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Inactivation of formyltransferase (wbkC) gene generates a *Brucella abortus* rough strain that is attenuated in macrophages and in mice

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ABSTRACT

Rough mutants of *Brucella abortus* were generated by disruption of *wbkC* gene which encodes the formyltransferase enzyme involved in LPS biosynthesis. In bone marrow-derived macrophages the *B. abortus* Δ*wbkC* mutants were attenuated, could not reach a replicative niche and induced higher levels of IL-12 and TNF-α when compared to parental smooth strains. Additionally, mutants exhibited attenuation *in vivo* in C57BL/6 and interferon regulatory factor-1 knockout mice. A *wbkC* mutant strains induced lower protective immunity in C56BL/6 than smooth vaccine S19 but similar to rough vaccine RB51. Finally, we demonstrated that *Brucella* wbkC is critical for LPS biosynthesis and full bacterial virulence.

1. Introduction

Brucellosis is a zoonotic disease caused by *Brucella* spp., a Gram-negative facultative intracellular cocobacilli that affect many species of animals and occasionally humans, resulting in heavy economic losses and human suffering [1]. *Brucella* survives and replicates inside both phagocytic and non-phagocytic host cells by entering in these cells through lipid-rafts [2]. Afterwards, bacteria are found within a compartment termed the *Brucella*-containing vacuole (BCV), which transiently interacts with early endosomes, escapes lysosome fusion and further fuses with membrane of the endoplasmic reticulum (ER), establishing a replicative organelle [3]. This intracellular process is dependent upon the *Brucella* type IV secretion system *virB* [4].

The basis for *B. abortus* strength as an inducer of acquired cellular resistance is likely attributable to its ability to interact with TLRs, stimulating IL-12 production which in turn stimulates NK and T cells to secrete IFN-γ [5]. It is also known that different LPS molecules interact differently with TLRs and stimulation of TLR by *B. abortus* LPS induces low TNF-α and IL-12 production [6]. Entry and replication events are also dependent on its LPS [2]. LPS is a major component of the outer membrane of Gram-negative bacteria, one of *Brucella* main virulence factors, and it has been a target for attenuating strains for vaccine development. LPS is composed of three distinct structural regions: lipid A (responsible for endotoxic properties), core oligosaccharide and distal O-antigen. However, LPS is synthesized as two separated components, lipid A/core, and the O antigen synthesized on a lipid carrier by enzymes encoded by *wb** gene cluster [7]. According to their colony morphology, *Brucella* strains differ into smooth, rough or intermediated/mucoid types [8]. In general, smooth *Brucella* strains have been reported as more virulent than the rough strains. Currently, there are two main strains in use as live attenuated *B. abortus* vaccines, *B. abortus* S19 (smooth) and *B. abortus* RB51 (rough).

B. abortus S19 is the most commonly used attenuated vaccine for the prevention of bovine brucellosis and is widely used in eradication campaigns worldwide. However, *B. abortus* S19 is virulent for humans and can induce abortion when administered in

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pregnant cattle [9]. Moreover, its smooth LPS hinder the discrimination between infected and vaccinated animals during immunoscreening procedures [10]. On the other hand, *B. abortus* RB51 is a rough strain that arose spontaneously after multiple passages of the virulent strain *B. abortus* S2308 in selective medium. In this strain, there was an interruption of the *wboA* gene due the insertion of an IS*711* element and mutation in more than one of the genes necessary for the expression of a smooth phenotype [8,11]. *B. abortus* RB51 is currently employed as vaccine for cattle brucellosis in the United States and other countries. It is avirulent in mice and cattle, retains the capacity to induce partial protection and cellular immunity, and does not interfere with diagnosis [12]. However, *B. abortus* RB51 has the limitation to be rifampicin resistant, the antibiotic of choice for brucellosis treatment in pregnant women, children, and brucellosis endocarditis cases [13]. And depending upon dosage and route of delivery, its effectiveness for prevention of abortion is variable [14]. Therefore, all efforts have been focused on searching better live rough attenuated vaccine that could be capable of inducing efficient cellular immunity and protection [8,15].

Early study has shown that in *B. melitensis* a *wbkC* homologous gene was predicted to be absolutely required for the O-side-chain production [7]. WbkC acts in the LPS biosynthesis pathway catalyzing the GDP-4-NH_2-4,6 dideoximanose conversion in GDP-4-formamido-4,6 dideoximanose, the monomeric unit of antigen-O presents in *Brucella* LPS [7]. Herein, we have disrupted the *wbkC* gene using gene replacement by double-recombination strategy in *B. abortus* S19 and S2308 strains in order to study its role in virulence, protection and intracellular multiplication in mice and in bone marrow-derived macrophages (BMDM).

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Bacterial strains and plasmids used in this study are listed in Table 1. All *B. abortus* strains were grown on Brucella Broth medium (BB) (Becton Dickinson, Sparks, MD, USA) or on plates of BB containing 1.5% Bacto Agar (Becton Dickinson, Sparks, MD, USA) at 37 °C. *Escherichia coli* strains were grown on Luria-Bertani (LB) medium (Invitrogen, Carlsdan, CA, USA). If necessary, the medium was supplemented with appropriate antibiotic as follows: ampicillin, 10 μg mL^{-1} and/or kanamycin, 25 μg mL^{-1} for *Brucella*; ampicillin, 100 μg mL^{-1} and/or kanamycin, 50 μg mL^{-1} for *E. coli*.

