MAYSA SERPA GONÇALVES

IMMUNOGENICITY AND EFFECTIVENESS OF VACCINES AGAINST BOVINE BRUCELLOSIS

LAVRAS – MG

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Thesis manuscript presented to the Federal University of Lavras (Universidade Federal de Lavras), as part of the requirements of the Graduate Program in Veterinary Sciences, with a concentration in Animal Health and Public Health, for the attainment of the title of Doctor.

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Ficha Catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Gonçalves, Mays Serpa.
Immunogenicity and effectiveness of vaccines against bovine brucellosis / Mays Serpa Gonçalves. - 2025.
142 p. : il.

Orientadora: Elaine Maria Seles Dorneles

Tese (Doutorado) - Universidade Federal de Lavras, 2025.
Bibliografia.

1. Brucellosis control. 2. S19. 3. RB51. 4. Systematic review. 5. Brucellosis vaccination. I. Maria Seles Dorneles, Elaine. II. Universidade Federal de Lavras. III. Título.

MAYSA SERPA GONÇALVES

IMMUNOGENICITY AND EFFECTIVENESS OF VACCINES AGAINST BOVINE BRUCELLOSIS

IMUNOGENICIDADE E EFICÁCIA A CAMPO DE VACINAS CONTRA A BRUCELOSE BOVINA

Thesis manuscript presented to the Federal University of Lavras (Universidade Federal de Lavras), as part of the requirements of the Graduate Program in Veterinary Sciences, with a concentration in Animal Health and Public Health, for the attainment of the title of Doctor.

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2025

À Santíssima Virgem Maria

AGRADECIMENTOS

Agradeço a Deus que possibilitou aos homens o dom da ciência e da inteligência, para crescimento enquanto indivíduo e sociedade.

Aos meus pais, por sempre terem a convicção que o estudo mudaria minha realidade e terem feito o impossível para que eu chegasse até aqui.

Ao meu esposo, Yohannes, por acreditar mais em mim que eu mesma e por ser o único com total ciência das minhas muitas limitações e dificuldades e carregar junto comigo esses fardos.

Aos demais membros da minha família, meus sogros, cunhada, sobrinhas, tios e primos que sempre estiveram na torcida e em oração por mim.

Ao sistema público de educação básica e superior e a cada cidadão brasileiro que custeou os meus estudos com o suor do seu trabalho.

À Universidade Federal de Lavras, na qual realizei graduação, mestrado e doutorado e se tornou mais que uma instituição, sendo uma segunda casa por 12 anos.

À minha orientadora, Profa. Elaine Dorneles, por ser exemplo diário de profissionalismo e humanidade e por ser fundamental no desenvolvimento da minha paixão pela pesquisa aplicada.

A todos os demais mentores que passaram pela minha caminhada - em especial Dr. Alessandro de Sá Gumarães, Prof. Dr. Geraldo Márcio da Costa, Prof. Dr. Javier Sanchéz, Dr. Flavio Sacchini, Profa. Dra. Gláucia Frasnelli Mian, Profa. Dra. Ana Paula Peconick, Prof. Dr. Christian Hirsch - que acreditaram em mim, incentivaram-me e de alguma forma me inspiraram a seguir o mesmo caminho.

A todos os integrantes dos Laboratórios Integrados de Sanidade Animal e Saúde Coletiva (LISASC) que fizeram os meus dias mais felizes e compartilharam diversas batalhas. Em especial aos meus amigos Alice, Amanda, Ana Beatriz, Ana Carolina, Ana Clara, Anna Cecília, Bruno, Carine, Dircéia, Eduarda, Erika, Glei, Jéssica, Marcilene, Maria Eduarda, Paula, Pedro, Rafaella, Vitória e Yasmim, grandes encontros que a vida acadêmica me proporcionou.

À Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig) pela bolsa concedida durante este período de doutorado e ao financiamento de projetos, ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e ao Emerging Leaders in the Americas Program (ELAP) pelas bolsas de doutorado sanduíche.

"Pouca ciência nos afasta de Deus, muita nos aproxima."

"A grandeza das ações humanas é proporcional à inspiração que as produz. Feliz é aquele que traz dentro de si um Deus, um ideal de beleza a que obedece: ideal de arte, ideal de ciência, ideal de pátria, ideal de virtudes evangélicas. São essas as fontes vivas dos grandes pensamentos e das grandes ações. Todas elas refletem a luz do infinito."

(Louis Pasteur)

ABSTRACT

Vaccination of production animals with Brucella spp. live strains is one of the major measures for brucellosis control worldwide, aiming both human and animal health. However, although vaccine strains have been widely studied, there are still many aspects for being clarified. This thesis contains four chapters: (1) “Efficacy of Brucella vaccine strains: S19, RB51 and Rev-1” and (2) “Does lipopolysaccharide morphology (smooth or rough) of Brucella abortus vaccine strains influence the potency or efficacy of the vaccine?”; (3) “Systematic review on the effectiveness of Brucella abortus S19 and RB51 vaccine strains in field studies”; and (4) “Short communication: Effects of age on the immune response induced by Brucella abortus S19 or RB51 vaccination in calves”. The principal scientific contributions were: (1) clarification of various aspects about dissociation of smooth Brucella strains and its use for bovine vaccination; (2) confirmation of the effectiveness of B. abortus S19 and RB51 vaccines in the field, particularly when combined with other control policies; and (3) observation of similar immunogenicity of the S19 and RB51 strains in calves vaccinated at different ages within the 3 to 8-month range.

Keywords: brucellosis control, S19, RB51, systematic review, brucellosis vaccination

RESUMO

A vacinação de animais de produção com cepas vivas de *Brucella* spp. é uma das principais medidas para o controle da brucelose em todo o mundo, visando à saúde humana e animal. No entanto, embora as cepas vacinais tenham sido amplamente estudadas, ainda há muitos aspectos a serem esclarecidos. Esta tese contém quatro capítulos: (1) "Eficácia das cepas vacinais de *Brucella*: S19, RB51 e Rev-1" e (2) "A morfologia dos lipopolissacarídeos (lisa ou rugosa) das cepas vacinais de *Brucella abortus* influencia a potência ou eficácia da vacina?"; (3) "Revisão sistemática sobre a eficácia das cepas vacinais de *Brucella abortus* S19 e RB51 em estudos de campo"; e (4) "*Short communication*: Efeitos da idade na resposta imune induzida pela vacinação de *Brucella abortus* S19 ou RB51 em bezerros". As principais contribuições trazidas por estes capítulos científicas foram: (1) esclarecimento de vários aspectos sobre a dissociação de cepas de *Brucella* lisa e seu uso para vacinação bovina; (2) confirmação da eficácia das vacinas *B. abortus* S19 e RB51 no campo, particularmente quando combinadas com outras políticas de controle; e (3) observação de imunogenicidade semelhante das cepas S19 e RB51 em bezerros vacinados em diferentes idades dentro da faixa de 3 a 8 meses.

Palavras-chave: controle da brucelose, B19, RB51, revisão sistemática, vacinação contra brucelose

IMPACT INDICATORS

This thesis, dedicated to the study of the effectiveness and immunogenicity of live *Brucella* spp. vaccines in cattle, presents relevant social, technological, and economic impacts, with direct effects on public health and the sustainability of Brazilian livestock production.

Brucellosis is a highly important zoonosis that affects both animal health and human populations. The confirmation of the efficacy of the S19 and RB51 vaccines, especially when used in combination with control policies, contributes to reducing the incidence of the disease in humans, increasing food safety, and protecting workers in the production chain, veterinarians, and consumers, thus generating direct social impacts.

In addition, the results reinforce the use of existing vaccine tools, optimizing immunization protocols and offering greater flexibility in sanitary management. The demonstration of similar immunogenicity at different ages of vaccination allows better adaptation to production realities, representing a technological advance applicable on a large scale, which constitutes the main technological and production impacts.

From an economic perspective, brucellosis causes significant losses in beef and dairy production, related to abortions, reduced fertility, and culling of animals. By consolidating field evidence of vaccine efficacy, this work contributes to reducing such losses and enhancing the competitiveness of national livestock, while also supporting access to markets that require controlled sanitary status.

The extension component of the study is manifested in the practical applicability of its results for farmers and official veterinary authorities. The impacted territory is national, with potential repercussions worldwide, and the beneficiaries include farmers, rural workers, and society in general. Faculty members, undergraduate and graduate students, farmers, and technicians directly participated in the project.

The impacts are mainly classified under the thematic areas of Health and Technology and Production of the National Extension Policy, and transversally under Work and Environment. Regarding the United Nations Sustainable Development Goals (SDGs), the research contributes to SDG 2 (Zero Hunger and Sustainable Agriculture), SDG 3 (Good Health and Well-Being), SDG 8 (Decent Work and Economic Growth), SDG 12 (Responsible Consumption and Production), and SDG 15 (Life on Land).

Thus, the results of this thesis transcend the academic sphere, providing support for public policies, technological advances, and social benefits, aligned with the One Health approach and sustainable development.

INDICADORES DE IMPACTO

A presente tese, dedicada ao estudo da eficácia e imunogenicidade de vacinas vivas de *Brucella* spp. em bovinos, apresenta impactos relevantes de ordem social, tecnológica e econômica, com reflexos diretos na saúde pública e na sustentabilidade da pecuária brasileira.

A brucelose é uma zoonose de grande importância e que compromete tanto a saúde dos animais quanto das populações humanas. A confirmação da eficácia das vacinas S19 e RB51, especialmente quando utilizadas em conjunto com políticas de controle, contribui para reduzir a ocorrência da doença em humanos, aumentar a segurança alimentar e proteger trabalhadores da cadeia produtiva, veterinários e consumidores, trazendo impactos sociais diretos.

Além disso, os resultados reforçam o uso de ferramentas vacinais já disponíveis, otimizando protocolos de imunização e oferecendo maior flexibilidade para o manejo sanitário. Além disso, a demonstração de imunogenicidade semelhante em diferentes idades de vacinação permite melhor adaptação às realidades produtivas, configurando avanço tecnológico aplicável em larga escala, sendo estes os principais impactos tecnológicos e de produção.

Considerando os impactos econômicos, a brucelose provoca perdas significativas na pecuária de corte e leite, relacionadas a abortos, queda de fertilidade e descarte de animais. Ao consolidar evidências de eficácia vacinal em campo, o trabalho contribui para a redução desses prejuízos e para a competitividade da pecuária nacional, além de favorecer o acesso a mercados que exigem status sanitário controlado.

O caráter extensionista do estudo se manifesta na aplicabilidade prática de seus resultados para produtores rurais e órgãos oficiais. O território impactado é nacional, com potencial repercussão também no resto do mundo, e o público beneficiado inclui produtores, trabalhadores rurais e a sociedade em geral. Estiveram diretamente envolvidos neste projeto docentes, estudantes de graduação e pós-graduação, produtores rurais e técnicos vinculados ao projeto.

Os impactos classificam-se principalmente nas áreas temáticas de Saúde e Tecnologia e Produção da Política Nacional de Extensão, e transversalmente em Trabalho e Meio ambiente. Em relação aos Objetivos de Desenvolvimento Sustentável da ONU, a pesquisa contribui para o ODS 2 (Fome Zero e Agricultura Sustentável), ODS 3 (Saúde e Bem-Estar), ODS 8 (Trabalho Decente e Crescimento Econômico), ODS 12 (Consumo e Produção Responsáveis) e ODS 15 (Vida Terrestre).

Assim, os resultados desta tese transcendem o campo acadêmico, oferecendo subsídios para políticas públicas, avanços tecnológicos e benefícios sociais, alinhados à abordagem de Saúde Única (One Health) e ao desenvolvimento sustentável.

SUMMARY

GENERAL INTRODUCTION

Brucellosis is a chronic disease that affects several animal species, including humans, and has worldwide distribution. *Brucella abortus* is the main species causing bovine brucellosis and leads to several economic losses, mainly due to abortions, stillbirth and infertility. In humans, brucellosis is a chronic and debilitating illness and is transmitted through ingestion of unpasteurized milk and dairy products or through contact with fetuses and abortion products from infected animals.

Vaccination of young bovine females (between 3 and 8 months) with S19 or RB51 live strains is one of the major measures for brucellosis control worldwide, aiming both human and animal health. S19 is a smooth vaccine strain, and thus, vaccine-induced antibodies interfere with serological tests, preventing differentiation between vaccinated and infected animals. RB51, on the other hand, is a rough mutant and does not induce detectable antibodies in serological tests, but it is resistant to rifampicin, complicating the treatment of human cases caused by vaccination accidents. Although the two vaccine strains have been widely used, they confer only 60-70% of protection against infection and abortion, value still far from being considered as ideal. In addition, many aspects of bovine vaccination with S19 and RB51 remain still unclear as whether there is or not an ideal age at vaccination for improving immune response and the effectiveness (field efficacy) of these strains.

Given that, the general objective of this thesis is to evaluate immunogenicity and the effectiveness vaccines against bovine brucellosis. In this sense, this manuscript brings four chapters: (1) “Efficacy of *Brucella* vaccine strains: S19, RB51 and Rev-1” and (2) “Does lipopolysaccharide morphology (smooth or rough) of *Brucella abortus* vaccine strains influence the potency or efficacy of the vaccine?”; (3) “Systematic review on the effectiveness of *Brucella abortus* S19 and RB51 vaccine strains in field studies”; and (4) “Short communication: Effects of age on the immune response induced by *Brucella abortus* S19 or RB51 vaccination in calves”.

CHAPTER I

In review process as chapter of the book ""Brucellosis: The Silent Threat to Livestock and Human Health" edited by Elsevier.

Efficacy of *Brucella* vaccine strains: S19, RB51 and Rev-1

Abstract

Brucellosis is a chronic disease that affects several animal species, including humans, and has worldwide distribution. *Brucella melitensis* and *Brucella abortus* are, respectively, the main species causing brucellosis in small ruminants and cattle, leading to several economic losses, mainly due to abortions, stillbirth and infertility. Vaccination of production animals with *Brucella* spp. live strains is one of the major measures adopted in brucellosis control programs worldwide. There are three attenuated vaccines officially recommended by the World Organization for Animal Health (WOAH): *B. abortus* strain 19 (S19) and *B. abortus* strain RB51, used for cattle, and the *B. melitensis* strain Rev.1, for small ruminants. This chapter will discuss the efficacy and effectiveness of these official vaccines, in addition to recommendations of trials and study designs used to obtain these epidemiological measures, characteristics of an ideal *Brucella* vaccine and the impacts of some variables on the immune response triggered by these vaccines.

