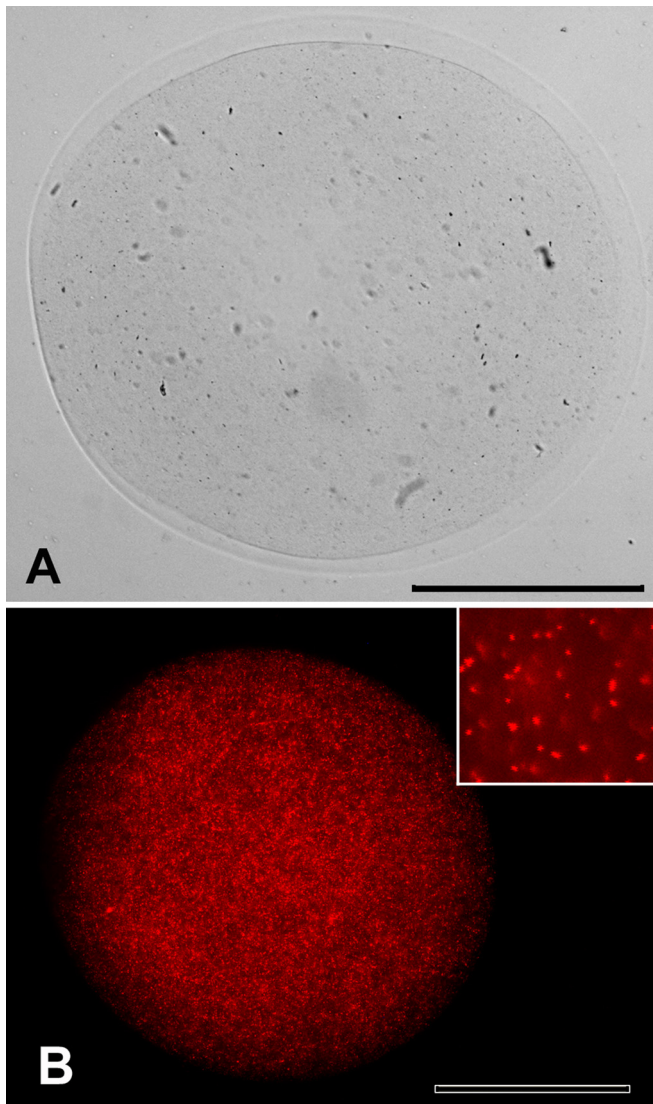
FIG 3 Light microscopic image of empty alginate capsules (A) and fluorescence microscopic images of alginate capsules containing *mCherry*-expressing *Brucella ovis* Δ*abcBA* (B). Inset, individual *mCherry*-expressing *Brucella ovis* Δ*abcBA*. Bars = 100 μm.

were divided into four groups for each mouse strain, namely, a control group (subcutaneous inoculation of sterile PBS), a group with subcutaneous immunization with empty alginate capsules, a group with subcutaneous immunization with the *B. ovis* Δ*abcBA* strain (10^8 CFU per mouse), and a group with subcutaneous immunization with the *B. ovis* Δ*abcBA* strain encapsulated with alginate (10^8 CFU per mouse). After 8 weeks, all mice were challenged with wild-type *B. ovis* (10^6 CFU per mouse); 2 weeks later, mice were euthanized as described above.

***In vivo* persistence of encapsulated *B. ovis* Δ*abcBA*.** To evaluate the persistence of alginate-encapsulated *B. ovis* Δ*abcBA*, three groups of male BALB/c mice ($n = 6$) were infected with 10^6 CFU per mouse of wild-type *B. ovis*, *B. ovis* Δ*abcBA*, or encapsulated *B. ovis* Δ*abcBA* by peritoneal injection. At 24 h postinfection, mice were euthanized and peritoneal macrophages were harvested using intraperitoneal injection of 10 ml of cold PBS, which was immediately aspirated from the peritoneal cavity and transferred to a 15-ml tube on ice. The aspirate was centrifuged at $400 \times g$ for 10 min at 4°C. The supernatant was discarded, and macrophages were suspended in a depolymerization solution (50 mM sodium citrate,

0.45% sodium chloride, and 10 mM MOPS) in order to disrupt capsules. After another centrifugation step and washing of the macrophages with PBS, 500 μl of sterile water was added, followed by vigorous vortex-mixing, serial 10-fold dilution in PBS, and plating for CFU counting. To evaluate systemic wild-type *B. ovis*, *B. ovis* Δ*abcBA*, and encapsulated *B. ovis* Δ*abcBA* infections in mice, spleens were collected for bacterial isolation at 24 h postinfection and results were compared among these groups.

Bacterial isolation. Fragments of spleen and liver were weighed and homogenized in tubes with 2 ml of sterile PBS, using a mixer (Ultra Stirrer; Biotech, Brazil). Each sample was prepared in serial 10-fold dilutions and plated for CFU counting in TSA with 1% hemoglobin. Plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 and were evaluated for 7 days. Protection index values were calculated by considering the difference in log CFU numbers in the spleens of nonvaccinated versus vaccinated mice at 2 weeks after the experimental challenge (i.e., log CFU unvaccinated − log CFU vaccinated).