2.2. Animals

A pair of IRF-1 $^{-/-}$ (interferon regulation factor-1 knockout) mice breeders was kindly donated by Dr. Luis F. Lima Reis from the Ludwig Institute for Cancer Research, São Paulo, Brazil. C57BL/6 mice were purchased from the Federal University of Minas Gerais. Both mouse strains were bred and maintained at the Department of Biochemistry and Immunology animal care facility, and used at 6–9 weeks-old of age.

2.3. Cloning, DNA sequencing, and gene disruption

Chromosomal mutants were generated from the parental *B. abortus* S2308 and S19 strains using the gene replacement strategy originally described in *Brucella* by Halling et al. [16]. *Brucella* genomic DNA extraction was performed as previously described [17]. The 970 bp *XhoI*/*XbaI* PCR-amplified fragment containing *wbkC* was obtained from the *B. abortus* genomic DNA. The primers used were: wbkCR, 5′-GCG **CTC GAG** TAC GAA TTG CAG CGC CT-3′; wbkCF, 5′-GCG **TCT AGA** GCC AGA AGC CTT TAT CAT CA-3′ and the amplification conditions were performed as follows: 94 °C for 2 min; 35 cycles including 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min; and a final extension of 72 °C for 10 min. The nucleotides in bold indicates cleavage site of the endonuclease enzymes *Xba*I and *Xho*I added to the *wbkC* primer sequences R (reverse) and F (forward), respectively in order to clone the DNA fragment previ ously digested with *Xho*I/*Xba*I into pBluescript KS II (+) (Stratagene, La Jolla, CA, USA) resulting in the pBlue:*wbkC* plasmid. A 1250-bp kanamycin resistance cassette (*kan*) was amplified with primers kanF 5′-GCG GCA TGC CGC TGA GGT CTG CCT C-3′ and kanR 5′-GCG GCA TGC GGG GAA AGC CAC GTT GT 3′ from the plasmid pUC4K (GE Healthcare) and it was cloned into the *Sph*I site in the middle of the gene *wbkC* of the pBlue:*wbkC* plasmid resulting in the pBlue:*wbkC-kan* construct. All constructed plasmids were sequenced using the MegaBACE 1000 (GE Healthcare, São Paulo, Brazil). The obtained clone was sequenced using a DYEnamic ET Dye Terminator kit (GE Healthcare), and the primers used were M13 reverse sequence, M13 universal sequence from GE Healthcare, and specific primers were purchased commercially. To prepare *B. abortus* S2308 competent cells, bacteria were grown in 100 mL of Brucella Broth overnight at 37 °C to the log phase (optical density at 600 nm, 0.8–1.0). The bacterial cells were harvested by centrifugation at 3290 × *g* for 10 min at 4 °C, and they were washed three times with cold apyrogenic water and resuspended in 1.0 mL of the same water. The aliquots were immediately used for electroporation. One to 10 μg of pBlue:*wbkC*-kan plasmid DNA was added to 0.05-mL aliquots of *B. abortus* competent cells and the electroporation was performed as previously described [17]. Colonies were plated on Brucella Broth agar plates containing kanamycin (25 μg/mL) and incubated at 37 °C for 3 days. The recombinant clones were selected in the presence of kanamycin and ampicillin. Ampicillin was used to differentiate deletions resulting from double recombination events (amp^s) from insertions resulting from a single recombination (amp^r).

Table 1
Bacterial strains and vectors used in this study.

Strain or plasmid	Characteristics	Source
E. coli Top 10 F	F-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ*M15* Δ*lacX74 recA1 ara*Δ*139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
Brucella strains		
B. abortus S2308	Wild type, smooth, virulent	Laboratory stock
B. abortus S19	Vaccine strain, smooth	Laboratory stock
B. abortus RB51	Rif^r, rough mutant of S2308	Laboratory stock
B. abortus virB9-	Mutation in type IV secretion system	Celli et al. [4]
B. abortus Δ*wbkC*	Kan^r, Δ*wbkC* mutant of S2308	This study
Plasmids		
pUC4K	ColE1, Amp^r Kan^r	GE Healthcare
pBluescript II KS (+)	ColE1, Amp^r	Stratagene
pBlue:wbkC-Kan	Amp^r-Kan^r, contains *wbkC*::Kan^r	This study

Kan^r: kanamycin resistance; Amp^r: ampicillin resistance.

2.4. Characterization of B. abortus ΔwbkC mutants

To provide genetic evidence that the *B. abortus wbkC* gene was interrupted by the kanamycin cassette, PCR and Southern Blot analysis were performed. PCR analysis was conducted with genomic DNA of *B. abortus ΔwbkC* mutant strains and the wild type. The specific primer sequences for *wbkC* and *kan* genes described above were used for PCR amplification. Southern blot analysis of *Nhe*I and *Dra*I digested genomic DNA with *wbkC* and *kan* probes labeled with AlkPhos Direct Labeling and Detection System (GE Healthcare, São Paulo, Brazil) was performed. An internal 600 pb *wbkC* fragment and an *amp* probe was used to confirm the lack of ampicillin resistance gene within the mutants. In order to confirm the rough morphology of the mutants, it was used the crystal violet method [18]. By this methodology, rough colonies take up the dye, whereas the smooth colonies do not. To confirm the lack of LPS O-chain in *ΔwbkC* mutant strains, Western Blot analysis was also performed as described before [7]. In this study, it was used mAbs O4F9 (IgG 2a) for S-LPS [19] or A68/24 D08/609 (IgG1) for R-LPS as primary antibodies (1:1000) (kindly donated by Dr. Axel Cloeckaert from INRA, France) and anti-mouse total IgG alkaline phosphatase labeled as secondary antibody (1:4000). The development of the reaction was performed using CDP-Star (GE Healthcare, São Paulo, Brazil).