1. Introduction

Controlling brucellosis in livestock (cattle, sheep, goat, and swine) is a priority of many countries, in order to avoid economic losses, but mainly to reduce the risk for human population, since this is one of the most important bacterial zoonosis worldwide [1–3]. Animal brucellosis control is basically achieved by two means: (1) eliminating positive animals from the herd, to avoid the transmission to healthy animals, and (2) improving immunity against *Brucella* spp. in healthy animals through vaccination [4,5]. Since1940's , this strategy – elimination of infected animals combined with vaccination – is the basement of many control programs, especially in endemic areas with moderate and high prevalence rates [4–7].

Currently, brucellosis vaccination is well stablished for cattle, sheep and goats, with three attenuated vaccines officially recognized by the World Organization for Animal Health (WOAH): (1) the smooth *Brucella abortus* strain 19 (S19), (2) the rough *B. abortus* strain

RB51, and (3) the *B. melitensis* strain Rev.1 [8]. However, other strains and types of vaccines were and have been tested and proposed as alternatives over the years [9].

In this chapter, we will discuss concepts as vaccine efficacy and effectiveness (field efficacy) and which trials and study designs are used to obtain these epidemiological measures, focusing on the WOAH official brucellosis vaccines (S19, RB51, and Rev.1). Additionally, we will discuss the characteristics of ideal vaccines for livestock brucellosis and of the experiments/studies to assess these vaccines, as well as the impacts of some variables on triggered immune response and vaccine efficacy.

2. How to evaluate vaccine efficacy

Differently from studies on human vaccines, epidemiological concepts and study designs are often misapplied in veterinary medicine, leading to limited or biased interpretation of the results [10]. Therefore, before planning or even reading studies on vaccine or vaccination evaluation, it is important to be aware of concepts as efficacy and effectiveness, as well as study designs and epidemiological measures used to assess these values.

Vaccine efficacy refers to the vaccine ability to protect the animal from infection or from the disease clinical signs (e.g. abortion, in bovine brucellosis). In veterinary medicine, vaccine efficacy is obtained through challenge studies (as known as clinical trials), in which vaccinated and unvaccinated animals may be compared under controlled conditions and after direct challenge with the target pathogen [10]. The assays can be initially performed using other animal models (as mice, rats, guinea pigs, etc.), in order to obtain preliminary results, subsequently validated in the target species. For bovine brucellosis, the murine model is well-stablished for studying vaccines, especially for S19 and Rev.1 strains, which have standardized protocols determined by the WOAH for evaluating immunogenicity, potency, and residual virulence [8]. Despite these protocols are not mentioned in the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals[8], they were already successfully applied to assess RB51 strains[11].

Although studies in laboratory models provide valuable insights into the expected outcomes as strain immunogenicity, subsequent tests of the vaccine in the target species are crucial to obtain vaccine efficacy. As a first step, the strain is usually evaluated under standardized conditions, using homogenous groups (same age, breed, weight, sanity conditions

etc.), controlled environment and systematized challenge procedures (strain, route, dose, etc.), following the international guidelines for randomized clinical trials in animals [12,13]. Considering specifically brucellosis vaccines, additionally to the conditions stated above, it is crucial that the animals are pregnant during the challenged, in order to also evaluate abortion as outcome, and that reference strains are used (such as *B. abortus* strain 544, as pointed by WOAH protocols), aiming to standardize and compare results from different trials. About the challenge procedure, there are no available protocols stating number of CFU, challenge route, and stage of pregnancy for challenge in clinical trials evaluating brucellosis vaccines. Nevertheless, for evaluating S19 and RB51 strains in cattle, most of the trials have been using 10^7 CFU by conjunctival route at the 6-7 months of pregnancy, which was considered a good challenge protocol [14]. All these cited statements will allow to the clinical trial to obtain reliable findings for brucellosis vaccination, providing an excellent perspective about how the vaccine works in ideal conditions (10,14). However, controlled trials are far from reflecting the reality of the field, which is usually very diverse. Thus, another parameter is necessary to assess the efficacy in the "real world", called effectiveness or field efficacy [15,16].

Vaccine effectiveness in veterinary medicine is an embracing concept related to the vaccine ability to protect from disease under field conditions. It is assessed by observational studies, in which the only intervention is the vaccination and the challenge occurs naturally, imposed by the environmental conditions where the experiment is being conducted [10]. As bovine brucellosis is a chronic illness, this type of study lasts years and must be conducted in herds with moderate prevalence. There are two main study designs to obtain effectiveness for brucellosis vaccines: (1) cohort studies in which vaccinated and unvaccinated animals in the same population are compared along time with natural exposition to the pathogen; and (2) prevalence panels, when all animals are vaccinated and the prevalence of the disease in the herd is compared at two different times, before and after vaccination, through two cross-sectional studies [17].

Despite many heterogeneities being expected in effectiveness studies, there are some points that should be considered when performing a field efficacy assay. First, if cohort design is applied, the groups (vaccinated and unvaccinated) must be as most homogenous as possible, considering characteristics as age of the animals, breed, housing, nutrition and other aspects (confounders) that can bias the results, or these factors must be controlled when the results are analyzed. Also, both vaccinated and unvaccinated animals must be in the same herd, to be exposed to the same level of challenge. Finally, if possible, only vaccination must be

implemented as control policy, both for cohort as prevalence panel studies, in order to assess the isolated effect of vaccination under field conditions, otherwise the value of effectiveness obtained will reflect the combo of policies adopted in the program (e.g. vaccination and test-and-slaughter) and not the isolated result of the vaccination [18].

Both vaccine efficacy and effectiveness are calculated using the same formula, however, the measure of association used to compare groups may be different depending on the study design. When the study is performed with two different groups (vaccinated and unvaccinated) followed and compared along time (clinical trials and cohort studies), the Relative Risk (RR, risk in the vaccinated population divided by the risk in the unvaccinated population) is the most important measure to be calculated. Conversely, for prevalence panels, Prevalence Ratio (PR, prevalence after vaccination divided by prevalence before vaccination) is the correct measure to compare herd prevalences before and after intervention [19]. Thus, vaccine efficacy and effectiveness will be obtained using one of these measures of association, by subtracting 1 from the RR or the PR and dividing by the RR or the PR [(RR-1)/RR or (PR-1)/PR) [19]. This value represents the vaccine efficacy or effectiveness for the proposed outcome in the studied population. Exemplifying, considering a vaccine with 90% of efficacy against infection (VE = 0.9), for every 10 vaccinated individuals, it is expected that 9 will be protected against disease in ideal conditions. For effectiveness, this value is expected to be lower than efficacy, once conditions are not ideal and other factors can influence the vaccine performance. Finally, it is worth mentioning that clinical trials and cohort studies provide higher levels of scientific evidence for efficacy and effectiveness trials, respectively. Therefore, if possible, these designs should be preferred in detriment of other types of studies [19].

3. Ideal vaccine for brucellosis

As mentioned before, efficacy refers to the vaccine capacity to protect individuals from infection or adverse effects caused by infection. Thus, for achieving this purpose, the vaccine must be capable of induce the immune system to respond adequately and create memory cells, without causing real disease [20]. However, some factors other than only efficacy are desirable considering brucellosis vaccines. These aspects will be following discussed, listing the characteristics of an ideal brucellosis vaccine.

The efforts to create a brucellosis vaccine started in the beginning of the 20th century when, initially, inactivated strains were proposed due its safety both for humans and animals.

However, live attenuated vaccines soon replaced inactivated vaccines, as they promote more complete and long-lasting immunity [17,21]. This happens mainly because live strains can multiply for limited periods in host macrophages, inducing a cell-mediated immune response, which is crucial for response against *Brucella* spp. [22,23]. In this sense, one of the most important characteristics of a brucellosis vaccine is being able of inducing a T helper type 1 immune response (Th1), with high production of IFN-γ, macrophages activation and cytotoxic activity of T CD8+ lymphocytes [9,24–26]. Until the present days, the most important brucellosis vaccines are attenuated strains, even though many efforts have been developed to produce other type of vaccines (as subunits, vector-delivered, DNA etc.) [8,9,26,27]. The important message in case of brucellosis vaccines is that, regardless of the technology, the vaccine must be able to induce a Th1 response to protect against the disease.

Indeed, antibodies seem to play a minor or secondary role in protection against brucellosis [22], and there is a poor relationship between circulating titers and protection [17]. Even so, *Brucella* spp. infections induces antibodies production, mainly IgM and IgG, which are used for diagnosis in livestock, being most of the brucellosis tests based on serology [28,29]. Considering the diagnosis, a very desirable characteristic of brucellosis vaccine is not inducing antibodies that are detectable in the classical serological tests for the disease, allowing Differentiating Vaccinated from Infected Animals (DIVA) and contributing to improve the control programs [9,17,26]. Not all vaccines used for brucellosis have this characteristic and, therefore, animals that were vaccinated with non-DIVA vaccines cannot be tested for a certain period after vaccination [8].

In consequence of the disease pathogeny, brucellosis vaccines must be able to protect from systemic and uterine infection [9], preferentially covering the animal entire life. The long-lasting protection is particularly important since vaccination of adult animals is not recommended for both cattle and small ruminants, due to the persistence of vaccinal antibodies, considering S19 and Rev. 1, but also due to the risk of abortions and bacteria excretion in the milk [8], in case of RB51 vaccination [30–32]. *Brucella* vaccines must be also able to protect against abortion and reproductive outcomes, which are the most important clinical signs of the disease and the primary cause of economic losses. Also, abortion products/post-partum vaginal discharges are the main source of *Brucella* spp. to the other animals in the herd, since it carry a great load of B. abortus [10^{14} colony forming units (UFC) per gram of material], which contributes to the spread of the disease [33,34]. Therefore, controlling abortion is a key measure

for break brucellosis transmission cycle and, thus, it is the main target of brucellosis vaccination.

It was already mentioned that a vaccine must be attenuated and do not cause disease in the vaccinated animal. In order to guarantee the absence of risk imposed by the attenuated brucellosis vaccines, WOAH standardized tests for safety and residual virulence for the officially recommended vaccine strains [8] (exception: for RB51 there is no recommendation of residual virulence test). In addition to being safe for the target species, it would be desirable for brucellosis vaccines to be non-pathogenic for humans, a characteristic that none of the currently used vaccines possess [9,17]. Hence, accidental exposure of humans to live attenuated vaccine strains, either during inoculation of animals or during vaccine production in laboratories, often results in clinical disease in these individuals, which contributes to the increase of brucellosis prevalence in humans and characterize brucellosis as an occupational disease [33,35]. This is another issue that justifies the efforts for finding a non-live and effective vaccine for brucellosis, however, inducting the necessary type of immunity to protect animals (Th1) is even more challenging with this type of vaccines.

Vaccine strains are inoculated many times in culture media and animal models for vaccine production along the years. Therefore, another characteristic to be considered for an ideal vaccine is its stability to remain attenuated and do not revert virulence. Likewise, the strains cannot lose virulence, otherwise they may become less immunogenic and stop inducing satisfactory immune response. To guarantee the adequate virulence, WOAH also preconizes potency tests in mice for the approved vaccine strains [8]. Finally, in addition to all characteristic listed, brucellosis vaccines should not be expensive [9], in order to the price not hamper control programs. This is a possible disadvantage of new vaccine technologies, since they tend to be more expensive.

In summary, an ideal brucellosis vaccine should (1) induce a lasting and strong Th1 immune response; (2) do not induce vaccinal antibodies detectable by the routine serological tests for brucellosis; (3) protect from systemic and uterine diseases, and, mainly, abortion; (4) not cause disease or abortion in vaccinated animal; (5) be eligible for revaccination of adult animals; (6) not be excreted in milk of vaccinated animals; (7) not be pathogenic for humans; (8) not revert or lose virulence, being stable; and (9) not be expensive.

4. History, characteristics and efficacy of the vaccine strains currently used for brucellosis control

Three brucellosis vaccine strains are recognized by the WOAH: the smooth *B. abortus* strain 19 (S19), the rough *B. abortus* RB51, and the smooth *B. melitensis* Rev.1, being the first two used for cattle vaccination, while the last one is preconized for vaccination of sheep and goats. There are no official vaccines for swine nowadays. All of these vaccines are used as live vaccines and are pathogenic for humans, being produced in authorized biosafety level 3 laboratories (BSL3) , under strict biosafety procedures [8]. It is worth to mention that the purpose of *Brucella* vaccines is protect animals from infection and abortion, and there is no effect of disease attenuation or reversion by inoculation of vaccine strains as therapy [9]. So, in the next sections, we will report the efficacy of brucellosis vaccine strains on protecting from infection and abortion, in addition to their history and characteristics.

4.1. S19 vaccine strain

Strain 19 was reported as a potential vaccine strain in 1930, by Dr. John Buck [36]. The strain was isolated from milk of a Jersey cow 7 years before (1923) and was initially virulent. However, the culture was accidently left out at room temperature for about a year or more and, after this period, when re-inoculated in guinea pigs, it was observed that the virulence was reduced compared to the previous tests. The given name was strain 19 because it was the 19th of the stock culture series isolated by Dr. Buck [17]. After that, S19 was tested as a live vaccine strain for cattle immunization, with good results [36,37] that culminated into its use in the field for the first time in 1941, in the United States of America (USA) [17].

S19 is a smooth *B. abortus* biovar 1 strain and its biological characteristics are stable, not being altered by several passages in culture media or animal models [38]. The most important characteristics of S19 are: low and stable pathogenicity, relatively high immunogenicity, and antigenicity [17]. Biochemically, S19 strain is very similar to any *B. abortus* biovar 1 strain, except by its independence of CO_2 for growth and sensitivity to thionine blue, penicillin and safranin O [39]. Another difference is that S19 is inhibited in the presence of erythritol, while the other biovar 1 strains are stimulated [38].

Regarding the immune response triggered, S19 vaccination induces a strong Th1 polarization in cattle, with proliferation of specific $CD4^+$ and $CD8^+$ T-cells. $CD4^+$ T-cells are the main source of IFN-γ and IL-17A, while $CD8^+$ T-cells present a strong cytotoxic activity through Granzyme B production. Memory cells of both populations are also induced by

vaccination. S19 also stimulates B memory cells, being IgG1 the most important subclass of produced antibodies [25,40].

The main disadvantage of the S19 strain is the induction of antibodies that cannot be differentiated from those induced by infection, in other words, S19 is not a DIVA strain. Also, in some cases, animals can develop antibodies persistence, which is directly related to dose, age at vaccination, method of administration (subcutaneous, conjunctival or intramuscular), and status of pregnancy [17]. To overcome this limitation, for many years researchers focused on testing different vaccine doses, routes, and ages at vaccination, in order to find the best strategy for reducing the duration of antibodies titers induced by vaccination.

Currently, S19 is recommended for vaccination of female calves, usually between 3 and 8 months of age, and studies indicate that there is no difference in the trigged immunity or vaccine efficacy within this period [41–43]. However, it is also known that the younger the calf is vaccinated, the faster the vaccinal antibodies will decrease [42–44]. Also, two out of every 100,000 vaccinated calves will have persistent S19 infection until adult life [45].