Histopathological assessments. Fragments of spleen, liver, and subcutaneous tissue from the vaccination site were fixed by immersion in 10% buffered formalin for 24 h. After fixation, tissues were processed for paraffin embedding. Four-micrometer sections were stained with hematoxylin and eosin. Occurrences of microgranulomas, necrosis, and thrombosis in the liver or microgranulomas in the spleen were scored as follows: 0, absent; 1, mild; 2, moderate; 3, severe. Therefore, the scores of lesions for the liver ranged from 0 to 9 (combined scores for the three distinct lesions), whereas those for the spleen ranged from 0 to 3 (referring to the intensity of inflammation). Livers and spleens from mice that were neither immunized nor challenged were used as controls.

Statistical analyses. CFU data were logarithmically transformed and then subjected to analysis of variance (ANOVA). Means were compared by Tukey's test. Histopathological scores were compared using the Kruskal-Wallis nonparametric test (GraphPad Prism version 5.0).

RESULTS

Immunization with *B. ovis* Δ*abcBA* induced mild protection against experimental challenge. In a preliminary evaluation, the subcutaneous and intraperitoneal routes of vaccination were compared and bacterial loads recovered from spleens and livers were measured 2 weeks after challenge with wild-type *B. ovis*. Bacterial loads in the livers were significantly lower in BALB/c mice immunized with *B. ovis* Δ*abcBA* by the subcutaneous ($P < 0.001$) or intraperitoneal ($P < 0.05$) route than in nonimmunized mice (Fig. 1A). Surprisingly, there were no significant differences in CFU numbers recovered from spleens from mice immunized by the subcutaneous or intraperitoneal routes, compared to nonimmunized mice (Fig. 1B). These results also indicated that the subcutaneous and intraperitoneal routes provided similar protection in mice.

Repeated subcutaneous vaccination with *Brucella ovis* Δ*abcBA* induced mild protection against experimental challenge. Because a single immunization resulted in only mild protection, a prime immunization by the subcutaneous route followed by two buster immunizations at 1-week intervals was evaluated. Repeated immunizations induced only mild protection (Fig. 2), which was similar to the findings for a single immunization (Fig. 1). Indeed, there were significant reductions in bacterial loads in the livers after immunization ($P < 0.001$), compared to nonimmunized mice (Fig. 2A). However, there was no statistical difference in bacterial loads recovered from the spleens of immunized mice versus nonimmunized mice (Fig. 2B).