2.5. Persistence of ΔwbkC mutant strains in C57BL/6 mice

Mice were injected intraperitoneally with 1×10^5 CFU of smooth *Brucella* S19 or S2308 or 1×10^8 CFU of rough *Brucella ΔwbkC* mutants or RB51 in 0.1 mL of phosphate buffered saline (PBS). To count residual *Brucella* CFU in the spleens of mice, 8 animals from each group were examined at 1, 2, 3, and 6 weeks post-infection. Spleens from individual animals were homogenized in PBS, 10-fold serially diluted, and plated on Brucella Broth agar. For *ΔwbkC* mutant strains culture, the BB agar containing kanamycin (25 mg/mL) was used. Plates were incubated at 37 °C, and the number of CFU was counted after 3 days. The experiments were repeated twice with similar results.

2.6. Virulence of ΔwbkC mutants in IRF-1 KO mice

Five groups of eight IRF-1 KO mice were injected i.p. with 1×10^6 CFU of either *B. abortus* S2308, S19, *ΔwbkC* S2308, *ΔwbkC* S19 or RB51 vaccine strains in 0.1 mL. Number of surviving mice was observed during 30 days post-infection. The experiments were repeated twice with similar results.

2.7. BMDM culture and infection

Bone marrow cells were isolated from femurs and tibias of 6–9-week-old C57BL/6 mice and differentiated into macrophages as previously described [20]. Infections were performed at a multiplicity of infection of 50:1. Bacteria were centrifuged onto macrophages at 400 × *g* for 10 min at 4 °C then incubating the cells for 30 min at 37 °C under 7% CO_2. Macrophages were extensively washed with HBSS to remove extracellular bacteria and incubated for an additional 90 min in medium supplemented with 100 μg/mL gentamicin to kill extracellular bacteria. Thereafter, the antibiotic concentration was decreased to 10 μg/mL. At each time point, samples were washed three times with HBSS before processing. To monitor *Brucella* intracellular survival, infected cells were lysed with 0.1% (vol/vol) Triton X-100 in H_2O and serial dilutions of lysates were rapidly plated onto Brucella Broth agar plates to count the number of CFU. The level of IL-12 (p40) and TNF-α in the supernatants of BMDM were measured by a commercially available ELISA Duoset kit (R&D Systems, Minnesota, MN).

2.8. Immunofluorescence microscopy

Bone marrow-derived macrophages from C57BL/6 mice were infected with a multiplicity of infection of 25:1. Infected cells grown on 12-mm glass coverslips in 24-well plates were fixed in 3% paraformaldehyde, pH 7.4, at 37 °C for 15 min at different time points. Cells were labeled by inverting coverslips onto drops of primary antibodies diluted in 10% horse serum and 0.1% saponin in PBS and incubating for 30 min at room temperature. The primary antibodies used for immunofluorescence microscopy were: cow anti-*B. abortus* polyclonal antibody and rat anti-mouse LAMP1 ID4B (Developmental Studies Hybridoma Bank, National Institute of Child Health and Human Development, University of Iowa, Iowa city, Iowa). Bound antibodies were detected by incubation with 1:1000 dilution of Alexa Fluor 488 goat anti-rat or 1:100 dilution of TexRed goat anti-cow (Jackson ImmunoResearch Laboratories, Suffolk, UK) for 30 min at room temperature. Cells were washed twice with 0.1% saponin in PBS, once in PBS, once in H_2O and then mounted in Mowiol 4-88 mounting medium (Calbiochem, Darmstadt, Germany). Samples were examined on a Zeiss LSM 510 laser scanning confocal microscope for image acquisition. Images of 1024 × 1024 pixels were then assembled using Adobe Photoshop 7.0. Intracelullar bacteria of at least 50 cells for each time point and each strain were counted in three independent experiments.

2.9. Protection induced by ΔwbkC mutants in IRF-1 KO and C57Bl/6 mice.

The challenge infection using the virulent strain *B. abortus* S2308 was performed in two mouse models, C57BL/6 and IRF 1 KO. Groups of 8-week-old-male C57BL/6 mice ($n = 8$ per group) were vaccinated intraperitoneally (i.p.) with 1×10^5 CFU of smooth strain *B. abortus* S19 or with 1×10^8 of rough strains *B. abortus* RB51, *ΔwbkC* 2308 or *ΔwbkC* S19, separately. A control group of 8 unvaccinated mice was injected i.p. with 0.1 mL of PBS. Six weeks after vaccination, mice were challenged i.p. with 1×10^6 CFU/mouse of the virulent *B. abortus* S2308 strain. C57BL/6 mice were euthanized by cervical dislocation 2 weeks after challenge and the bacterial loads in their spleen were determined. Using the IRF 1 KO mouse model, protection was observed in groups of 8-week-old-male IRF-1 KO ($n = 8$ per group). These mice were vaccinated with the same strains and doses as mentioned above for the C57BL/6 experiment. Six weeks after vaccination, mice were challenged i.p. with 1×10^6 CFU/mouse of the virulent *B. abortus* S2308 strain. The number of surviving mice was observed during 30 days after challenge. The experiments were repeated twice with similar results.

2.10. Statistical analysis

Student's *t*-test was used to analyze the data for bacterial clearance and protection experiments. The other experiments were analyzed by two-way ANOVA with Bonferroni post-test.