The recommended dose for calves by WOAH is 5–8 × 10^{10} viable organisms, by subcutaneous route. The organization also states that a reduced dose, from 3 × 10^{8} to 5 × 10^{9} CFU (colony-forming unit), can be administrated subcutaneously in adult animals to reduce chances of interference on serological diagnosis and improve control strategies. Nevertheless, this approach is highly discouraged since pregnant animals may abort and excrete the bacteria in the milk [8]. In addition, the probability of developing persistent antibodies is also higher in sexually mature animals, especially in pregnant cows [46]. In terms of diagnosis, animals vaccinated with S19 during calfhood must be tested for brucellosis only after the 20th month of age, to avoid false positive results due the presence of vaccine antibodies. The immunity conferred by S19 is lasting and one dose of S19 vaccine during calfhood (around 6 months) is enough to protect from brucellosis until the fifth pregnancy [47], which generally is productivity-life-long immunity for the target species.

Along the years, many studies performed challenge trials to evaluate S19 vaccine efficacy for protecting cattle from brucellosis caused by *B. abortus*. In general, studies pointed out that the efficacy of S19 reduced dose (10^{9} CFU) to protect from infection [72% of vaccine efficacy (VE), 95% Confidence Interval (CI): 58-81%] is very similar to protection from abortion (VE: 75%, 95% CI: 48-88%), according to a systematic review with metanalysis on controlled studies using S19 vaccination [14]. In addition, this same systematic review showed that animals vaccinated with reduced dose exhibited four times less risk of abortion when compared to the control group, while, for the regular dose (10^{10} CFU), the value was only 1.89.

These results suggest that reduced dose would be more effective for controlling abortion [14] than the full-dose, though WOAH currently recommendation remains the full dose.

The available literature also indicates that the S19 strain is effective to control bovine brucellosis in the field, in herds with moderate and high prevalences, with several studies pointing out to more than 80% effectiveness [18]. However, it is important to mention that many studies that evaluated S19 effectiveness also implemented other control policies (as elimination of positive animals) during the study, hampering the exclusive evaluation of vaccine performance [18]. Nevertheless, it is indubitable that S19 is effective and contributes to brucellosis control, since the strain has been the most used brucellosis vaccine worldwide, playing a critical role in the achievement of brucellosis free status in most of the countries that are so far considered brucellosis-free. Some authors suggest that S19 strain is also effective for protecting from *B. melitensis* infection [48], being used in Russia for protecting small ruminants [49], however the English literature in this topic is much scarcer and evidence is not as consistent as for protection against *B. abortus* infection.

4.2. RB51 vaccine strain

RB51 strain is a mutant strain developed in 1982 by Dr. Gerhardt Schurig's group [50]. The strain was obtained by isolating rough colonies from serial passages of the virulent smooth *B. abortus* strain 2308 on culture media with varying concentrations of rifampicin or penicillin. Thereafter, it was observed that RB51 strain was stable, not reverting virulence by *in vitro* and *in vivo* passages. The name RB51 comes from "R" standing for "rough", "B" for "*Brucella*" and 51 is an internal laboratory nomenclature [51]. RB51 was proposed as a rough vaccine strain in 1991 and, since 1996, it is an official vaccine for bovine brucellosis control in the USA.

The rough and stable phenotype of RB51 strain is due to an IS711 element interrupting the gene (*wboA*), which encodes a glycosyl transferase responsible for O-side chain synthesis, leading to a lack of its expression [52]. Biochemically, RB51 resembles the parental smooth strain 2308 and is able to metabolize erythritol. The strain also presents low pathogenicity and good immunogenicity [51]. However, RB51 is resistant to rifampicin and does not induce antibodies detectable by routine serological tests, which encumbers the diagnosis and treatment of human cases caused by accidental exposure to this vaccine [53].

RB51 also induces a strong Th1 immune response in cattle, with proliferation of $CD4^+$ and $CD8^+$ T-cells. In the same way as for S19, $CD4^+$ T lymphocytes are the main responsible for IFN-γ and IL-17A, whereas $CD8^+$ T cells produce mainly granzyme B and perforin. RB51

vaccination also induces $CD4^+$ and $CD8^+$ T memory cells, but it does not induce significative number of memory B cells, when compared to S19 [25].

Being a rough strain, RB51 does not induce the production of antibodies against LPS O-chain , which is the main target of most serological tests used for diagnosis of brucellosis [29,54]. This feature makes RB51 a DIVA vaccine and vaccinated animals can be tested any time after vaccination, which is the main advantage of the use of this vaccine strain and leads it to be adopted in several countries. The DIVA characteristic of RB51 is especially important in regions with very low prevalence of brucellosis, eradication zones and to manage outbreaks of the disease, allowing the simultaneous use of the two main strategies for brucellosis control and eradication: vaccination and test-and-slaughter policies.

RB51 is recommended for vaccination of calves between 4 and 12 months of age with 1–3.4 × 10^{10} viable organisms, by subcutaneous route [8]. There is no evidence of differences in the immune response triggered in calves by vaccination within this period [41]. Vaccination or revaccination of adult animals with RB51 is allowed in some countries, the booster vaccination increase the immunity induced by prime vaccination by stimulating the proliferation of $CD4^+$ and $CD8^+$ effector and memory T-cells and increase of IFN-γ production, both for animals vaccinated with S19 or RB51 at calfhood [25]. Vaccination or revaccination of adult animals with RB51 could be an important strategy for brucellosis control in areas with a low vaccination coverage or in outbreaks not only for increasing the immune response of previously vaccinated animals, but mainly for increase the herd immunity. Moreover, calfhood RB51 vaccinated animals have a decrease in immunity around four years after vaccination and must be revaccinated by this time [55]. One of the concerns of the use of RB51 in adult animals is the risk of abortion and spread of the vaccine strain through the milk after vaccination of pregnant cows [8]. Many studies evaluated the risk of abortion induce by RB51 and showed that it is very low, around 0.5%, which is very much lower than the risk of abortion by brucellosis itself [51,56–58]. There are some reports on the excretion of RB51 in milk of adult vaccinated cows [31,32] and human infection by RB51 associated with the consumption of the raw milk [59–61]. This risk can be minimized by milk pasteurization, which destroys all *Brucella* spp. strains [62], however there are no consensus so far about the public health associated with vaccination of adults with RB51.

RB51 is the most recent among the brucellosis vaccines recommended by WOAH [8], however, many studies have evaluated its efficacy and a metanalysis pointed out that the protection conferred by RB51 is equivalent to the given by S19 strain, with 69% (95% CI: 39-

84%) efficacy against abortion and 57% (95% CI: 31-73%) against infection, using the regular dose (1–3.4 × 10^{10} CFU) [14]. The role of RB51 on controlling brucellosis in field points out for effectiveness greater than 90% especially with adoption of other control measures [63–66]. Regarding large-scale eradication programs, RB51 was adopted as vaccine strain after most of the brucellosis free countries achieved this status. Even though, RB51 was reported as a successful complementary tool for brucellosis eradication in regions of Portugal (Azores region) [58] and Spain (Extremadura region) [57]. Thus, likewise S19, there is consistent scientific evidence to state that RB51 strain is effective for controlling brucellosis infection and abortion under both controlled and field conditions. The protection against *B. melitensis* conferred by RB51 strain in cattle is unknown.

4.3. Rev.1 vaccine strain

Rev.1 strain is a biovar 1 mutant obtained from the *B. melitensis* 6056 strain by Dr. Elberg and Dr. Faunce, in the mid-1950's. The initial idea was to develop a streptomycin-dependent strain and control its growth after animal inoculation by the co-injection of the antimicrobial agent [67]. The streptomycin-dependent strain was developed but results of subsequent tests were not as promising in guinea pigs as in mouse [68]. So, the dependent strain was cultured in the absence of the drug and new mutant colonies were observed, being streptomycin independent. These strains showed a good level of attenuation when compared to a virulent strain in mice and guinea pigs and conferred a promising protection level from brucellosis in these animals. Thus, further studies lead to the isolation of a stable colony from this culture and other studies were performed to evaluate the performance of new vaccine – now named Rev.1 – in mouse, guinea pigs and goats, showing satisfactory results [69–71]. From 1960's, this vaccine started to be used to prevent animal brucellosis and currently is the preferential strain for sheep and goat immunization for the control of *B. melitensis* infection [8].

Biochemically, Rev.1 strain is very similar to the other *B. melitensis* strains and has a smooth phenotype. A difference when compared to other *B. melitensis* field strains is its susceptibility to high concentrations of fuchsin and thionin [39]. It is also resistant to 2.5 μg/mL of streptomycin and susceptible to 5 IU of penicillin G, which allows its differentiation from field strains [70,72]. Cellular immune response induced by Rev.1 vaccine is not yet well stablished in the target species (sheep and goat). A study in goats observed that, unlikely the S19 and RB51 strains in cattle, $CD4^+$ T-cells seems not to be the most important cells proliferating after Rev.1 vaccination. Also, IFN-γ seems to be mainly produced by γδ T cells, not by $CD4^+$ T cells and there is no information about the role of $CD8^+$ T cells on the immune

response of goats and sheep after vaccination [73]. Regarding antibodies production, Rev.1, as S19, is not a DIVA strain and induces long-lasting antibodies that are detectable in the serologic tests, mainly when the subcutaneous route is used and adult animals are vaccinated [74]. Alternatively, conjunctival route can be used, triggering similar protection, but with reduced magnitude and persistence of vaccinal antibodies [8,75].

Differently from cattle vaccination, Rev.1 strain is used for protecting both male and female small ruminants from brucellosis. According to the WOAH, independently of the route (subcutaneous or conjunctival), the vaccine dose must be $0.5–2.0 \times 10^9$ viable microorganisms and should be administrated to lambs and kids from 3 to 5 months of age [8]. Five months is the limit of age for minimizing antibody response and not compromising diagnosis. Reduced doses are not recommended for sheep and goats, since it confers a significantly lower protection [8,75]. For some endemic areas, vaccination of the whole population can be considered, even for adult animals, but the vaccine strain can be eliminated in the milk and in the vaginal discharge when pregnant animals are vaccinated [76,77]. Thus, for control / eradication programs, conjunctival is the preferential route for both pregnant and non-pregnant animals, to avoid side-effects, such as vaccinal strain shedding and persistent vaccinal antibody titers. However, even when conjunctival route is adopted, diagnosis with serological tests should be carefully conducted.

In terms of efficacy trials, the literature for Rev.1 is scarcer compared to S19 and RB51. However, in goats, vaccination of non-pregnant animals showed 93% (95% CI: 55-99%) of efficacy for protecting against infection after experimental challenge with the virulent strain *B. melitensis* H38 [78]. This same study also observed the superiority of Rev.1 in relation to S19 on protecting goats from *B. melitensis*. Another study was conducted in ewes vaccinated at 4-5 months of age and then experimentally challenged in the first pregnancy (83-85 days of pregnancy). The results showed around 70% (95% CI: 34-88%) of efficacy (values calculated by the authors of this chapter) for protection against abortion using the full dose ($0.5–2.0 \times 10^9$ CFU), regardless of the route (subcutaneous or conjunctival). Protection against infection was 56% (CI 95%: 21-60%) and 37% (CI 95%: 3-59%) for subcutaneous and conjunctival routes, respectively, using the same dose. It is worth to mention that the authors of this study considered as protected from infection animals that did not abort, excreted *Brucella* in the uterine discharge (at the parturition day and in the day after) or were negative by culture of all evaluated tissues after slaughter, but differentiation between vaccinal and challenge strain was not performed [75], which could decrease the vaccine efficacy values. Rev.1 was also shown to protect rams

vaccinated at 3 months-old and revaccinated at 14 months-old against infection with *B. ovis*, with 67% efficacy (95% CI: 34-83%, values calculated by the authors of this chapter) [79].

Regarding field efficacy and large-scale eradication programs, Rev.1 performance is very debated and problems as vaccine quality, vaccination coverage, interference of vaccinal antibodies in diagnosis and abortion in pregnant animals are pointed. Despite these problems, in general, it is possible to state that Rev.1 vaccination is important for brucellosis control and eradication programs and that, after vaccination, a significant reduction of brucellosis in the vaccinated flocks is observed [72].

4.4. Other live vaccines

As mentioned, vaccine strains other than the three WOAH recommended ones have been tested and even used for animal brucellosis control and eradication. The most used strains and their characteristics will be described.

In Russia, besides S19, two live vaccines are used for cattle vaccination in different ages: the *B. abortus* SR82 and *B. abortus* 75/79 [49]. SR82 is a biovar 6 strain, has a smooth-rough phenotype, and its main characteristics are: low virulence, reduced agglutinogenic properties, immunogenicity and protection against infection [80]. In addition, animals can be tested 3–6 months after vaccination and, as disadvantages, it can cause abortion. The vaccine was field implemented in 1982 in Russia, being its most used vaccine strain and the one related to the eradication of brucellosis in many regions of the country. The vaccine was also tested with promising results in reindeer, buffalo, elk, yaks, and pigs [81].

B. abortus strain 75/79-AB is a mutant used for cattle vaccination in Russia obtained in 1979 from a cow vaccinated twice (in 1974 and 1976) with strain 82. Subsequently, the strain was posteriorly selected by multiple passages in guinea pigs, heifers and nutrient media. Final strain presents rough phenotype and vaccinated cattle completely lost agglutinins and complement fixation antibodies up to 90 days after vaccination. It is reported that the strain does not cause abortions, is not virulent in guinea pigs, is not excreted in milk, and is effective to protect from infection, in both in controlled and field conditions [82].

China has three licensed live vaccines for animal brucellosis control: *Brucella suis* strain S2, *B. melitensis* strain M5, and *B. abortus* strain A19 [83]. S2 was obtained from a *B. suis* bv.1 strain isolated from an aborted fetus of pig in 1952, by China Institute of Veterinary Drug Control [27,84,85]. The virulent strain was passed multiple times on media and a naturally attenuated mutant was obtained and tested in guinea pigs, mice, sheep, pigs, and rhesus monkeys. Further tests in guinea pigs, goat, pigs, pregnant goat, pregnant sheep, pregnant

swine, and rabbit showed satisfactory immunity against brucellosis conferred by S2. Biochemically, S2 is a smooth *Brucella*, CO_2 independent, resistant to thionin and susceptible to fuchsin, exhibits an extreme urease activity, and agglutinates with monovalent specific anti-bovine serum (A) but not with monovalent specific anti-goat serum (M).