Encapsulation enhanced protection induced by *Brucella ovis* Δ*abcBA* against experimental challenge in mice. Alginate capsules were evaluated by light microscopy, whereas *B. ovis* Δ*abcBA* expressing *mCherry* was encapsulated with alginate and evaluated by
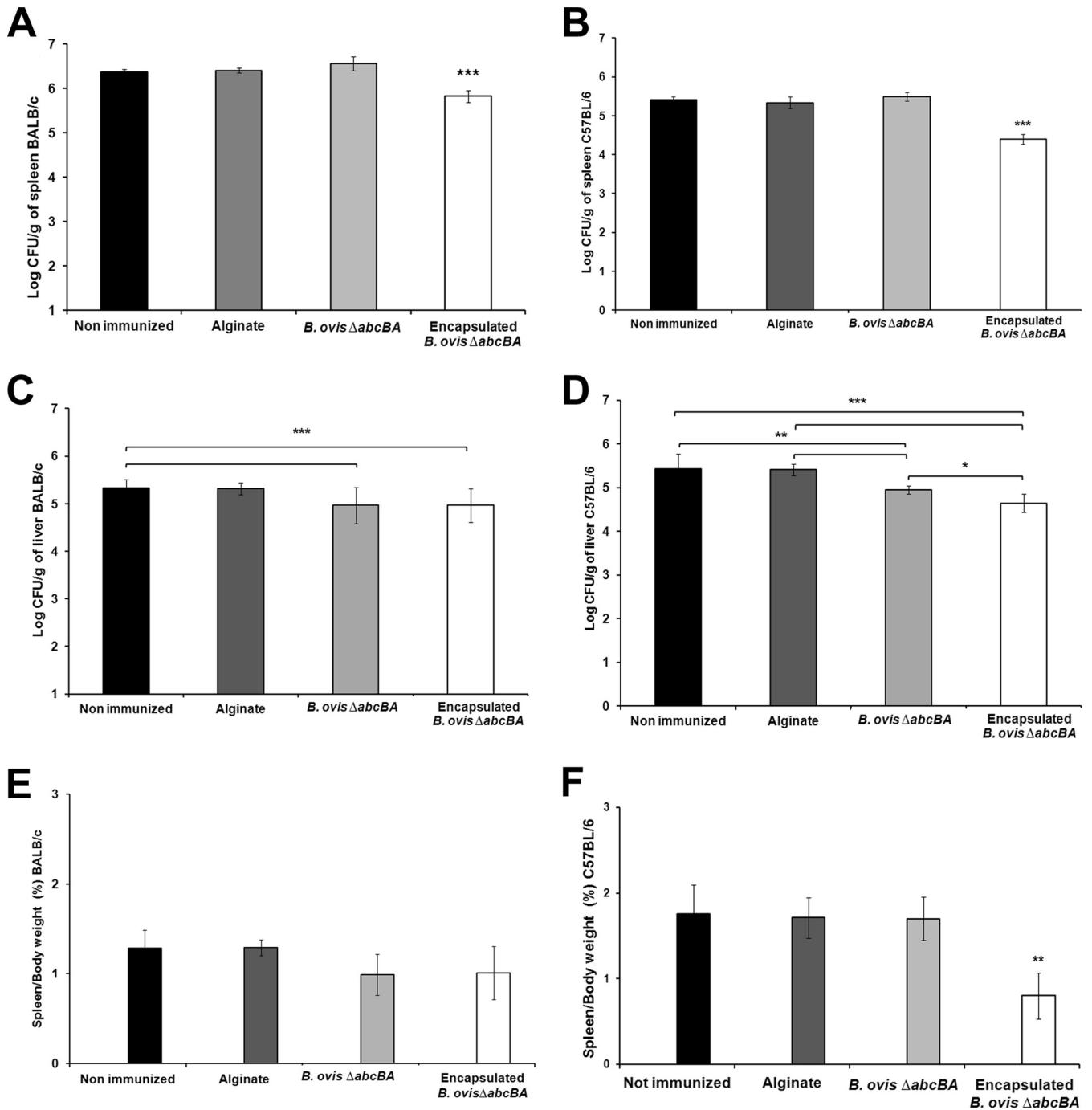
FIG 4 (A to D) Numbers of CFU of *Brucella ovis* recovered from spleens of BALB/c (A) and C57BL/6 (B) mice and livers of BALB/c (C) and C57BL/6 (D) mice subcutaneously inoculated with sterile PBS or sterile alginate capsules or immunized with *B. ovis* Δ*abcBA* or encapsulated *B. ovis* Δ*abcBA* and then challenged with wild-type *B. ovis*. (E and F) Spleen/body weight ratios of BALB/c (E) and C57BL/6 (F) mice. Bars represent the mean and standard deviation of results for 5 mice per group. Raw data were logarithmically transformed prior to ANOVA, and means were compared by Tukey's test. Statistical differences between groups are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

fluorescence microscopy. Empty capsules and capsules containing *B. ovis* had similar sizes and shapes. These capsules were spherical and ranged from 300 to 800 μm in diameter (Fig. 3A). Abundant red fluorescent *mCherry*-expressing *B. ovis* Δ*abcBA* was observed inside the alginate capsules, confirming that *B. ovis* was efficiently encapsulated (Fig. 3B).

Considering the strong attenuation and poor persistence of the *B. ovis* Δ*abcBA* strain in mice (22), this candidate vaccine strain was encapsulated in alginate microcapsules, to overcome the lack of persistence. BALB/c and C57BL/6 mice were subcutaneously immunized with *B. ovis* Δ*abcBA* encapsulated in sterile alginate, thus providing a system for gradual release and uptake of antigens by the host.

TABLE 1 Protection induced by *Brucella ovis* Δ*abcBA* encapsulated with alginate in BALB/c and C57BL/6 mice experimentally challenged with wild-type *B. ovis*[a]

Mouse strain	Vaccine strain	Route/dose (CFU)	No. of immunizations	Challenge	Protection index[b]
BALB/c	Encapsulated *B. ovis* Δ*abcBA*	s.c./1×10^8	1	*B. ovis* (i.p./1×10^6 CFU)	0.54
C57BL/6	Encapsulated *B. ovis* Δ*abcBA*	s.c./1×10^8	1	*B. ovis* (i.p./1×10^6 CFU)	1.01

[a] s.c., subcutaneous; i.p., intraperitoneal.
[b] Protection index was calculated considering the difference in CFU numbers (log) in the spleens of nonvaccinated and vaccinated mice at 2 weeks after experimental challenge, i.e., log CFU unvaccinated − log CFU vaccinated.