3. Results

3.1. B. abortus ΔwbkC are rough mutants

After disrupting the *wbkC* gene with the kanamycin resistance cassette, *B. abortus ΔwbkC* mutants where double recombination event took place were successfully obtained. The *wbkC* gene disruption was confirmed by PCR (data not shown) and by Southern blot analysis (Fig. 1). A PCR analysis was conducted on genomic DNA with specific primers to amplify *wbkC* gene from *Brucella* parental strains and respective mutants. For Southern blot analysis, genomic DNA was isolated from *B. abortus* S2308 and S19 parental strains and *B. abortus ΔwbkC* mutants and digested with *Dra*I and

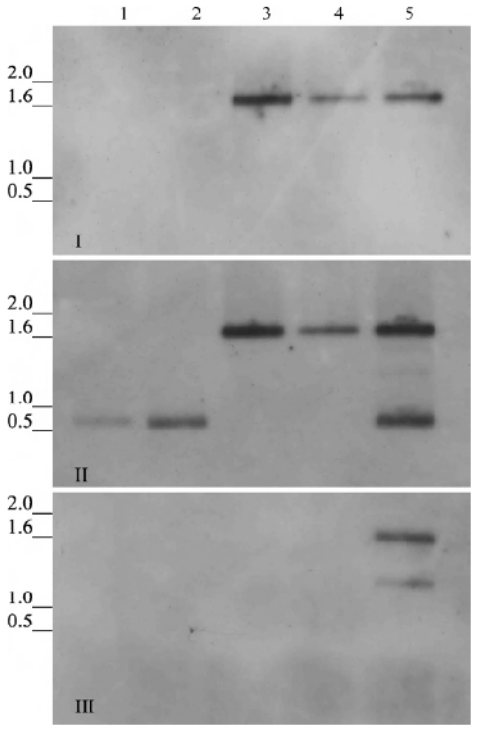

Fig. 1. Molecular characterization of *Brucella abortus ΔwbkC2308* and *ΔwbkCS19* mutant strains. Southern blot analysis of *ΔwbkC* strains was performed. Genomic DNA of *B. abortus* 2308 (1), S19 (2) *wbkC2308* (3), *wbkCS19* (4) and strain where a single recombinant event took place (5) were restriction endonuclease digested and probed with the *kanamycin* (I), *wbkC* (II) and *ampicillin* (III) genes.

*Nhe*I, simultaneously. DNA digested was hybridized with *kan* probe (Fig. 1I) and one band of approximately 1800 bp was observed for the mutants *B. abortus ΔwbkC* S2308 or *ΔwbkC* S19. When the same membrane was hybridized with the probe for *whkC* gene (Fig. 1II), we observed one band of approximately 600 bp in parental strains *B. abortus* S2308 or S19, and another band of 1800 bp in *ΔwbkC* S2308 or *ΔwbkC* S19 mutant strains that correspond to the kanamycin resistance gene integrated into the *wbkC* gene. The strain where a single recombination occurred presented both bands (600 pb and 1800 pb) which indicate the presence of two *wbkC* gene copies, one functional and one interrupted with the kanamycin resistance gene. Also only this strain where a single recombination event took place was recognized when a probe for ampicillin resistance gene was used (Fig. 1III). It presented two bands that resulted from cleavage of ampicillin gene when the DNA was digested to perform the Southern blot. Southern blot analysis confirmed the disruption of the *wbkC* gene in the *ΔwbkC* 2308 and *ΔwbkC* S19 mutants. Characterization by crystal violet colony staining also confirmed that the mutants *B. abortus ΔwbkC* 2308 and *ΔwbkC* S19, and the vaccine strain *B. abortus* RB51 were all morphologically rough strains as they absorbed the dye, which was not observed for smooth parental strains (data not shown). Additionally, crude extracts of the obtained mutants, the parental strains, and the vaccine rough strain *B. abortus* RB51, were analyzed by Western blot. The immunoblotting with mAbs 04F9 (IgG2a) revealed S-LPS presence only in *B. abortus* S2308 and S19 strains (Fig. 2A, lanes 1 and 3). On the other hand, the mutants and the RB51 strains were not recognized by the anti-S-LPS antibodies, once this mAb recognize antigen-O specifically, confirming that the antigen-O was altered in the mutants. However, Western blot analysis with mAbs A68/24 D08/609 (IgG1) specific for R-LPS of *Brucella* revealed high molecular mass ladder-like pattern only in *B. abortus* S2308 and S19, which was absent in rough strains. On the other hand, *B. abortus* RB51, *ΔwbkC* 2308 and *ΔwbkC* S19 have shown low molecular mass ladder-like pattern when probed with mAb specific for R-LPS (Fig. 2B) what was expected considering the absence of the O-chain.

3.2. *B. abortus ΔwbkC are attenuated in C57BL/6 and IRF-1 KO mice*

To verify if there are differences in virulence of the mutants compared to parental strains and the rough vaccine strain *B. abortus* RB51, we compared the bacterial persistence in C57BL/6 and IRF-1 KO mice inoculated i.p. with these strains. In C57BL/6 mice, the rough mutants were significantly attenuated ($p < 0.001$) at all time points observed when compared to parental strains and they were undetected by 3 weeks post-infection (Fig. 3). The rough vaccine strain RB51 load on mouse spleens was reduced to almost two logs by 3 weeks post-infection but it was completely eliminated at 6 weeks post-infection. Regarding testing these strains in IRF-1 KO mice, all infected mice with *B. abortus* S2308 died in 16 days.