The strain was introduced in China in a pilot trial in 1960s and, since then, has been used in the country for immunization mainly of pigs, but also cattle, goat and sheep, being also adopted in other countries along the years [84]. The vaccine is allowed in China for vaccination of male and female cattle, goat, sheep, and pigs. Oral (diluted in the drinking water), subcutaneous and intramuscular routes are suitable for goats, sheep, and pigs, while for cattle, small fat-tail sheep, and pregnant animals in general, only oral route is allowed. Reported protection against infection varies according to route and species, from 60 to more than 90% [27,84]. China national experience also supports the effectiveness of the vaccine in these species (pigs, cattle, goat and sheep), observing decrease in brucellosis prevalence comparing pre and post-immunization rates, especially using oral route [83,84].

B. melitensis M5 strain was developed in 1962 by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The attenuated strain was obtained from the sheep virulent smooth strain *B. melitensis* M28 by several passages in chicken, acriflavine treatment and 90 generations of chicken embryo fibroblasts. The main advantage of this vaccine is the immunization by aerosol, providing protection for sheep and goat for almost one year. In 1970, the strain was massively used in China for controlling disease in these hosts, with good results. As disadvantages, M5 has: (1) it is the most virulent among the authorized brucellosis vaccines in China, especially to humans, leading to high seroreaction, (2) it is unstable and mutations often occurs, and (3) it is not a DIVA strain. Because of these characteristics, M5 is only occasionally used in epidemic areas [85].

B. abortus strain A19 has a historical correlation with strain S19, with 99.9% of homology. The main difference is the presence of the gene for erythritol metabolization in A19, which is absent in S19, conferring to the last one greater attenuation. A19 has been used in China since 1950 for cattle immunization and is reported as having a stable virulence and immunogenic, conferring good protection. As S19, A19 is a smooth strain and induces antibodies detectable in the serological tests [86,87].

5. Factors that may impact efficacy and other vaccination aspects on brucellosis

A vaccine must be able to induce immunity without causing disease. Therefore, a vaccine strain is the primary contact with the microorganism in order to train the immune system for responding quicker and more effectively when/if the animal is exposed to the virulent pathogen. For this purpose, the vaccine must be able not only to induce immune response, but more importantly, to induce the production of memory cells (T and B lymphocytes). For brucellosis, since antibodies do not play a central role in protective immunity, the principal role of a good brucellosis vaccine is to induce T $CD4^+$ and T $CD8^+$ memory and effector cells, the latter ones will proliferate, produce cytokines and hamper disease.

Many factors can impact immune response to vaccination, changing the magnitude and the type of response and memory acquisition. In this section, we will discuss those more important aspects involving brucellosis vaccination, which can be classified as intrinsic host factors, vaccine and administration factors. It is worth to reiterate that, in vaccination studies, only one factor must be evaluated at a time (e.g. different ages, but same dosage, sex, route etc.) to avoid bias, otherwise it is not possible to understand which factor is leading to the observed (or not) differences.

5.1. Intrinsic host factors

Each animal is a complex organism, and variations are expected, since many individual characteristics influence the establishment of the immune response. Here, we will discuss the main animal intrinsic characteristics that are known to impact brucellosis vaccination, which are: age at vaccination, sex, and status of pregnancy.

Age impacts vaccination mainly in the extreme of life, e.g. young and old animals [88] but, since brucellosis vaccines are administered to livestock animals, young animals are the principal issue. In order to protect the individual and, consequently, improve herd immunity, calves, lambs, kids and piglets must be vaccinated as soon as possible; however, two factors can impact neonate immunity: maturity of the immune system and interference of maternal antibodies acquired from colostrum administration [88,89]. For cattle, it was observed that antibodies production is impaired if animals are vaccinated before 75 days of age compared to older animals, being an indicative that the immune system is not enough mature to react adequately [90,91]. In addition, the older is the animal, the stronger and long-lasting is the antibody production, supporting that there is really an impact of the maturity of immune system [43], even though humoral is not the target response for brucellosis. However, within the age range recommended for calves vaccination, studies suggest that there is no difference in the

cell-mediate immune response in the protective effect conferred by S19 and RB51 strains [41–43].

Sex also influence on immunity and this phenomenon is called sexual dimorphism immunity [88,92]. Although males and females have the same immune organs and cells, response to microorganisms and self-antigens are different. For example, females tend to lead better with infectious diseases than males, inducing higher and prolonged humoral and cell-mediate immune responses, in addition to higher innate response. On the other hand, females are more susceptible to immune-mediated diseases [92]. Vaccination with S19 strain may induce localized infection in the genital tract of males, while, in females, persistent infections are rare. Therefore, since males do not play as an important role for brucellosis transmission as females, vaccination of bulls and male calves are not recommended [8]. Females, in contrast, are the vaccination target to break transmission chain, since aborted fetus and post-abortion or post-partum secretions have a very high number of *Brucella* spp., and, associated to excretion in colostrum and milk, are the main source of *Brucella* spp. to other animals in the herd [33]. Regarding swine brucellosis, males tend to have more persistent infections caused by field strains than females, with lesions in the reproductive tract [8]. Thus, in this case, the relevance of disease in males justify their vaccination. In fact, in China where *B. suis* S2 vaccine is largely used, vaccination is performed in both sexes [84].

It is commonly known that immune response is different in pregnant and non-pregnant animals, since it is influenced by hormonal alterations and a fetus tolerance must be established. However, there is also a difference considering stages of pregnancy. Brucellosis vaccination of pregnant animals, although could lead to abortion in cattle and small ruminants, can be suggested and effectively used in some cases, as stated before. Thus, the understanding of the best stage of pregnancy for vaccination is very relevant. In cattle, pregnant animals vaccinated with S19 tends to retain vaccinal antibodies longer than non-vaccinated animals, indicating an influence of pregnancy on humoral immunity [46]. Regarding efficacy against infection, a study compared protection in cows vaccinated with S19 at first, second and third trimester of gestation: vaccine only confers significant protection when performed in the second trimester of pregnancy (55% of efficacy), while first and third trimesters groups were not different from non-vaccinated group [93].

5.2. Vaccine and administration factors

Vaccine quality is a crucial point for brucellosis control programs [7] and, for reaching results as close as possible of the ideal, vaccine production and vaccination management must be standardized and performed according to the factory recommendations. Production of the official vaccines are regulated and there are WOAH standardized production protocols and tests for assessments of its immunogenicity, potency, residual virulence and safety, in order to assure its excellence [8]. At field level, factors as vaccine expiration time and storage conditions, before and during vaccination procedures, are also fundamental and highly related to the vaccine performance on inducing adequate immunity, and thereby, must be strictly followed. On the other hand, vaccination procedures, as dose and route of inoculation, may change according to the vaccination strategy.

Attenuated vaccines must mimic infection for inducing immune system to respond and generate memory. This is directly related to the dose – or number of viable microorganisms (in case of live vaccines) – inoculated in the animal. A very low dose probably will be easily neutralized by innate mechanisms of immune response, avoiding activation of adaptative response and memory induction. Also, for achieving a high Th1 immune response, which is expected in the case for brucellosis, vaccine strains must be phagocytosed and multiply in antigen presenting cells. Specially for live vaccines, an inoculum with too many cells can be a challenge for the animal, causing colonization and disease, even though vaccine strains are attenuated.

Generally, determination of vaccine dose usually is performed during early stages of vaccine development, by a combination of optimal immunogenicity and safety [20]. For brucellosis, before the RB51 strain development, several studies were performed with reduced doses of S19 strain, aiming to minimize impacts in serological diagnosis. In fact, in these studies, it was observed the effect of dose in magnitude and lasting of antibodies titers in cattle [94,95], without prejudicing immunity. In fact, a metanalysis on S19 efficacy showed better performance of reduced dose when compared to full dose to protect from infection and abortion [14]. In contrast, regarding Rev.1 vaccination for small ruminants, studies showed that reduced dose is not as effective as the full dose, therefore it is not recommended [75].

In addition to dose, the route of administration can impact immune response to vaccination. Microorganisms have preferential entering sites that favors colonization and the immune system respond in different ways according to the inoculation site [20,96]. Since vaccines for brucellosis are adopted for livestock animals, a balanced association between bacteria replication, immune response and management practices must be achieved. For

brucellosis, route of administration seems to have low impact of vaccine efficacy [97], however it influences on seroconversion and can be used for minimizing impact of S19 vaccinal antibodies in diagnosis. Subcutaneous administration induces more lasting antibodies than intradermal or conjunctival routes [95,96]. In normal situations, the preconized route for cattle is subcutaneously [8], due its effectivity allied to the suitability for administration in field, especially for mass vaccination. The administration route can favor strain colonization and, for S2 strain vaccination in cattle, subcutaneous vaccination was abolished because of its high virulence when administrated through this via, being only allowed by oral route in China [84].

6. Final considerations: beyond vaccine efficacy

Animal brucellosis is a complex disease, and, as largely discussed, vaccine efficacy can be influenced by many factors. There are different options of brucellosis vaccine strains, and more are in development and testing phases. This highlights the importance of disease and the compromise of the scientific community with its control and eradication worldwide.

Brucellosis vaccines have been extensively studied for more than 100 years and there are still many questions to respond. Therefore, choosing a vaccine for a control/eradication program of animal brucellosis is far from a simple task. Many other aspects than vaccine efficacy must be considered, and this process requires careful assessment of advantages and drawbacks of each vaccine option. Aspects regarding farm, region and country context must not be neglected if the intention is to have a successful control or eradication program. Hence, there is not a best vaccine strain, but circumstances that they fit better.

In this scenario, extremely relevant aspects to taking into consideration is the availability of the strain in the country or region, the country legislation and vaccine cost, as financial affordability is very desirable for a brucellosis vaccine to be included in a control program. Since most of brucellosis control programs are based in mandatory and massive vaccination, price is a very sensible issue, even more if the costs of vaccine and vaccination must be covered by farmers. In general, live vaccine strains are less expensive than other technologies, especially since brucellosis vaccines usually do not have adjuvants; but still, some strains cost more than others and it should be cautiously considered for adoption on a control program.

Another important aspect is the objective of the vaccination approach, such as control in endemic or epidemic areas, eradication in a region or country, achievement of free status for

a farm, etc. It is important to highlight that vaccination is a key and a very important tool of control phase of programs but used as the only control tool will be not able to effectively eradicate brucellosis. In endemic areas, vaccination of all young animals (females, in cattle case) associated to test-and-slaughter policy (elimination of positive animals), is the most common strategy adopted. However, even for this approach, vaccination management must be aligned with diagnosis strategy, mainly when non-DIVA strains will be employed. In these cases, limitation of age for vaccination must be strictly respected and animals can only be tested on a time after decrease of vaccinal antibodies, otherwise many false-positive animals will be detected, burdening farmers and government.

If the aim is brucellosis eradication in a low prevalence area or even a farm, DIVA strains are the recommended vaccines, since it is possible to test animals immediately after vaccination, accelerating the decision-making process. DIVA strains can also be used in outbreaks and neighbor areas, with massive vaccination of animals of all ages, without prejudicing the serological diagnosis of infected animals. Reduce doses and other routes can be considered as alternatives for DIVA strains, taking into consideration the risks of vaccination of adult animals. Vaccination of pregnant cows with RB51 must be carefully thought out, since there is a chance of excretion of the bacteria in milk and monitoring and milk pasteurization must be used.

In conclusion, brucellosis is a complex and dynamic disease, which directly impact choosing a vaccination approach. Hence, decision-makers must be aware of all aspects of a vaccine strain before designing a brucellosis control plan and recognize that no approach will be perfect, but the most appropriate considering the circumstances.

Figure 1. Glossary of terms on veterinary vaccination.

Glossary	
Vaccine efficacy	Vaccine ability to protect the animal against infection or against the disease clinical signs, evaluated by studies with experimental challenges, i.e. clinical trials.
Vaccine effectiveness	Vaccine ability to protect against infection or against the disease clinical signs under field conditions, assessed by observational studies, with natural challenge, e.g. cohort, prevalence panels.
Immunogenicity	Vaccine ability to induce an immune response. It can be used as an indirect parameter to evaluate vaccine efficacy, may be measured by immune systems products, e.g. interferon gamma

Potency	Viability or infectivity of a vaccine strain, related to the number of bacteria inoculated required to stimulate immune system
Residual virulence	Vaccine capacity to replicate in the host response and to stimulate immune system, related to the time interval necessary to eliminate the vaccine strain from the animal, known as clearance, e.g. 5 weeks.
DIVA	Vaccine property that allows differentiating vaccinated from infected animals, i.e. do not induce production of antibodies detectable in serological tests used for diagnosis, e. g. RB51.

Table 1. World Organisation for Animal Health (WOAH) official brucellosis vaccines: characteristics and efficacy.

Vaccine strain	Phenotype	Characteristics that allow differentiation from field strains	Target species	Sex	Age at vaccination	Route	Dose (CFU/mL)	Vaccine efficacy	Reference
B. abortus S19	smooth	Independence of CO_2; sensitivity to thionine, penicillin and safranin O; inhibited by erythritol	cattle	female	3 – 8 months	subcutaneous	$0.3–5 \times 10^9$ (reduced dose)	72% (CI95%: 58-81%)[a] 75% (CI95%: 48-88%)[b]	[14]
							$5–8 \times 10^{10}$	47% (CI95%: 29-60%)[b]	[14]
B. abortus RB51	rough	Agglutination in acriflavine; resistance to rifampicin	cattle	female	Young and adult animals	subcutaneous	$1–3.4 \times 10^{10}$	57% (CI95%: 31%-73%)[a] 69% (CI95%: 39%-84%)[b]	[14]

B. melitensis Rev1	smooth	Susceptibility to high concentrations of fuchsin and thionin; resistance to 2.5 μg/mL of streptomycin and susceptible to 5 IU of penicillin G	goat	female	adult	subcutaneous or conjunctival	0.5–2 × 10^9	93% (CI 95%: 55-99%)[a**]	[78]
			sheep	female	4-5 months	subcutaneous	0.5–2.0 × 10^9	56% (CI95%: 21-60%)[a**] 70% (95% CI: 34-88%)[b**]	[75]
			sheep	female	4-5 months	conjunctival	0.5–2.0 × 10^9	37% (CI95%: 3-59%)[a**] 70% (95% CI: 34-88%) [b**]	[75]
			sheep	male	3 months	Subcutaneous	0.5–2.0 × 10^9	67% (95% CI: 34-83%)[a**]	[79]

CFU: Colony forming unit; 95% CI: Confidence interval of 95%; a: protection against infection; b: protection against abortion; ** Calculated by this chapter authors.

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CHAPTER II

Published at: Veterinary Immunology and Immunopathology (doi: 10.1016/j.vetimm.2025.110950)

Does lipopolysaccharide morphology (smooth or rough) of *Brucella abortus* vaccine strains influence the potency or efficacy of the vaccine?