Bacterial loads were significantly reduced ($P < 0.001$) in spleens from both BALB/c and C57BL/6 mice immunized with encapsulated *B. ovis* Δ*abcBA*, compared to mice immunized with nonencapsulated *B. ovis* Δ*abcBA* or nonimmunized controls (Fig. 4A and B). Bacterial loads in the livers were significantly reduced in BALB/c and C57BL/6 mice immunized with encapsulated or nonencapsulated *B. ovis* Δ*abcBA* ($P < 0.001$), compared to nonimmunized controls (Fig. 4C and D). Furthermore, spleen weights were significantly lower ($P < 0.001$) in C57BL/6 mice immunized with encapsulated *B. ovis* Δ*abcBA* than in nonimmunized mice or mice immunized with nonencapsulated *B. ovis* Δ*abcBA* (Fig. 4E and F). Protection index values for encapsulated *B. ovis* Δ*abcBA* in experimentally challenged BALB/c and C57BL/6 mice are described in Table 1.
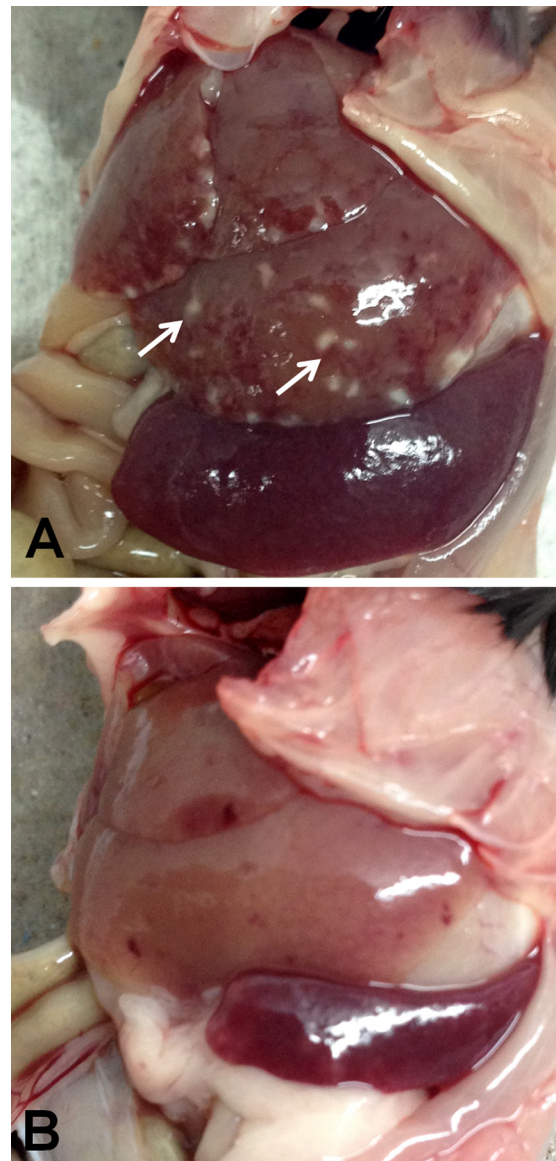
Immunization with encapsulated *Brucella ovis* Δ*abcBA* prevents lesions in spleens and livers of experimentally challenged mice. Spleens of C57BL/6 mice immunized with encapsulated *B. ovis* Δ*abcBA* did not develop splenomegaly, compared to nonimmunized mice (Fig. 5). In the livers of nonimmunized BALB/c (data not shown) and C57BL/6 mice, there were firm white nodular lesions approximately 0.3 cm in diameter (Fig. 5A), which histologically corresponded to microgranulomas. Interestingly, BALB/c (data not shown) and C57BL/6 mice immunized with encapsulated *B. ovis* Δ*abcBA* did not develop lesions in their spleens (Fig. 5B).

In the livers, there was a multifocal, randomly distributed, inflammatory infiltrate composed of neutrophils, lymphocytes, and macrophages, characterizing microgranulomas associated with moderate multifocal necrosis and thrombosis. A moderate amount of fibrin and numerous neutrophils in the intima and media of portal blood vessels, characterizing vasculitis, were observed in nonimmunized BALB/c (data not shown) and C57BL/6 (Fig. 6A) mice. In BALB/c (data not shown) and C57BL/6 mice immunized with encapsulated *B. ovis* Δ*abcBA*, there were only few microgranulomas in the livers (Fig. 6B).

There was a moderate, multifocal, inflammatory infiltrate composed of epithelioid macrophages and neutrophils in the marginal zone and red pulp of nonimmunized mice, characterizing moderate, multifocal, pyogranulomatous splenitis (Fig. 6C). In contrast, both mouse strains immunized with encapsulated *B. ovis* Δ*abcBA* had just rare neutrophils in the red pulp (Fig. 6D).

Scores for hepatic and splenic lesions in BALB/c mice immunized with encapsulated *B. ovis* Δ*abcBA* were significantly lower than scores for nonimmunized mice ($P < 0.01$) (Fig. 7A and B). Similar results were obtained for C57BL/6 mice ($P < 0.05$) (Fig. 7C and D).