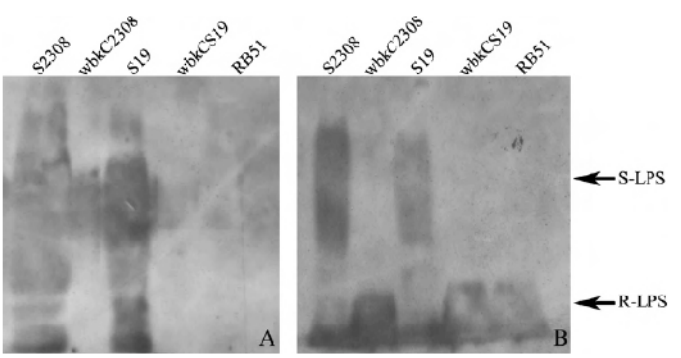

Fig. 2. Characterization of LPS morphology by Western blot. Western blot analysis using mAbs 04F9 (IgG 2a) for smooth LPS (A), or mAbs AG8/24 D08/G09 (IgG1) for rough LPS (B) was performed. (A) Only *B. abortus* S2308 and *B. abortus* S19 had positive results for anti-smooth LPS mAb. *B. abortus wbkC2308*, *wbkCS19* and RB51 were negative for this antibody reaction. (B) *B. abortus* S2308 and *B. abortus* S19 had the typical high molecular ladder-like pattern and all rough strains presented only low molecular ladder-like pattern.

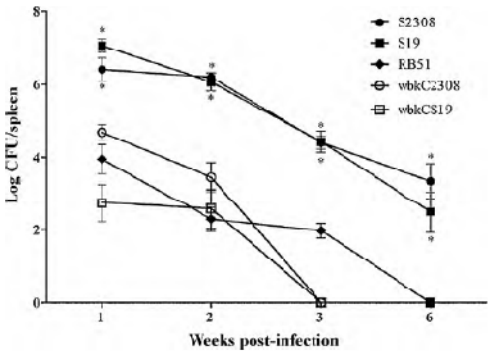

Fig. 3. Persistence of *B. abortus* Δ*wbkC* S2308 and Δ*wbkC* S19 mutant strains in C57BL/6 mice. Eight mice of each group were infected i.p. with a dose of 10^6 CFU of smooth strains (S2308 and S19) and 10^8 CFU of rough strains (Δ*wbkC* S2308, Δ*wbkC* S19 and RB51). Spleens were removed 1, 2, 3 and 6 weeks after infection and the CFU number was determined by serial dilutions and plating. The values correspond to means ± standard deviations. The asterisks indicate statistically significant differences between the results obtained for the groups that received the parental strains and the results obtained for the groups that received the mutant strains ($p \leq 0.001$).

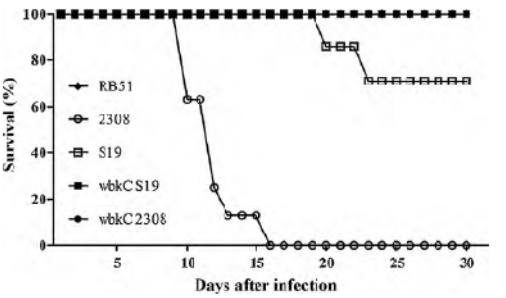

Fig. 4. Virulence of *B. abortus* Δ*wbkC* S2308 and Δ*wbkC* S19 in IRF-1 KO mice. Eight mice for each group were infected with 1×10^5 CFU of the smooth strains (S2308 and S19) and 1×10^8 of the rough strains (Δ*wbkC* S2308, Δ*wbkC* S19 and RB51). Mice survival was observed during 30 days after infection.

After 30 days, it was observed that 70% of the *B. abortus* S19 group survived. However, all mice inoculated with the mutants or RB51 group survived during the period of time studied (Fig. 4).

3.3. Intracellular survival of *B. abortus* Δ*wbkC* mutants and production of pro-inflammatory cytokines by infected BMDM

BMDM were infected with the *B. abortus* Δ*wbkC* rough mutants and compared to the parental smooth strains (Fig. 5A and B). The parental smooth strain *B. abortus* 2308 was able to replicate and survive inside macrophages, while Δ*wbkC 2308* mutant decreased its intracellular number at 24 and 48 h post-infection (Fig. 5A). For the parental smooth strain *B. abortus* S19 and the mutant Δ*wbkC S19* (Fig. 5B), both had a prominent intracellular number decrease at 24 h post-infection; however, S19 recovered and replicated, while Δ*wbkC S19* mutant maintained its intracellular number at 48 h after infection. At later time points, after 48 h, the intracellular numbers of both mutants kept on decreasing and the same profile was observed for the *B. abortus* RB51 strain (data not shown). Meanwhile parental strains replicated inside the macrophages, maintaining their intracellular CFU number.

Because different LPS molecules can interact differently with TLR [21] and it is known that stimulation of TLR-4 by *B. abortus* LPS induces low TNF-α and IL-12 production, we evaluated IL-12 (Fig. 5C) and TNF-α (Fig. 5D) production in supernatants of infected cells. *B. abortus* Δ*wbkC 2308* mutant produced higher levels of IL-12 at 24 and 48 h post-infection compared to the its parental strain *B. abortus* 2308. The same profile was observed for *B. abortus* Δ*wbkC S19* mutant ($p < 0.01$). A significant accumulation of TNF-α was observed into supernatants of all rough *Brucella* strains infected cells, which in each case was significantly higher than cells infected with smooth parental strains.