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Abstract

Brucella abortus exhibits the dissociation phenomenon, in which naturally smooth samples lose the O chain of lipopolysaccharide (LPS) and become rough, associated with changes in colony shape, culture characteristics, cell morphology, immunological reactions, biochemical reactions and, possibly, virulence. However, the significance and impact of S-R dissociation in cultures (*in vitro*) or even *in vivo* is unclear, especially considering that rough samples have already been isolated from clinical samples in different hosts and, also, are successfully used as vaccine strains. Thus, the objective of this study was to review the literature on *Brucella* spp. LPS to better understand the impact of the LPS morphology in *B. abortus* in the vaccinal efficacy. The available information indicates that is undeniable that LPS is related to virulence modulation and inducing immunity in the natural hosts of *Brucella* spp. However, the continuous emergence of rough variants *in vivo* (infection) or *in vitro* (cultivation of the microorganism) suggests that this phenotype is part of the biology of the agent and may confer some survival advantage to the bacteria. In fact, for some samples, the permanent or temporary loss of the O chain (O-PS), whether natural or induced, did not necessarily imply a decrease in virulence, immunogenicity, or post-challenge induced protection, since results in both directions were observed in the literature, depending mainly on the parental samples used and the silenced genes. Thus, it is concluded that the emergence of variants related to the smooth/rough LPS of a sample of *B. abortus* does not necessarily imply changes in the virulence/immunogenicity of that sample and, consequently, in vaccine potency or efficacy, in case of vaccine strains.

Keywords: LPS, bovine brucellosis, vaccination, *rough* Brucella, *smooth* Brucella

1. Introduction

Brucellosis is a bacterial zoonosis caused by microorganisms of the genus *Brucella*, which affects a wide variety of animal species including humans (Corbel et al. 2006). Despite the disease had been eradicated in several countries, it continues to be one of the most economically important zoonoses in the world (WOAH 2022a; Laine et al. 2023). In 1895, the pathologist L. F. Bernhard Bang associated one of the agents of brucellosis with cases of abortions in cattle (Nicoletti 2002), and, since then, the disease has been reported in different species of domestic (goats, sheep, pigs, dogs, cats) and wild animals (camelids, antelopes, buffalo, bison, elk, baboons, frogs) (WOAH 2022a).

Currently, the genus *Brucella* includes thirteen known species, differentiated according to their phenotypic and genotypic characteristics and host preference: *B. melitensis*, *B. suis*, *B. abortus*, *B. neotomae*, *B. canis*, *B. ovis*, *B. ceti*, *B. pinnipedialis*, *B. inopinata*, *B. papionis*, *B. vulpis,* and *B. nosferati* (Whatmore and Foster 2021; Hernández-Mora et al. 2023). The genus is spread worldwide and species infecting domestic animals are responsible for significant losses in livestock, in addition to representing substantial burdens to public health (Scholz et al. 2018). In addition to these species, the International Committee on Systematics of Prokaryotes had recently reclassified the genus *Ochrobactrum* into *Brucella* (Hördt et al. 2020). The species of this genus, unlike the "traditional" *Brucella* species, are frequently isolated from the environment and considered opportunistic pathogens, affecting animals and humans (Ashford et al. 2020).

Among the traditional species of *Brucella* spp., only *B. canis* and *B. ovis* have a naturally rough colonial morphology (R), i.e., they do not have the O side chain (O-PS) in the external portion of the lipopolysaccharide (LPS) (Alton et al. 1988). In general, rough strains have lower zoonotic importance compared to the smooth strains (S), such as *B. melitensis*, *B. abortus* and *B. suis*, although *B. canis* also causes disease in humans (Whatmore and Foster 2021). *B. abortus*, which mainly affect cattle and buffaloes, stands out as one of the species of greatest importance in many countries, both for animals and humans. Due to its importance, many control programs have been implemented to control *B. abortus*, which are based mainly on the test-and-slaughter policy and on the vaccination (Zhang et al. 2018).

Although naturally smooth, *B. abortus* strains can exhibit the phenomenon of dissociation, that is associated with changes in the morphology of the colonies, culture characteristics, cell morphology, immunological reactions, biochemical reactions, and possibly virulence (Marshall and Jared 1931; Henry 1933; Braun 1946; Alton et al. 1988; Turse et al. 2011; Pei et al. 2014). Likewise, the vaccine strain *B. abortus* S19 can also dissociate and there is not a consensus in the literature regarding the maximum level of dissociation that should be present in vaccine batch (1%, 5%, and 15%) (Alton 1951; Alton et al. 1988; WOAH 2022b). Although these recommendations exist, no studies so far have clearly answered whether and how dissociation would influence the potency/efficacy of this vaccine. Thus, considering the lack of scientific information on this matter, this literature review was focused on highlighting the significance (*in vitro* and *in vivo*) of smooth (S) and rough (R) LPS in the context of natural infections and vaccinations, centering the discussion in *B. abortus* strains. Since dissociation is

a phenomenon that makes smooth strains permanently or temporarily rough, this study considered the knowledge accumulated from both naturally rough *Brucella* spp. strains and laboratory-obtained rough mutants, investigating how the temporary or permanent acquisition of this phenotype influenced aspects mainly related to the virulence, pathogenicity and immunogenicity of these isolates.

2. Morphology and structure of *B. abortus* and its LPS

Brucella abortus is a Gram-negative bacterium, shaped like short bacilli or coccobacilli, measuring 0.5-0.7 x 0.6-1.5 μm, non-motile, non-sporulating, and with positive results in the urease, catalase, oxidase, and nitrate reduction tests (Alton et al. 1988). Seven biovars or biotypes of *B. abortus* are known (1, 2, 3, 4, 5, 6, and 9) (WOAH 2022a). It is a facultative intracellular bacterium that multiplies mainly inside macrophages of its hosts (Celli 2019). Generally, the strains are strictly aerobic, however, certain biovars require an atmosphere containing CO_2 for primary isolation (Alton et al. 1988). Biotypes are differentiated according to CO_2 requirements, H_2S production, sensitivity to thionine and basic fuchsin dyes, and the type of antigens present in the surface (WOAH 2022a). *B. abortus* is classified as a biosafety level 3 microorganism, grows well at a temperature of 37 °C in culture media such as Tryptose or Tryptic Soy, producing colonies with smooth edges, translucent, pale and with 1-2 mm in diameter (Alton et al. 1988).

Liposaccharide is vital for the structural and functional integrity of the outer membrane of Gram-negative bacteria and contains several well-conserved domains, being one of the primary targets of mammalian innate immunity. In *Brucella* spp., for practical purposes, the main relevance of LPS is associated with virulence/pathogenicity/immunogenicity and vaccination/diagnosis. *B. abortus* has the O-PS antigen in their LPS composition, therefore strains are naturally smooth, although rough isolates are frequently observed under different culture conditions and have also been isolated causing infection in animal hosts (Marshall and Jared 1931; Henry 1933; Turse et al. 2011; Mancilla et al. 2012). The S-R (smooth-rough) dissociation of *B. abortus* strains occur spontaneously and continuously in the laboratory. In liquid medium, rough strains may present the formation of a film, sediment and fluid supernatant (Henry 1933). Rough colonies (R) grow faster, are more opaque and larger compared to smooth parental colonies (S), being also described with capacity of spontaneous agglutination (Plastridge and McAlpine 1930; Marshall and Jared 1931).

The *B. abortus* LPS is composed of three domains: the lipid A, the central oligosaccharide (core), and the O antigen or O side chain (O-PS) (Figure 1), the latter being completely or partially absent in rough strains. The O-polysaccharide (O chain or O-PS) of the smooth type of *Brucella* (S-LPS) is an unbranched homopolymer generally with an average chain length of 96 to 100 glycosyl subunits (Bundle et al. 1989). The O-polysaccharide is linked to a central oligosaccharide (core) composed of several sugars, which, in turn, is linked to the lipid A that is also formed by sugars, in addition to a hydrophobic region (Cardoso et al. 2006). Normally, the hydrophobic region of lipid A constitutes the external coating of the outer membrane and is responsible for many of the endotoxic properties attributed to the bacterial

LPS, which conversely are absent in the LPS of *Brucella* spp. (Bundle et al. 1989). Thus, *Brucella* spp. has a peculiar non-classical LPS compared to classical LPS of enterobacteria (Cardoso et al. 2006). *B. abortus* LPS exhibit chemical differences that are not restricted to the O chain but also present in the core region and lipid A, although the skeleton of this structure is preserved (Freer et al. 1995).

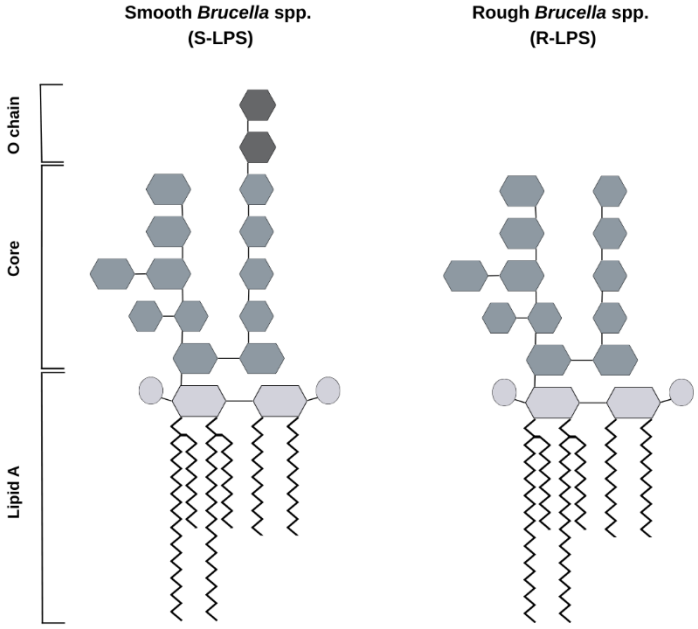

Figure 1 - Schematic structure of the lipopolysaccharide (LPS) of *Brucella* spp. smooth (S), which has the O-polysaccharide intact, and rough (R), which lacks this O side chain.

3. Dissociation mechanisms

The spontaneous dissociation of smooth into rough (S-R) strains has been observed in different situations *in vitro*, such as microbiological culture (Henry 1933; Braun 1946) and cell culture (Turse et al. 2011), as well as *in vivo*, both in laboratory animals (guinea pigs and mice) (Marshall and Jared 1931; Henry 1933; Turse et al. 2011) as in natural hosts (Henry 1933; McEwen 1940; Mancilla et al. 2012). Studies from the first half of the 20th century showed that long cultivation, cultivation in liquid medium (broth), oxygen limitation, high concentration of bacteria in culture (competition for nutrients) and acidic or basic pH, favor the dissociation process of smooth strains (McAlpine et al. 1929; Marshall and Jared 1931; Henry 1933; Braun 1946; Gerhardt 1958). In this sense, Mancilla et al. (2012) discuss two hypotheses for *in vitro* dissociation: (i) the energetic cost of O chain synthesis is not worth it given the absence of selection pressure; and (ii) smooth strains have genetic instabilities in genes involving the synthesis and expression of the O chain (O-PS).

The composition of the culture medium appears to be associated with different dissociation rates, although presence of metabolites or nutrient deficiencies in old cultures

seems not to be related with the phenomenon (Henry 1933; Braun 1946). On the contrary, high growth rates (shorter lag phase duration) appear to determine a higher percentage of dissociation (Braun 1946). Likewise, low oxygen tension and acidic pH promote S-R dissociation, with accumulation of rough cells in cultures that reach the stationary phase (Altenbern et al. 1957). Among several factors, dissociation is a continuous and spontaneous phenomenon and its levels *in vivo* and *in vitro* varies according to the strain, within the same species of the genus *Brucella* (Marshall and Jared 1931; Henry 1933; Mancilla et al. 2012), or even from different clones obtained from the same isolate (Henry 1933; Braun 1946). Thus, *in vitro* dissociation rates appear to be more associated with characteristics inherent to the strain/clone being cultivated, and can also be influenced by cultivation conditions, especially those that affect growth and viability rates, such as inoculum concentration, pH and oxygen tension. Based on the evidence in the literature, it is also possible to assume that all *B. abortus* strains isolated from cattle can produce rough clones, although some isolates are more prone to dissociate than others.

Although the causes of S-R dissociation of *Brucella* spp. strains have not been fully elucidated, recent advances have shed light on the genetic mechanisms involved in this event. In fact, the increasing number of sequenced *Brucella* spp. genomes and recent advances in bioinformatics have enabled a better understanding of the genetic mechanisms involved in the biological processes of this genus (Rajendhran 2021). In this sense, among the most studied genes, those linked to the synthesis and export of LPS stand out (Cloeckaert et al. 2000; Godfroid et al. 2000; Moriyón et al. 2004; Cardoso et al. 2006; Haag et al. 2010) (Table 1 and Figure 2).

To date, 11 genes related to the synthesis and transport of the O chain of *Brucella* spp. have been identified, mainly located in two regions of the genome, *wbo* and *wbk* (Cardoso et al. 2006; Mancilla 2016; Stranahan and Arenas-Gamboa 2021) (Table 1 and Figure 2). The GC% content of these two regions are lower compared to the rest of the genome of *Brucella* spp., suggesting a lateral (horizontal) acquisition of these regions that probably occurred before the microorganism have become intracellular (Godfroid et al. 2000; Chain et al. 2005). The *wbo* region is composed of the genes *wbo*A and *wbo*B, part of a genomic island GI-2, and encodes O-polysaccharide glycosyltransferases (Rajashekara et al. 2008). The *wbk* region consists of genes required for the synthesis of N-formyl-perosaminase, the polymerization of O-PS and its transport (ABC transporters) (Godfroid et al. 2000). Homologous recombination in the *wkb* region, especially in the *wbk*A gene, has been observed occurring spontaneously *in vitro* and *in vivo*, being indicated as one of the main factors responsible for the dissociation of smooth strains of *B. melitensis* (Mancilla et al. 2012). In fact, most variants resulting from S-R dissociation in the laboratory appear to occur through the deletion of the *wbk*A gene and the GI-2 genomic island (*wbo*), or by mutations in the mannose genes involved in the synthesis of the LPS core (Mancilla et al. 2010; Turse et al. 2011). Homologous recombination is one of the mechanisms responsible for intra- or inter-specific polymorphisms in bacteria and is also implicated in S-R dissociation. Studies carried out by Mancilla et al. (2012) suggested that the *rec*A gene may be involved in recombination events in the *wbk* region, although it is not the only gene responsible. In fact, mutations in the *int* gene, which is located in the GI-2 genomic island and is associated

to the encoding of a putative phage integrase, demonstrated the stabilization of the smooth phenotype in the *B. abortus* strain 2308 (Mancilla et al. 2010). However, it did not completely eliminate S-R dissociation, which suggests the presence of an additional mechanism. Corroborating with this hypothesis, results obtained by Turse et al. (2011) showed that the emerging rate of rough variants in culture is 2-3 log higher than the rate of mutations related to antimicrobial resistance, indicating that this phenomenon is, in fact, associated with more than one *locus*.