Encapsulated *Brucella ovis* Δ*abcBA* induced granuloma formation in subcutaneous tissue at inoculation site. Grossly, mice immunized with encapsulated *B. ovis* Δ*abcBA* developed swelling at the inoculation site (approximately 3 mm in diameter), which

FIG 5 Representative macroscopic lesions in the livers and spleens of C57BL/6 mice not immunized (A) or immunized with encapsulated *Brucella ovis* Δ*abcBA* (B) and then challenged with wild-type *B. ovis* (both images are from challenged mice), showing splenomegaly and numerous hepatic granulomas in nonimmunized C57BL/6 mice (white arrows) and neither hepatic granulomas nor splenomegaly in C57BL/6 mice immunized with encapsulated *B. ovis* Δ*abcBA*.
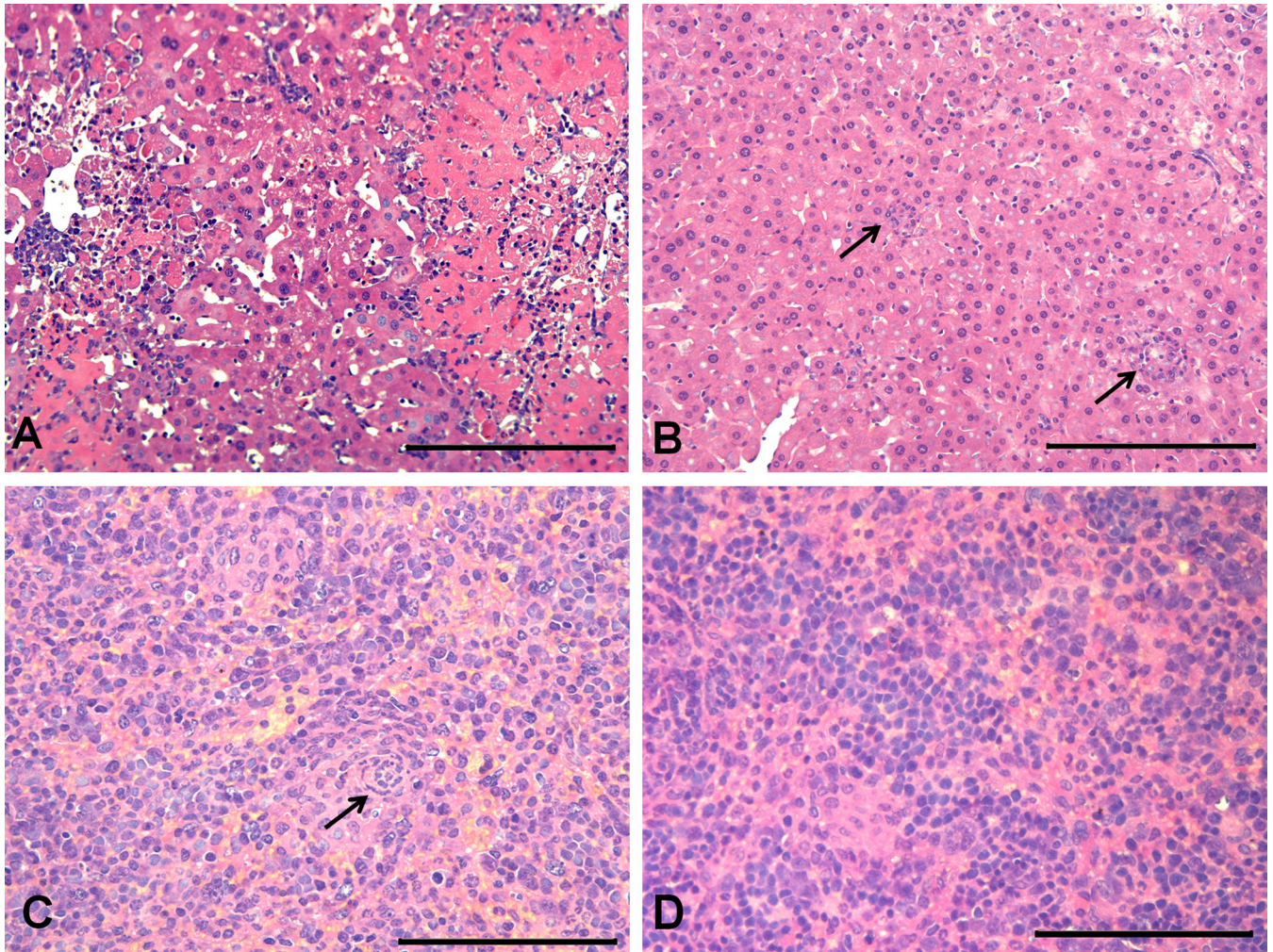
FIG 6 Representative microscopic changes in the livers and spleens of C57BL/6 mice not immunized or immunized with encapsulated *Brucella ovis* Δ*abcBA* and challenged with wild-type *B. ovis* (all micrographs are from challenged mice). (A and B) Numerous microgranulomas associated with necrosis in the liver of a nonimmunized C57BL/6 mouse (A), compared to only scarce microgranuloma (black arrows) in the liver of a C57BL/6 mouse immunized with encapsulated *B. ovis* Δ*abcBA* (B). Hematoxylin and eosin staining. Bars = 100 μm. (C and D) Microgranulomas (black arrow) in the spleen of a nonimmunized C57BL/6 mouse (C) and rare neutrophils in the spleen of a C57BL/6 mouse immunized with encapsulated *B. ovis* Δ*abcBA* (D). Hematoxylin and eosin staining. Bars = 50 μm.

histologically corresponded to an inflammatory infiltrate composed of numerous epithelioid macrophages, lymphocytes, and multinucleated giant cells, surrounded by fibroblasts and fibrocytes (Fig. 8A). Interestingly, mice inoculated with nonencapsulated *B. ovis* Δ*abcBA* (not shown) or empty sterile alginate capsules did not develop any lesions (Fig. 8B).