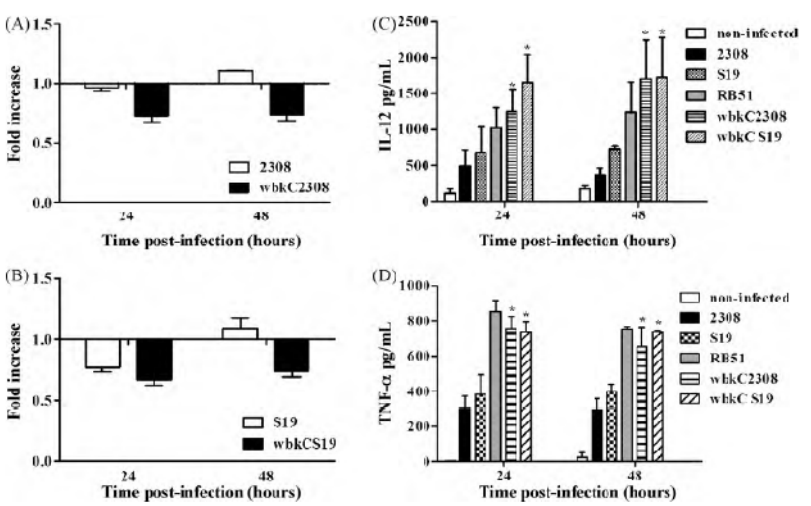

Fig. 5. BMDM infection with Δ*wbkC* S2308 and Δ*wbkC* S19 mutants and pro-inflammatory cytokine production. BMDMs infected (MOI:50) with *B. abortus 2308* or Δ*wbkC2308* mutant (A) and *B. abortus S19* or Δ*wbkCS19* mutant (B) were lysed and intracellular CFUs enumerated at different times after inoculation. In both graphs, data are presented as relative fold increase over 2-h data. All CFU/mL (colony forming units) values were divided by the corresponding 2-h data. Analysis of IL-12 (C) and TNF-α (D) secretion was measured by ELISA from the supernatant of macrophages at 24 and 48 h after infection. (*) Significantly different compared to parental strain ($p < 0.01$).

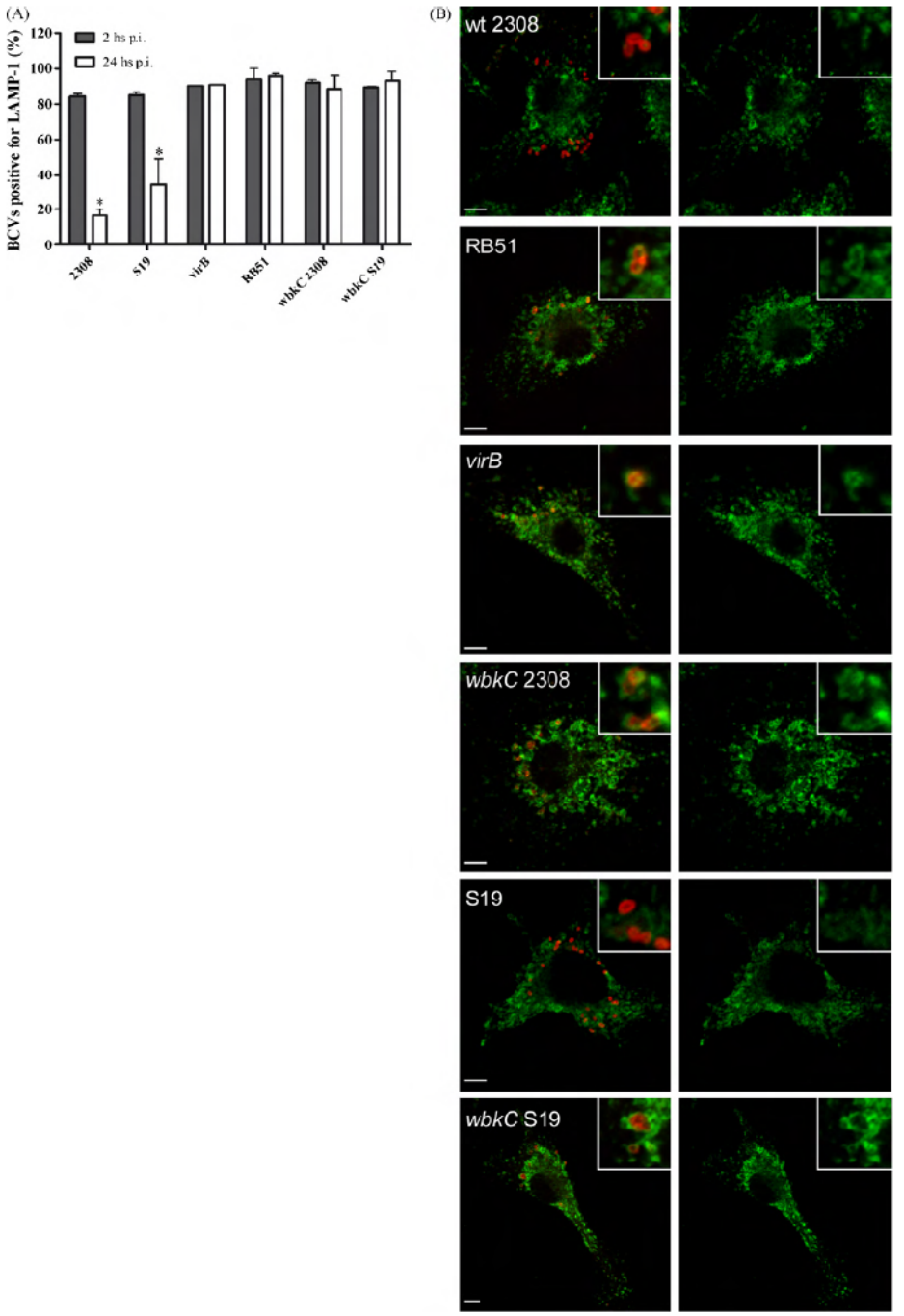

Fig. 6. Intracellular localization of *B. abortus* Δ*wbkC* mutants and parental strains in BMDM. Macrophages infected with *B. abortus 2308*, *B. abortus S19*, Δ*wbkC* 2308 mutant, Δ*wbkC* S19 mutant, RB51 or Δ*virB9*. (A) Quantification of the percentage of bacteria BCVs that contain LAMP 1 by confocal immunofluorescence microscopy. The difference between wild type and mutant were statistically significant at 24 h ($p < 0.001$). Data are means from tree different experiments. (B) Representative confocal images of BMDM 24 h post-infection with wild type *B. abortus 2308*, S19, RB51, or Δ*wbkC* mutants. *B. abortus* is labelled in red and LAMP-1 in green. Scale bars are 5 μm.