The O chain is an immunodominant antigen of smooth strains, to which most host antibodies are directed in case of vaccination/infection. Because of that, studies aiming to obtain DIVA (Differentiating Infected from Vaccinated Animals) vaccines have evaluated the role of several genes in the synthesis and expression of the O chain in the LPS, in order to obtain rough mutants to be evaluated as vaccine candidates. These studies showed that the deletion of the *wbo*A, *wbo*B, *wbk*A, *wbk*B, *wbk*D, *wbk*E, *wbk*F, *wzt*, *per*, *pgm*, *gmd* and *manBcore* genes from smooth strains of *Brucella* spp. (mainly *B. abortus* 2308 and *B. melitensis* 16M) leads to the emergence of rough mutants from smooth parental strains, in which the expression of O-PS is completely absent (Winter et al. 1996; Godfroid et al. 2000; Ugalde et al. 2003; González et al. 2008; Barrio et al. 2009; Smith et al. 2019). However, the silencing of the *wa*** and *wzm* genes demonstrated that these genes are probably liked only to the transport of the O chain to the surface of the bacteria, since the mutants obtained were able to synthesize the antigen, but it was accumulated in the cytoplasm of the bacterial cell instead to be expressed in the outer membrane (González et al. 2008; Barrio et al. 2009).

In general, the emergence of rough strains from smooth *B. abortus* strains is more common than the opposite. However, a study evaluating 62 rough strains of *B. abortus* (obtained from 33 isolates) revealed the reversion back to the smooth phenotype after prolonged cultivation in liquid medium containing glycerin-dextrose (Henry 1933). Likewise, the use of the *B. abortus* 45/20 vaccine strain as a attenuated rough vaccine, evaluated in Europe as a possible replacement for vaccination with S19, was discontinued due to the *in vivo* reversion of the rough phenotype back to smooth (McEwen 1940). Therefore, the significance of the S-R dissociation or the reversion of this condition (R-S) in culture or even *in vivo* is unclear, but the available evidence points to a non-unidirectional instability (S-R) in the expression of the O-PS inherent to the *B. abortus* species. In other words, usually rough strains emerge from smooth strains, but the opposite is also true. In the latter case, it can be assumed that the rough parental strain does not have a deficiency in any of the genes related to the synthesis or expression of the O chain, but rather, the environmental conditions (natural or artificial) suppressed the expression of these genes.

Taken together, the available results indicate that dissociation (expression of the rough phenotype) is a common and transient phenomenon in *B. abortus*, probably triggered by environmental conditions and regulated by yet unknown epigenetic factors. In this sense, studies aiming to understand how environmental changes influence gene expression (RNAseq) and DNA alterations would be of great value, including methylation, post-translational modifications of histones and non-coding RNAs, considering that epigenetics involves the

492 study of heritable changes in gene expression, which occur without changes in the underlying
493 DNA sequence.

494 **Table 1** - Genes associated with synthesis and export of membrane lipopolysaccharide (LPS)
495 in *Brucella* spp.

Gene	Locus tag*	Product	Function
LpxC	BMEI0586	dp-3-o- [3-hydroxymyristoyl] n-acetylglucosamine deacetylase	Lipid A synthesis
LpxD	BMEI0831	udp-3-o- [3-hydroxymyristoyl] glucosamine n-acyltransferase	Lipid A synthesis
LpxA	BMEI0833	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase	Lipid A synthesis
LpxB	BMEI0835	Lipid-A-disaccharide synthase	Lipid A synthesis
KdsA	BMEI0850	2-dehydro-3-deoxyphosphooctonate aldolase	Lipid A synthesis
wboB	BMEI0997	mannosyltransferase	O chain synthesis
wboA	BMEI0998	glycosyltransferase	O chain synthesis
HtrB	BMEI1115	lipid a biosynthesis lauroyl acyltransferase	Lipid A synthesis
lpxE	BMEI1212	phosphatidylglycerophosphatase b	Lipid A synthesis
*wa**I*	BMEI1326	lpsa protein	Core synthesis
wbkE	BMEI1393	mannosyltransferase c	O chain synthesis
manAOA	BMEI1394	mannose-6-phosphate isomerase	Core synthesis
manBOA	BMEI1395	mannose-1-phosphate guanylyltransferase	Core synthesis

manCOA	BMEI1396	phosphomannomutase	Core synthesis
wbkA	BMEI1404	mannosyltransferase	O chain synthesis
gmd	BMEI1413	GDP-mannose 4,6-dehydratase	O chain synthesis
per	BMEI1414	perosamine synthetase	O chain synthesis
wzm	BMEI1415	Transport permease protein (rfbd) of the system of exportation of O antigen	Export of the O chain from the cytoplasm to the cell membrane
wzt	BMEI1416	O-antigen export system ATP-binding protein (rfbb)	Export of the O chain from the cytoplasm to the cell membrane
wbKB	BMEI1417	Unknown product	Unknown function
wbkC	BMEI1418	GDP-mannose 4,6-dehydratase / GDP-4-amino-4,6-dideoxy-D-mannose	O chain synthesis
wbkF	BMEI1426	putative undecaprenyl-phosphate alpha-n-acetylglucosaminyltransferase	O chain synthesis
wbkD	BMEI1427	UDP-4-dehydro-6-deoxy-2-acetamido-d-glucose-4-reductase	O chain synthesis
pgm	BMEI1886	phosphoglucomutase	Core synthesis
KdsB	BMEI1904	3-deoxy-manno-octulosonate cytidylyltransferase	Lipid A synthesis
*wa**II*	BMEII0053	Mg (2+) transport protein C ATPase	Core synthesis
*wa**III*	BMEII0682	Oxacillin resistance-associated protein fmtc	Core synthesis

manBcore	BMEII0899	phosphomannomutase	Core synthesis
manCcore	BMEII0900	mannose-6-phosphate isomerase / mannose-1-phosphate guanylyltransferase (gdp)	Core synthesis
LpxK	BMEII1028	tetraacyldisaccharide 4'-kinase	Lipid A synthesis
KdtA	BMEII1029	3-deoxy-D-manno-octulosonic acid transferase	Lipid A synthesis
*wa**IV*	BMEII1134	amidase	Core synthesis

*_Brucella melitensis_ 16M – (chromosome 1 and 2: AE008917.1 and AE008918.1)

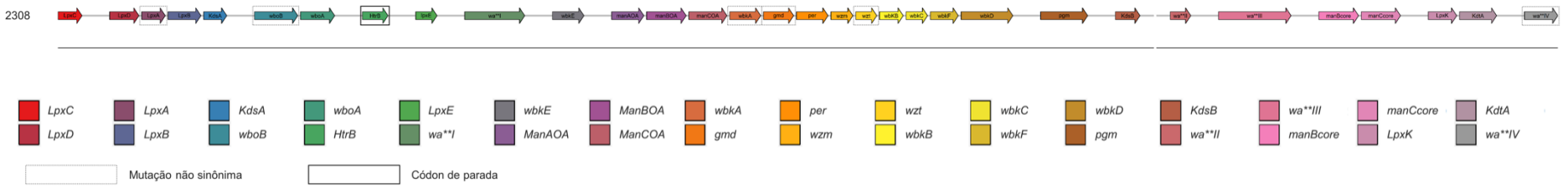

Figure 2 - Schematic representation of the genes associated with lipopolysaccharide (LPS) synthesis and export present in the *Brucella abortus* 2308 sample.

4. Vaccines and vaccination against bovine brucellosis

Vaccination of female cattle with live *B. abortus* vaccines and test-and-slaughter approach are the two main measures to control brucellosis in several countries worldwide (Zhang et al. 2018). Among the available vaccines, the *B. abortus* biovar 1 S19 and RB51 strains are the most used, since they are the official vaccines recommended by the World Organization for Animal Health (WOAH) for bovine brucellosis control (WOAH 2022a).

The S19 vaccine is a smooth, attenuated and stable vaccine strain that promotes a long-lasting immune response in cattle (Manthei 1960). However, it triggers immunity that can interfere with the serological diagnosis of the disease, since it is not possible to differentiate the antibodies induced by vaccination from those induced by the disease (Dorneles et al. 2015a). In consequence, the use of the S19 vaccine is recommended only to females between three and eight months of age, in order to reduce the vaccinal interference in brucellosis diagnosis (WOAH 2022a). The persistence of vaccinal antibodies in the sera of the vaccinated animals is directly related to the age at vaccination, with more persistent and higher titers in older animals (Gilman and Wagner 1959; King and Frank 1961; Manthei 1968; Gonçalves et al. 2025b).

The RB51 vaccine is also attenuated, it was obtained from a pathogenic strain passed several times in culture medium with subinhibitory concentrations of rifampicin and penicillin, resulting in a stable rough and rifampicin-resistant strain (Schurig et al. 1991). Due to its rough morphology, this vaccine does not induce antibodies detectable in conventional serological tests and therefore does not interfere with routine disease diagnostic (Dorneles et al. 2015a). Consequently, although it is recommended by WOAH for females between 3 and 12 months-old (WOAH 2022a), it can be used in female cattle of any age, both for primo vaccination in calves, as well as in adult animals not immunized in the appropriate age range (Dorneles et al. 2015b). RB51 can also be used for revaccination, enhancing brucellosis immunity in adult females previously vaccinated with RB51 or with S19 (Dorneles et al. 2015b).

Although vaccination of cows can be adopted in some cases, calf immunization is the centerpiece of any brucellosis control program, performing very well on reducing the disease prevalence, especially in the disease control phase (Dorneles et al. 2017). Both vaccines (S19 or RB51) are effective in protecting vaccinated animals against *B. abortus* infections and abortions (Dorneles et al. 2015a) and their efficacy was recently recalculated in a systematic review and meta-analysis (Oliveira et al. 2021). In this study, it was observed that when the dose of 10^9 colony forming units (CFU) is administered, the S19 vaccine has 75.09% [95% confidence interval (95% CI): 48.08–88.05] of vaccine efficacy against the occurrence of abortions and 72.03% (95% CI: 57.70–81.50) against the occurrence of infection in cattle (Oliveira et al. 2021). On the other hand, RB51, at a dose of 10^{10} CFU, has a vaccine efficacy of 69.25% (95% CI: 39.48–84.38) against the occurrence of abortions and 57.05% (95% CI: 30.90–73.30) against infection (Oliveira et al. 2021). Another systematic review also demonstrated that S19 and RB51 vaccine strains are effective in filed to prevent brucellosis, mainly when adopted together with test-and-slaughter control policy, decreasing the prevalence of the disease in the herds (Gonçalves et al. 2025a).

Although effective and used to prevent bovine brucellosis in several countries around the world for decades, to date, most of the knowledge about the protective immune response against *B. abortus* infection/vaccination comes from studies using a mouse model. In contrast, there is a limited amount of information about the immunological mechanism by which the vaccines against *B. abortus* confer protection in cattle (Dorneles et al. 2015c). In mice, S19 and RB51 have been shown to induce a strong Th1 (T helper 1) cellular immune response with production of IFN-γ and specific CD8+ cytotoxic cells, without IL-4 production (Zhan et al. 1995; Vemulapalli et al. 2000a, 2000b; He et al. 2001; Pasquali et al. 2001; Andrews et al. 2006; Fu et al. 2012). In cattle, there is evidence that specific cell-mediated immune components are stimulated after vaccination with S19 or RB51, with IFN-γ production and an increase in CD4+ or CD8+ cells (Hu et al. 2009; Singh et al. 2012; Dorneles et al. 2014, 2015b). Furthermore, lymph node cells from cattle vaccinated with S19 or RB51, as well as murine cells, exhibit a significantly higher proliferation rate compared to unvaccinated animals, after *in vitro* stimulation with protein fractions from *Brucella* spp. or γ-irradiated *B. abortus* 2308 (Stevens et al. 1994; Palmer et al. 1997). Antibodies, although important from a diagnostic point of view, are considered secondary in the protective response against infection (Nicoletti 1990; Dorneles et al. 2015d).

5. Evaluation of vaccines against bovine brucellosis

The objective of vaccination is to reduce the number of susceptible individuals in the population. Consequently, the success of any vaccination program depends mainly on the efficacy of the vaccine used and vaccinal coverage in the target population. The efficacy of vaccines against brucellosis can be verified using three different criteria: (i) using laboratory animals, extrapolating the results to the target species; (ii) using natural hosts under controlled conditions, comparing the results of unvaccinated controls with vaccinated animals; (iii) using field conditions where pre- and post-vaccination prevalence and/or vaccinated and unvaccinated subpopulations are compared (Nicoletti 1990). In experimental studies of vaccine efficacy, vaccinated and unvaccinated controls receive a known infectious dose of a virulent strain of *B. abortus* (usually 544, 2308, or VRI3) at the most susceptible period (mid-gestation), and protection is measured by the vaccine's ability to prevent abortion (Oliveira et al. 2021). In field studies, on the other hand, animals are maintained in an infected herd with brucellosis to be exposed to natural challenge and two observational studies designs can be adopted: cohort or prevalence panels. Cohort studies are characterized by comparison between vaccinated and the non-vaccinated animals, considering the incidence of abortions and infections in the two groups along the time, while in prevalence panel design, the prevalence of abortion and infection in the herd is compared before and after vaccination of all animals (Gonçalves et al. 2025a).

Another important aspect related to the success of brucellosis control programs is the quality of the vaccine used. Although the cost of the vaccine is only a fraction of the total cost of a control program, its quality directly and considerably affects its outcome. The assessment of the quality of live brucellosis vaccines is generally based on *in vitro* criteria, including

584 physicochemical and microbiological tests related to purity, dissociation, pH determination,
585 moisture content, and viable bacterial count (Alton et al. 1988; WOAH 2022a). The WOAH
586 also recommends periodic tests in mice (*in vivo*) to evaluate the master seed of the strain used
587 in the manufacture of brucellosis vaccines (Miranda et al. 2015; WOAH 2022a). These tests in
588 mice are used to evaluate the efficacy/potency and residual virulence of vaccine strains
589 (Miranda et al. 2015; WOAH 2022a). Recently, the use of molecular tests to monitor vaccine
590 seeds has also been suggested, mainly tests based on the evaluation of the genetic signature of
591 vaccine seeds, based on the determination of alleles present in 16 *loci* with tandem repeats in
592 the genome of the strain (Dorneles et al. 2013; Miranda et al. 2013). Regarding the post-
593 vaccination immune response, although there is no correlate of protection, there is a consensus
594 in the literature that the desired/protective response against *B. abortus* infection is given by a
595 cell-mediated immune response, with strong induction of IFN-γ production and fundamental
596 participation of CD4+ and CD8+ lymphocytes, this being the immune response triggered by
597 the B19 and RB51 vaccines against bovine brucellosis (Dorneles et al. 2014, 2015b).