Alginate capsules enhanced persistence of *Brucella ovis* Δ*abcBA* in peritoneal cavities and spleens of BALB/c mice. Enhanced protection by encapsulated *B. ovis* Δ*abcBA* (Fig. 4) is hypothetically due to slow release of the vaccine strain, which overcomes its strong attenuation. In order to compare the persistence of *B. ovis* Δ*abcBA* with that of encapsulated *B. ovis* Δ*abcBA*, the total numbers of CFU recovered from intraperitoneal washes and spleens of infected BALB/c mice were evaluated at 24 h postinfection. The total numbers of CFU isolated from the intraperitoneal cavities (Fig. 9A) or the spleens (Fig. 9B) of encapsulated *B. ovis* Δ*abcBA*-infected mice were significantly higher ($P < 0.05$ and $P < 0.01$, respectively) than those from mice inoculated with nonencapsulated *B. ovis* Δ*abcBA*.

DISCUSSION

Currently there is no specific *B. ovis* vaccine available, which, considering the significance of *B. ovis* infection for the sheep industry, makes the development of such a vaccine a relevant scientific and technological challenge. In this study, we employed an encapsulation technology to overcome the poor performance of a candidate vaccine strain that has limited persistence in mice (22). Our results demonstrated that the subcutaneous route of immunization had a performance that was similar to that of the intraperitoneal route. The subcutaneous route is the most frequently used for human and animal vaccinations, although the intraperitoneal route has been used in experimental studies involving *B. ovis* (8), *Brucella abortus* (27), and *B. melitensis* (28) mutants as vaccine candidates in mice. Therefore, both immunization routes were evaluated in this study.

Live attenuated vaccines tend to provide the best results against *Brucella* infections (29, 30), which prompted us to develop a live attenuated vaccine against *B. ovis*. Considering the limited protec-
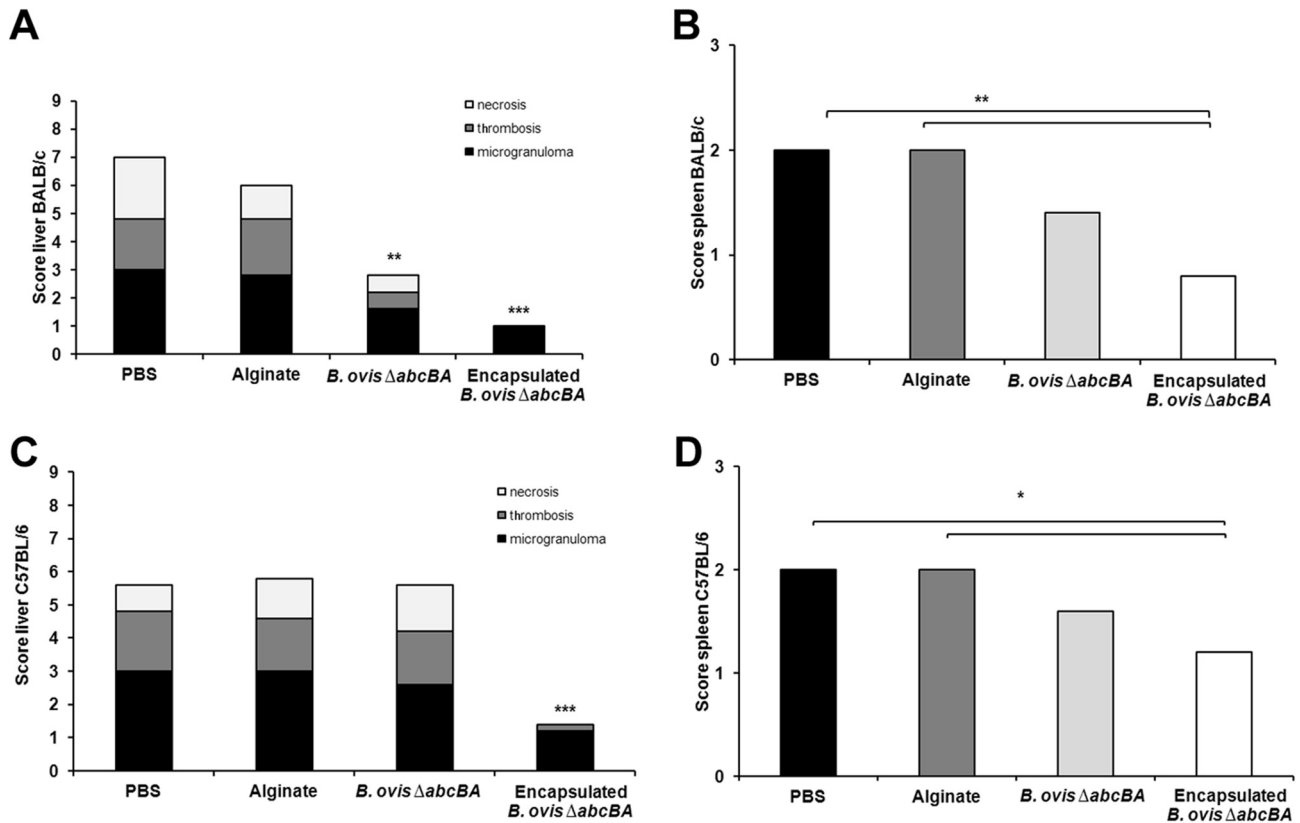
FIG 7 Scores for lesions in the livers of BALB/c (A) and C57BL/6 (C) mice and the spleens of BALB/c (B) and C57BL/6 (D) mice that were not immunized (inoculated with PBS or sterile alginate, as indicated) or were immunized with nonencapsulated or encapsulated *Brucella ovis* Δ*abcBA* and were challenged with wild-type *B. ovis*. Means were compared with the Kruskal-Wallis nonparametric test. Significant differences between nonimmunized controls (PBS and alginate) and immunized groups are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