Table 2
Protective immunity induced by immunization with the mutant strains *B. abortus* Δ*wbkC* S2308 and Δ*wbkC* S19 in C57BL/6 mice.

Vaccine	Log_{10} CFU of *B. abortus* S2308 in spleen (mean ± SD)[a]	Log_{10} units of protection
PBS control	5.14 ± 0.14	
B. abortus S19	4.45 ± 0.50	1.70[b]
B. abortus RB51	5.31 ± 0.35	0.84[b]
B. abortus ΔwbkC2308	5.57 ± 0.48	0.58[b]
B. abortus ΔwbkCS19	5.90 ± 0.35	0.24

[a] Mice were immunized with PBS, 10^5 CFU of *B. abortus* S19 or 10^8 CFU of the other strains. Six weeks later were challenged i.p. with 10^5 CFU of *B. abortus* 2308 and spleen CFU was enumerated 2 weeks after challenge.
[b] Significantly different compared to the PBS control group ($p \leq 0.05$).

3.4. Intracellular localization of Brucella ΔwbkC mutants in BMDM

Upon entry, wild type *B. abortus* establishes a replicative niche, acquiring endoplasmic reticulum markers (calnexin, calreticulin, PDI). While mutants, as the Δ*virB*, maintain late endosomal/lysosomal marker (LAMP 1), fuse with lysosomes and are eliminated. Immunofluorescence analysis of infected BMDM showed that unlike wild type bacteria, over 80% of Δ*wbkC* mutant BCVs retained LAMP-1 (Fig. 6A and B). Both Δ*wbkC* mutants showed the same profile as both the vaccine rough strain RB51 and the Δ*virB* mutant.

3.5. Protective efficacy of B. abortus ΔwbkC mutants against B. abortus 2308 challenge

To evaluate the potential use of Δ*wbkC* mutants as vaccine candidates, the protection level induced in mice against virulent challenge infection was assessed. The degree of vaccine efficacy in C57BL/6 mice was determined by subtracting the mean CFU/spleen recovered from mice after 6 weeks of vaccination and challenged with 2308 from the mean CFU/spleen recovered from non-vaccinated but challenged control mice (PBS). At this time, mice immunized with *B. abortus* S19, RB51 and Δ*wbkC S2308* mutant strain had significantly ($p < 0.05$) fewer splenic *Brucella* than non-immunized animals (Table 2). However, *B. abortus* Δ*wbkc* S19 mutant strain conferred no significant protection compared to the control group. This is probably due to the fact that this mutant came from the parental strain S19 that is already attenuated, so the rough LPS introduced an additional attenuation, inhibiting sufficient bacterial growth to induce protective immunity. The protection tested in IRF-1 KO mice was carried out following the same protocol as for C57BL/6 mice, but instead of counting CFU in mouse spleens, the survival of the animals was observed during 30 days post-challenge. Therefore, *B. abortus* Δ*wbkC* S19 mutant showed lower level of protection (60% survival) against virulent challenge in IRF-1 KO mice and the Δ*wbkC* S2308 mutant (80% survival) showed similar level of protection as the vaccine strains S19 and RB51 (Fig. 7).

4. Discussion

B. abortus is the causative agent of human and animal brucellosis, and many research groups around the world have been dedicating their efforts in isolation, identification and characterization of new antigens and virulence factors. Early observations that rough *B. abortus* strains are attenuated and do not agglutinate with antibody elicited by smooth bacteria led to the concept of *Brucella* rough vaccine [15] avoiding the problem of serological interference presented by smooth strain vaccines. Therefore, all efforts have been focused on searching better live rough attenuated vaccine that could be able of inducing an efficient cellular immunity and protection [8,15].

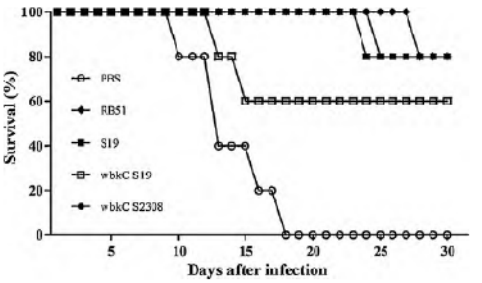

Fig. 7. Protection induced by *B. abortus* Δ*wbkC* S2308 or Δ*wbkC* S19 immunization in IRF-1 KO mice. Mouse groups were immunized i.p. with 10^8 CFU of the Δ*wbkC* mutants and the vaccine strain RB51 and with 1×10^5 of the vaccine strain S19. The control group received 100 μL of PBS i.p. Six weeks after immunization mice were challenged with 1×10^6 CFU of *B. abortus* S2308 virulent strain. Mice survival was observed during 30 days after infection.

In a tentative of developing a *B. abortus* rough vaccine strain, the *wbkC* gene was chosen to be disrupted in our study. Two Δ*wbkC* mutants were generated by gene replacement using double recombination strategy and they were termed *B. abortus* Δ*wbkC* S2308 and Δ*wbkC* S19. *WbkC* gene disruption confirmed by Southern blot analysis resulted in rough mutant strains. Lack of LPS O-side chain was also confirmed by crystal violet staining and immunoblotting assays. Earlier study by Godfroid et al. [7] has also shown that wbkC is required for the O-side chain production in *B. melitensis* 16 M strain. Further, by mass spectrometry analysis we could verify that *wbkC* gene mutation did not interfere in lipid A biosynthesis in *B. abortus* (data not shown).