598

599 **6. Biological significance of dissociation: comparison between smooth and rough**
600 **strains**

601 This topic will discuss the possible impacts of dissociation on the pathogenicity and
602 virulence of *Brucella abortus*, by comparing smooth and rough strains of different species of
603 *Brucella* spp. and considering naturally rough strains and rough mutants strains obtained in
604 laboratory. It is worth remembering that this approach was chosen due to the lack of studies in
605 the scientific literature that compared the impact of different levels of dissociation on the
606 potency/efficacy of vaccines against brucellosis in the target species or even in an animal model,
607 especially for the S19 vaccine. It is important to mention that this approach is supported
608 considering that it is a live vaccine, which multiplies in the host after inoculation for a few
609 weeks (Cheville et al. 1996), mimicking a natural infection without causing disease. Initially,
610 evolutionary issues related to the presence of the O chain in the LPS of microorganisms of the
611 genus *Brucella* spp. will be discussed, followed by the likely impact of dissociation on the initial
612 colonization events and on the intracellular survival of the agent, and finally, the results
613 obtained from the infection/vaccination of laboratory animals and natural hosts.

614 In other Gram-negative bacteria, the O chain of the LPS typically has a large antigenic
615 variation (e.g. *Escherichia coli*) (Liu et al. 2019), unlike what is observed for *Brucella* spp.,
616 which presents a very homogeneous O-PS in the comparison between and among its species
617 (Cardoso et al. 2006). This could be explained by considering that the intracellular niche
618 occupied by this pathogen does not allow much exchange of genetic material with other
619 bacteria, consequently limiting the lateral acquisition of DNA (Mancilla 2016). In fact, it has
620 been proposed that naturally rough (*B. ovis* and *B. canis*) and naturally smooth strains evolved
621 to become pathogens from soil microorganisms, a process in which the lateral acquisition of
622 genes related to O-chain synthesis was crucial (Whatmore and Foster 2021). However, the
623 coevolution of the pathogen with its hosts has modulated this evolution towards increasingly
624 promoting a chronic and silent (intracellular) infection. In this context, it is important to

consider that, during speciation, *B. canis* and *B. ovis* followed different paths from the smooth strains, and although they lost O-PS, they did not become less pathogenic for their natural hosts. This aspect suggests that O-PS, despite being immunodominant for the humoral response and influencing virulence, is not the unique factor that makes bacteria of the genus *Brucella* pathogenic for animals and humans.

Indeed, the naturally rough strains also cause reproductive problems in their primary hosts, likewise the naturally smooth species (WOAH 2022a). A critical difference between these strains is that *B. canis* is the only naturally rough zoonotic strain (Corbel et al. 2006), and can eventually produce severe disease in humans (Gul et al. 2009). Similarly, it is worth considering that *Yersinia pestis,* another Gram-negative bacteria, acquired an evolutionary advantage due to the loss of O-PS, causing more persistent infection in its hosts (Singh et al. 2020). Further confirming this hypothesis, outer membrane proteins (*Omps*) have been implicated as decisive in the pathogenicity of naturally rough strains and differentially expressed comparing *B. canis* and *B. ovis* with *B. abortus* and *B. melitensis* (Cloeckaert et al. 2000; González et al. 2008). These findings suggest that outer membrane proteins (*Omps*) could complement the absence of the O-PS in the establishment of chronic infection. Likewise, cell wall components other than O-PS have also been implicated in resistance to lysis by the complement system and antimicrobial peptides in *B. canis* and *B. ovis*, with the participation of the LPS core oligosaccharide side chain in these events being suggested (Stranahan and Arenas-Gamboa 2021). Furthermore, even when deficient in O-PS, *B. canis* and *B. ovis*, as well as smooth strains of *Brucella* spp., use scavenger receptors (SR-A/CD36) to enter macrophages, using the endocytosis pathway that favors the formation of the vacuole containing *Brucella* and the chronic infection of these cells (Martín-Martín et al. 2010). Thus, all these points listed show that the definitive loss of O-PS throughout the evolutionary process of *B. canis* and *B. ovis* did not make them less virulent or capable of causing disease in their natural hosts, since compensatory mechanisms are used for the successful establishment of chronic intracellular infection.

Considering laboratory-obtained rough mutants, studies involving infection of J774.A1 macrophages with rough strains of *B. melitensis* showed that these strains can cause cellular toxicity, which was essential for the release of the bacteria from the macrophages. It was supposed that dissociation is involved in the dissemination of the microorganism, which would occur with release into the medium and subsequent reinfection in other cells (Pei et al. 2014). Previous studies by this same group also showed that rough *Brucella* spp. strains induced the formation of pores in the membranes of macrophages, capable of causing necrosis of these cells (Pei et al. 2006). Both studies indicate that the emergence of rough variants from smooth strains of *Brucella* spp. could facilitate the dissemination of the microorganism in the host and consequently contribute to the virulence of the pathogen. Furthermore, it is hypothesized that this dissociation may be induced by acidic pH within phagosomes (Boschiroli et al. 2002). Another point is that it has been shown that rough *Brucella* spp. is also capable of establishing an intracellular niche in macrophages and inducing the production of cytokines and chemokines in cell culture, resulting in a strong pro-inflammatory response (Rittig et al. 2003; González et al. 2008; Pei et al. 2008). Indeed, *Brucella* spp. rough cells induce greater amounts of several

chemokines [CXC (GRO-alpha, IL-8) and CC (MIP-1alpha, MIP-1beta, MCP-1, RANTES)], as well as pro- (IL-6, TNF-alpha) and anti-inflammatory (IL-10) cytokines released by challenged monocytes compared to smooth strains (Rittig et al. 2003), suggesting that there is no impairment of the immunogenicity of these strains associated with the loss of O-PS. The results obtained from experimental infection of mice with rough mutants of *B. abortus* and *B. melitensis* showed different results depending on which genes linked to the synthesis and expression of the O chain were silenced: some rough mutants were cleared more rapidly than the smooth parental strains (Ugalde et al. 2003; Turse et al. 2011), while others showed similar performance to that observed for the smooth strains, including the *B. melitensis* Rev. 1 vaccine strain (González et al. 2008). Likewise, the results of potency tests performed in mice showed that some rough mutants of *B. melitensis* (5/14) presented protection similar to the Rev. 1 vaccine strain in mice (González et al. 2008), although they exhibited lower levels of protection than the Rev. 1 vaccine in sheep (Barrio et al. 2009).

The inoculation of guinea pigs with 13 different strains of *Brucella* spp., from which rough and smooth variants were obtained, showed that the smooth strains were generally more capable of causing generalized infection in the animals than the rough strains of the same origin (Marshall and Jared 1931). However, it was also observed that some groups of animals inoculated with smooth strains showed few signs of infection, as well as some groups of animals inoculated with some rough strains showed apparent infection, suggesting that the discrepancies between the virulence profiles could not be explained only by the composition of the LPS (presence or absence of the O chain) (Marshall and Jared 1931). This argument is corroborated by considering that the silencing of the same gene (*per*) in two different parental strains of *B. melitensis* (16M and H38) resulted in different levels of protection induced by the rough mutants in mice; the mutant obtained from strain H38 showed protection similar to the vaccine strain Rev. 1, while the rough mutant obtained from strain 16M did not induce protection against challenge (González et al. 2008).

Results in natural hosts show that inoculation of different animal species (pigs, cattle, goats, sheep and chickens) with 100 different isolates of *Brucella* spp. with different dissociation rates, containing up to 15% of rough organisms, showed the re-isolation of only smooth strains (Henry 1933). Similarly, the results obtained by Mancilla et al. (2013) showed that the higher degree of S-R dissociation in the *B. melitensis* Rev. 1 vaccine (average 4.27%) was not associated with greater attenuation of this strain in a mouse model, after challenge with a virulent strain (*B. melitensis* H38). This strain was compared to a mutant strain with a lower level of dissociation (*B. melitensis* Rev. 2), obtained from the stabilization of the *int* gene on the GI-2 island (95% less dissociation). Altogether, these results suggest that smooth strains would be more capable of colonizing and establishing infection niches in animals than rough parental strains, even when the inoculum contains different degrees of dissociation.

Nevertheless, although smooth strains seem to be more effective in causing infection, when considering vaccine parameters, rough morphology does not seem to harm vaccine potency and efficacy. In fact, results of the RB51 strain for potency and residual virulence in mice (Miranda et al. 2015) and for efficacy and induced immune response in the target species (cattle) (Dorneles et al. 2015b; Oliveira et al. 2021) showed no difference comparing RB51 to

S19 strain. In addition, it is worth noting that RB51 has been successfully used for several years in several countries to prevent bovine brucellosis, which also attests its efficacy as vaccine strain.

7. Final considerations

Although it is undeniable that the LPS is one of those responsible for modulating virulence and inducing immunity in the organism of natural hosts of *Brucella* spp., the continuous emergence *in vivo* of rough variants during infection, or *in vitro,* during cultivation of microorganisms, suggests that this phenotype is part of the biology of the agent and may confer some survival advantage to the bacteria. In fact, for some strains, the permanent or temporary loss of the O-PS, either occurring naturally or induced, does not necessarily imply a decrease in virulence, immunogenicity, or post-challenge induced protection, since results in both directions have been observed in the literature, depending mainly on the parental strain used and the silenced genes.

It is also important to consider that the speciation of the genus *Brucella* occurred from the coevolution of the pathogen with different hosts, according to the species. Hence, the *Brucella* species nowadays considered as naturally rough (O-PS) (*B. canis* and *B. ovis*) have lost the O chain in this process, but not the ability to cause disease and reproductive problems in their natural hosts, probably due to compensatory factors that are not yet fully understood (e.g. presence of a side chain in the LPS core and outer membrane proteins). In contrast, for the classic smooth species, coevolution and establishment of an intracellular replication niche occurred without the permanent loss of the O-PS. However, there appears to be a dynamic balance during infection, and especially in laboratory culture, in the expression of the O chain, which is regulated positively or negatively depending on the medium. In this sense, the knowledge accumulated to date strongly indicates that differences in the dissociation rates observed for different isolates of *B. abortus*, such as the vaccine S19 strain, are the result of epigenetic changes, which do not necessarily imply loss of efficacy or immunogenicity for vaccines, even for stable rough strains, such as RB51. Thus, it is possible to say that the alterations in LPS morphology and their consequences are part of a complex process that is still poorly understood for the genus *Brucella* as a whole. Consequently, the central question highlighted by this review is at the frontier of knowledge, for which it is not yet possible to have a conclusive answer with the literature available so far.

8. Conclusions

The significance of S-R dissociation in culture (*in vitro*) or even *in vivo*, considering that rough strains have already been isolated from clinical samples in different hosts, is not clear. However, considering that changes in LPS can occur in both directions (S-R and R-S) and *in vivo* and *in vitro*, it is reasonable to assume that the emergence of these variants is a response of the agent to the environment (selection pressure), but is not primarily related to virulence/immunogenicity, which could be secondarily affected by the strain in question from which the variant arose. In other words, the emergence of variants related to smooth/rough LPS

749 from a strain of *B. abortus* probably does not present changes in the immunogenicity and
750 efficacy of that strain as a vaccine.

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CHAPTER III

Published at: Vaccine (doi.org/10.1016/j.vaccine.2024.126649)

Systematic review on the effectiveness of *Brucella abortus* S19 and RB51 vaccine strains in field studies

Short running title: Effectiveness of *Brucella abortus* vaccines

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Abstract

Brucella abortus S19 and RB51 are the most used vaccines to control bovine brucellosis worldwide; therefore, this study aimed to perform a systematic review on the effectiveness of these two vaccine strains in field studies. The literature review was conducted on April 3rd 2020 on six databases (CABI, Cochrane, PubMed, Scielo, Scopus and Web of Science) and included papers published between 1976 and 2016. The search strategy recovered a total of 5,846 papers on databases and 6 papers were included due to specialists' suggestions. After selection, 17 papers were included, in which 33 trials were identified. Most trials [63.63% (21/33)] used

prevalence panel design (cross-sectional), while the others were cohort studies. S19 strain was used in most of the trials [75.76% (25/33)], mainly by subcutaneous route [84.00% (21/25)] and in adult cattle [76.00% (19/25)]. RB51 strain was administrated only by the subcutaneous route and in both young and adult animals. For case definition, complement fixation [60.60% (20/33)] and rivanol [30.30% (10/33)] were the most used tests. Twenty of the 33 trials (60.61%) showed significant effect of vaccination on brucellosis control, with lower incidence of infection in the vaccinated groups (in cohort trials) or reduced prevalence after vaccination (in prevalence panels); however, the great heterogeneity observed among the studies precluded a meta-analysis from the data extracted. In addition, most trials [57.57% (19/33)] adopted other control measures (test-and-slaughter or isolation of positive animals from the herd) in association with vaccination, which harmed the better understand of the isolated effect of vaccination for brucellosis control in field in these studies. In conclusion, the result from this review strongly suggests that both S19 and RB51 vaccine strains are effective in reducing brucellosis incidence in both calves and adults, as well as abortion rates, mainly when associated to other control policies.

Keywords: bovine brucellosis, vaccination, observational studies, natural challenge.

1. Introduction

Bovine brucellosis is a zoonotic disease of major importance, especially in developing regions, as Latin America, Asia and Africa [1]. The disease is mainly caused by *Brucella abortus* and responsible for significant economic losses in livestock, due to decrease in milk and meat production, disposal of infected animals, depreciation of the activity and reproductive clinical signs, mainly abortions, stillbirth and infertility [2,3].

Vaccination is a central point in the control and prevention of bovine brucellosis and the vaccine strains S19 and RB51 are the most used worldwide [4], being both effective in protecting vaccinated animals against infection and abortion [5]. S19 is an attenuated *B. abortus*

53 biovar 1 strain that induce antibodies that cannot be differentiated from those induced by
54 infection, restricting vaccination to calves aged between 3 to 8 months, since vaccination
55 antibodies decrease over time [6]. RB51 is also an attenuated *B. abortus* biovar 1 strain,
56 however, as it does not express the O-side chain lipopolysaccharide on its membrane, it does
57 not induce antibodies detected by routine serological tests [7].