tion observed in the first experiment in this study, BALB/c mice were immunized three times by the subcutaneous route, with intervals of 1 week between immunizations, but the results were strikingly similar to those from the first experiment. This limited protection by *B. ovis* Δ*abcBA* could be attributed to its low level of

persistence in mice (22). Therefore, an encapsulated formulation was used for immunization, which clearly improved the protective potential of *B. ovis* Δ*abcBA*. Experimental findings support the hypothesis that bacterial encapsulation modifies antigen uptake and processing (31, 32). For instance, a live attenuated *B. abortus*
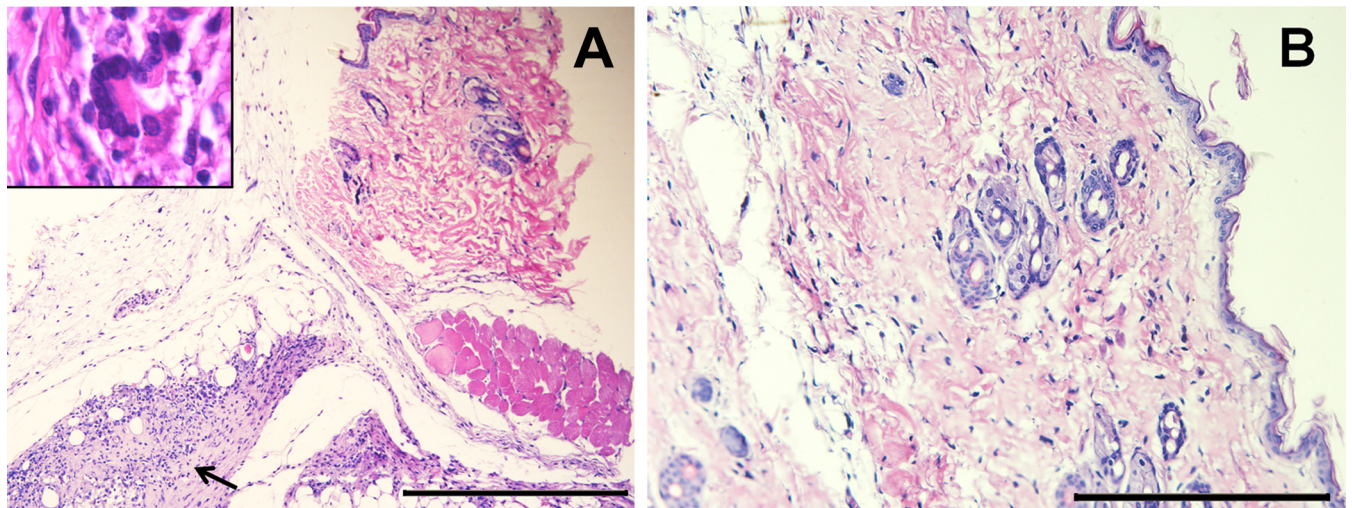