To evaluate the persistence of the mutants, *in vivo* assays were performed in C57BL/6 and IRF-1 knockout mice. The mutant strains were cleared faster than the parental strains in C57BL/6 mice even though the dose used for the rough mutants was 100 times higher. Three weeks after infection, the mutant strains had already been cleared in C57BL/6 mice. The Δ*wbkC* mutants showed reduced persistence when compared to the rough vaccine strain *B. abortus* RB51 which was completely cleared within 6 weeks post-infection. At that time, the parental smooth strains still had more than 3 log of CFU. Ko et al. [22] have previously demonstrated that IRF-1 KO mice are an important tool to determine the level of *Brucella* virulence and to evaluate *Brucella* mutants for attenuation. They reported that survival of IRF-1 KO mice can be correlated to virulence of *Brucella* strains, the criteria of *Brucella* virulence among several strains can be based on the rapidity of death in IRF-1 KO mice. Therefore, we have compared the virulence of our Δ*wbkC* mutants with the virulent strain *B. abortus* S2308 and the vaccine strains *B. abortus* S19 and RB51 in IRF-1 KO mice. Δ*wbkC* mutants showed the same reduced virulence as *B. abortus* RB51 and a lower virulence when compared to *B. abortus* S19 and S2308. All mice infected with the Δ*wbkC* mutants survived after infection, in contrast no animals infected with *B. abortus* S2308 survived after 16 days. In general, strains containing alterations in LPS are less virulent when compared to the wild type strain, except *Brucella ovis* and *Brucella canis* that are naturally rough virulent bacteria [23].

Brucella can infect macrophages and entry in these cells is essential for bacterial replication and survival in animals. Changes in the LPS structure can interfere with bacterial entry into host cells and some authors described that smooth LPS is an essential virulence factor for intracellular survival [24]. The rough strain *B. abortus* RB51 has a low persistence *in vivo* and cannot replicate as the smooth parental strain inside macrophages [11]. However, it has been shown that genetically characterized rough mutants have not lost their ability to replicate intracellularly even without

the entire antigen-O structure [25]. Our experiments with bone marrow-derived macrophages demonstrate that Δ*wbkC* mutants showed a reduced rate of replication inside these cells. Additionally, as observed with *B. abortus* RB51 strain, Δ*wbkC* mutants remained in LAMP-1-positive compartments and were eventually eliminated. This is consistent with previous reports showing that the vaccine strain *B. abortus* RB51 had a low persistence *in vivo* and cannot replicate inside macrophages [25]. As reported before, the smooth LPS and consequently its antigen-O are important for entry and early stages of BCV development. The LPS O-side chain is involved in the inhibition of early fusion events between *Brucella suis*-containing vacuoles and lysosomes in murine macrophages [2]. Also, Δ*wbkC* mutants had the same intracellular fate observed for the Δ*virB* mutant. The Brucella type IV secretion system has also been shown to be required for late maturation events necessary for the biogenesis of an ER derived replicative organelle in BMDM [4].

The presence of the O-side chain on LPS structure has also been reported to influence pro-inflammatory cytokine production [26]. These authors have shown that rough *Brucella* strains induce higher production of pro-inflammatory cytokines than smooth strains. IL-12 is involved in the development of Th1 responses, which *in vivo* are critical for the elimination of *Brucella* [27]. Higher IL-12 and TNF-α production by BMDM infected with Δ*wbkC* rough mutants might be one of the reasons for their faster elimination by infected cells when compared to parental smooth strains.

Ko et al. [22] also showed that utilization of a high dose of the rough vaccine strain *B abortus* RB51 (5×10^7 CFU) elicited a higher level of protection against *B. abortus* 2308 challenge when compared to lower doses. So, we used 1×10^8 CFU dose for the rough strains in the protection experiments using C57BL/6 mice. These results showed that *B. abortus* Δ*wbkC* 2308 conferred similar protection as the currently used rough vaccine RB51, but both induced lower protection compared to the smooth vaccine strain *B. abortus* S19.

As previously demonstrated [22] IRF-1 KO mice maintain an adaptative immunological memory necessary for protection, that is dependent on the level of bacteria virulence and dose of immunization. Therefore, we performed the protection experiment in IRF-1 KO mice and the *B. abortus* Δ*wbkC* 2308 mutant could prevent death of 80% of challenged mice, inducing similar protection as *B. abortus* RB51 or *B. abortus* S19. However, the *B. abortus* Δ*wbkC* S19 mutant showed no protection in C57BL/6 mice and low protection in IRF-1 KO mice.

As a conclusion, we can assume that *wbkC* gene is required for LPS O-side chain biosynthesis and virulence of *Brucella abortus*. Further, the Δ*wbkC* mutants behave intracellularly as most rough *Brucella* mutants. The *B. abortus* Δ*wbkC* 2308 mutant generated in this study showed similar protection as the current available rough vaccine *B. abortus* RB51, having the advantage of not being rifampicin resistant and the identity of the attenuation is known, which is still not completely known for RB51. Although *B. abortus* Δ*wbkC* 2308 mutant does not confer the same level of protection when compared to the smooth vaccine strain *B. abortus* S19, it has the advantage of not interfering with serological diagnosis of infected animals. However, due to this moderate protection efficacy the Δ*wbkC* 2308 has to be further evaluated.

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