FIG 8 Microscopic changes at the immunization sites of mice, showing a granuloma in the hypodermis of a mouse immunized with encapsulated *Brucella ovis* Δ*abcBA* (black arrow) (A) and no histopathological changes in the skin of a mouse inoculated with empty alginate capsules only (B). Inset, multinucleated giant cell associated with epithelioid macrophages. Hematoxylin and eosin staining. Bars = 200 μm.
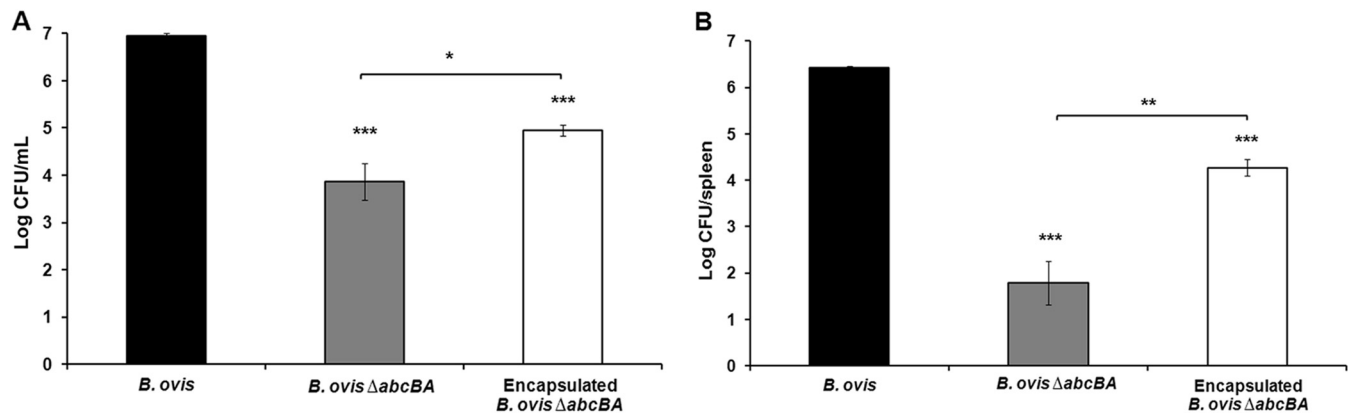
FIG 9 Numbers of CFU of wild-type *B. ovis*, *B. ovis* Δ*abcBA*, and encapsulated *B. ovis* Δ*abcBA* recovered from intraperitoneal macrophages (A) and spleens (B) of male BALB/c mice at 24 h postinfection. Raw data were logarithmically transformed prior to ANOVA, and means were compared by Tukey's test. Significant differences in CFU numbers between *B. ovis* Δ*abcBA* and encapsulated *B. ovis* Δ*abcBA* and between wild-type *B. ovis* and the other groups are indicated. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

S19 *vjbR* mutant encapsulated with alginate conferred greater protection than nonencapsulated *B. abortus* S19 in mice (33). Similarly, a live attenuated *B. melitensis vjbR* mutant strain provided enhanced protection against a *B. melitensis* challenge in mice (34). These previous studies clearly indicated that prolongation of antigen persistence in the host is beneficial for the development of anti-*Brucella* immunity (35). Indeed, our results clearly demonstrated that encapsulated *B. ovis* Δ*abcBA* persisted longer than the nonencapsulated strain in the peritoneal cavity and spleen. Therefore, the third experiment in this study evaluated the effects of *B. ovis* Δ*abcBA* encapsulation with alginate, which resulted in improved protection against an experimental challenge in the mouse model. To the best of our knowledge, this is the first time that a live attenuated *B. ovis* mutant strain encapsulated with alginate has been tested as a vaccine candidate.

Bacterial loads and lesions in spleens and livers from BALB/c and C57BL/6 mice immunized with encapsulated *B. ovis* Δ*abcBA* were significantly reduced, demonstrating that encapsulation improved immunogenic efficacy against *B. ovis* infections in mice under the conditions of this study. These results are in good agreement with previous reports demonstrating improved protective efficacy of alginate-encapsulated *B. abortus* S19 Δ*vjbR* in BALB/c mice (33) and alginate-encapsulated live RB51 in red deer (36).

Although the *B. melitensis* Rev-1 vaccine strain provides some protection against *B. ovis* infections in sheep (12), this vaccine cannot be used in areas free of *B. melitensis*, since Rev-1 retains pathogenic potential for animals and humans (14). Therefore, a specific live attenuated vaccine is highly desirable for controlling *B. ovis* infections, particularly because a vaccine strain developed on a *B. ovis* background would not pose any occupational risk for personnel involved in animal vaccination and handling, since, in contrast to *B. melitensis*, which has the greatest pathogenic potential for humans, *B. ovis* has no zoonotic potential (3). This study indicates that *B. ovis* Δ*abcBA* has potential as a vaccine strain. Although the mouse model has been extensively used to validate live *Brucella* vaccine-induced protection (37), results cannot be directly extrapolated to other host species, since even different mouse strains influence the protection results (38). Indeed, we have recently demonstrated that the *B. ovis* Δ*abcBA* strain induces humoral and cellular immune responses in rams that are indistin-

guishable from those triggered by the parental wild-type strain (24). Thus, the results of the present study encouraged us to pursue additional studies to demonstrate the potential of *B. ovis* Δ*abcBA* as a vaccine candidate in rams, which are the preferential hosts for this pathogen. Such experiments are under way, with highly encouraging unpublished preliminary results.

Immunization with encapsulated *B. ovis* Δ*abcBA* is capable of inducing protection, leading to reductions in bacterial loads and lesions in the spleens and livers of experimentally challenged BALB/c and C57BL/6 mice. These results support the notion that this strain has potential as a vaccine candidate for rams.

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