58 The establishment of the efficacy (proportion of vaccinated individuals protected by the
59 vaccine in controlled clinical trials) against a clear outcome (abortion and infection) and
60 effectiveness (vaccine efficacy measured by observational studies under field conditions) of
61 these vaccines widely used in the control of bovine brucellosis is important for the definition
62 of the appropriate level of vaccination coverage to break the transmission chain of a disease [8].
63 A solid consensus on the efficacy and effectiveness of bovine brucellosis vaccines must be built
64 for the optimization of vaccination schemes, including brucellosis control modeling for
65 endemic regions. Therefore, a meta-analysis recently conducted by our research group from
66 controlled clinical trials using experimental challenge showed that S19 and RB51 have similar
67 efficacy and that a dose of 10^9 CFU (colony forming units) for S19 and 10^{10} CFU for RB51 are
68 the most suitable for the prevention of abortion and infection caused by *B. abortus* in cattle [5].
69 However, the effectiveness, efficacy in the real world, assessed from field studies using natural
70 challenge for bovine brucellosis vaccines remains to be determined. Thus, the aim of this study
71 was to perform a systematic review on field trials used to determine the effectiveness of S19
72 and RB51 in cattle.

73 **2. Material and methods**

74 The guidelines of PRISMA statement (Preferred Reporting Items for Systematic
75 Reviews and Meta-Analysis) were adopted in this review (Supplementary Table S1) [9].

2.1.Strategy of search and selection of the studies

The selected keywords were investigated within all the sections from papers (title, abstract and full text) on April 03rd, 2020 in the following databases: CABI, Cochrane, PubMed, Scielo, Scopus and Web of Science. Briefly, the PICO (population, intervention, comparison, and outcome) included cattle, vaccination with *B. abortus* S19 and/or RB51, natural challenge, prevalence/incidence of infection and/or occurrence of reproductive clinical signs, without restrictions regarding the time when the studies were published. An overview of the search terms used is shown in the Supplementary Table S2.

In the first stage of selection, the studies were selected based on their titles (MMO and RSA). Then, two reviewers (MMO and RSA), independently, evaluated the abstracts. Subsequently, full text of the papers selected based on the abstract were evaluated in terms of their relevance and by means of inclusion/exclusion criteria. When the two reviewers disagreed, a third one (EMSD) was responsible for the final decision. Further, the reference lists of the selected papers were reviewed to find pertinent studies not identified during the initial search. Specialists in vaccination against brucellosis were also consulted (JG and APL) to include manuscripts that did not return from databases search.

2.2.Inclusion and exclusion criteria

The following characteristics were considered for the inclusion of articles: (*i*) approach on *B. abortus* vaccination of cattle using S19 or RB51, (*ii*) non-experimental challenge, (*iii*) observational studies and (*iv*) assessment of disease prevalence/incidence or disease clinical signs. Articles focusing on (*i*) brucellosis caused by other *Brucella* species than *B. abortus*, (*ii*) genetics, immunology, microbiology, or vaccine safety, (*iii*) vaccine efficacy assessed by experimental studies, (*iv*) involving other animal species, (*v*) that performed experimental or no challenge, (*vi*) evaluated effectiveness on long-term control programs, or (*vii*) written in languages other than English, Spanish, French and Portuguese were excluded. In the quality

level assessment, the following criteria were used to select the paper included by eligibility: data on vaccination (dose, route and number of vaccinations), post-vaccination assessment (time interval, tests used and number of animals) and natural challenge (prevalence at vaccination) must be available, and the data must be original (not presented elsewhere). Full inclusion and exclusion criteria are shown in the Supplementary Table S3.

2.3.Type of studies

Cross-sectional and cohort studies were included. Case-control, case series and case reports were excluded, as well as experimental studies, conference proceedings, book, book chapters and reviews.

2.4.Data extraction

Data was extracted from papers by one of the reviewers (MMO) and then checked for accuracy by other two reviewers (MSG and EMSD). Disagreements regarding data extraction among the reviewers were solved by consensus. Extracted data included: first author, year of the publication, location where studies were performed (when available), breed of the animals (when available), type of herd (beef or dairy) (when available), number of animals used, number of animals per group, animals' age at vaccination, vaccine strain(s), vaccine dose(s), vaccine route(s), number of vaccinations (when applicable), interval between vaccinations (when applicable), number of herds assessed, field challenge (initial prevalence before vaccination), diagnostic test(s) used, interval between vaccinations and assessment of outcome(s), assessed outcomes (prevalence reduction, disease incidence, occurrence of reproductive clinical signs, etc.), and other policies used for disease control (when applicable). As a study can comprise multiple trials, an entire manuscript was referred to as a "study", whereas each single tested vaccination regimen in a study was referred to as a "trial".

2.5.Statistical analysis

125 Relative risk (RR) for cohort trials and prevalence ratio (PR), as well as the 95%
126 confidence intervals (CI) for both measures, were calculated using the R software version 4.2.2
127 [10] with aid of the package 'epiR' [11]. For trials that showed zero outcome events after
128 vaccination (prevalence panels) or in the vaccinated groups (cohort), CI was calculated by
129 moving one non-event to event in intervention groups/panel (e. g. vaccinated group (n=10) on
130 a cohort study showed zero positive animals after evaluation period: CI was calculated using 9
131 negative and 1 positive events) [12]. In addition, CI values were obtained only for those trials
132 that provided the absolute number of cases and animals at risk. Since brucellosis is a chronic
133 disease, prevalence after vaccination was calculated considering only animals at risk (not
134 previously infected) in the trials in which positive animals were not withdraw of the population.
135 Vaccine effectiveness (VE) was obtained by the attributable fraction and multiplying by 100 to
136 obtain the percentage value (%) [13].

137 **3. Results**

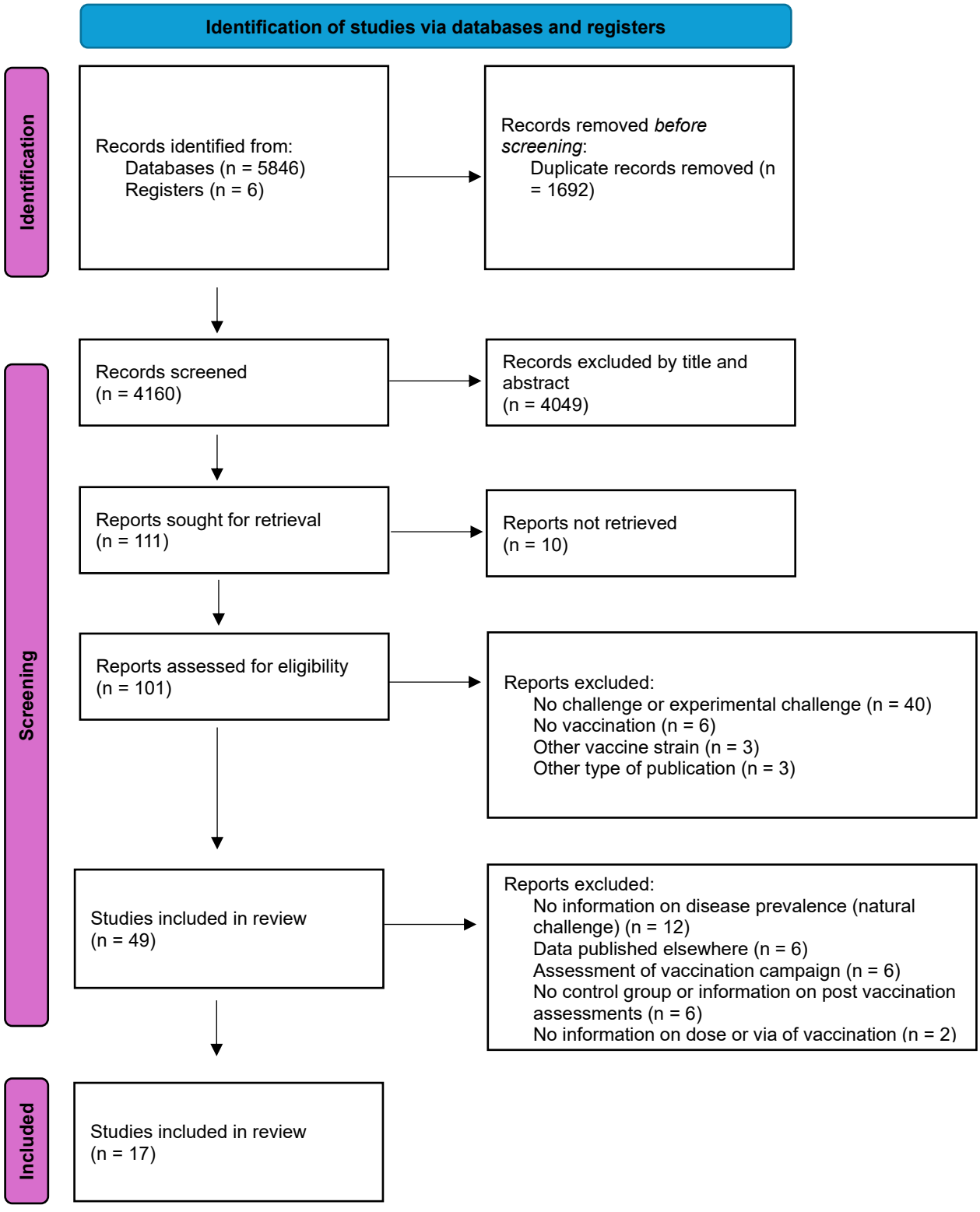
138 *3.1.Selected studies*

139 The literature review included papers published between 1976 and 2016. The search
140 strategy adopted identified a total of 5,846 papers on databases and 6 papers were included due
141 to specialists' suggestions (APL and JG); 1,692 duplicates were excluded, and 101 full texts
142 were screened for eligibility. Subsequently, 49 articles were assessed by quality and 17 were
143 included and data synthesis appraisal, after a thorough review (Figure 1 and Supplementary
144 Table S4). The temporal distribution of the selected papers showed that 10 of the 17 papers
145 [58.82% (10/17)] were published before 1990, whereas 7 [41.17% (7/17)] were published after
146 this date until 2016. Selected publications originated from nine different countries (Table 1),
147 mainly from the United States [29.41% (5/17)] and Mexico [23.53% (4/17)].

148 Of the 17 selected studies, 11 [64.71% (11/17)] conducted a single trial, while six
149 [35.29% (6/10)] studies comprised at least two trials, reaching a total of 33 assessed trials (Table

1 and Figure 2). Studies were divided in more than a trial according to the following criteria: levels of field challenge (different prevalence levels); adopted study design (cohort or prevalence panel; duration of the studies); vaccination strategies (different vaccine strains, doses or routes); and employment or not of other control measures along with vaccination. Detailed information about how the trials were determined in the six studies that performed multiple trials are available in Supplementary Table S5.

Figure 1. PRISMA flowchart used in the selection of the studies for this systematic review on the effectiveness of Brucella abortus S19 and RB51 vaccine strains.



200 **Table 1 –** General information about the 33 trials (17 studies) selected in this systematic review on effectiveness of bovine brucellosis vaccines (S19 and RB51),
201 1976-2016.

First author, year	Study design	Country	N Herd[a]	Herd type	Animal age	Vaccination						Field Challenge[d] (%)
						N Control[b]	N Vac[c]	Strain	Dose	Route	Booster	
Al-Khalaf, 1992	PP[e]	Kuwait	33	D[f]	Adults	NA[g]	12000	S19	3 x 10^9	SC[h]	S19	7.90
Bahena, 2003	PP	Mexico	1	D	Adults	NA	67	S19	3 x 10^9	SC	S19	8.22
Caetano, 2016*	PP	Portugal	5	B[i]	> 4 months	NA	1763	RB51	1-3.4 x 10^{10}	SC	RB51	39.31
Caetano, 2016*	PP	Portugal	5	B	> 4 months	NA	1637	RB51	1-3.4 x 10^{10}	SC	RB51	0.49
Cantú, 2007	Ch[j]	Mexico	1	B	Adults	35	392	RB51	4 x 10^9	SC	No	8.70
Cardeña, 2009	Ch	Mexico	NI[k]	NI	> 6 months	88	88	RB51	0.03-5 x 10^{10}	SC	No	5.00
Crawford, 1978	Ch	USA	2	B/D	Adults	284	281	S19	3 x 10^9	SC	No	14.27
Crawford, 1988	PP	NI	6	B	Adults	NA	360	S19	3 x 10^9	SC	No	34.72
Enright, 1984*	PP	USA	2	B	Adults	NA	322	S19	2-3 x 10^9	SC	No	14.90
Enright, 1984*	PP	USA	3	B	Adults	NA	1664	S19	2-3 x 10^9	SC	No	3.54
Enright, 1984*	PP	USA	2	B	> 12 months	NA	223	S19	2-3 x 10^9	SC	No	36.77
Enright, 1984*	PP	USA	2	B	> 12 months	NA	719	S19	2-3 x 10^9	SC	No	6.54
Herr, 1984*	PP	South Africa	1	B	Adults	NA	1058	S19	1.9-3.8 x 10^9	SC	No	10.00
Herr, 1984*	PP	South Africa	2	B	Adults	NA	1282	S19	1.9-3.8 x 10^9	SC	No	9.09
Herr, 1984*	PP	South Africa	1	B	Adults	NA	140	S19	1.9-3.8 x 10^9	SC	No	7.70
López, 2007	PP	Mexico	1	D	Adults	NA	241	RB51	3 x 10^9	SC	RB51	15.35
Lord, 1998*	Ch	Venezuela	1	NI	3-8 months	40	90	S19	5 x 10^9	SC	No	2.00
Lord, 1998*	Ch	Venezuela	1	NI	3-12 months	40	75	S19	5 x 10^9	SC	No	39.00
Lord, 1998*	Ch	Venezuela	1	NI	3-8 months	40	150	RB51	5 x 10^9	SC	No	2.00
Lord, 1998*	Ch	Venezuela	1	NI	3-12 months	40	75	RB51	5 x 10^9	SC	No	39.00
Lord, 1998*	Ch	Venezuela	1	NI	3-12 months	40	60	RB51	5 x 10^9	SC	RB51	39.00
Nicoletti, 1976*	Ch	USA	1	NI	Adults	143	314	S19	4-12 x 10^{10}	SC	No	2.13
Nicoletti, 1976*	Ch	USA	1	NI	Adults	143	290	S19	5 x 10^9	C[l]	S19	2.13
Nicoletti, 1976*	PP	USA	2	NI	Adults	NA	1384	S19	4-12 x 10^{10}	SC	No	2.46
Nicoletti, 1976*	PP	USA	1	NI	Adults	NA	182	S19	4-12 x 10^{10}	ID[m]	No	2.75
Nicoletti, 1976*	PP	USA	1	NI	Adults	NA	436	S19	4-12 x 10^{10}	SC	No	13.75
Nicoletti, 1976*	PP	USA	1	NI	Adults	NA	439	S19	0.2-0.6 x 10^9	SC	No	13.75

Nicoletti, 1979*	PP	USA	85	NI	Adults	NA	54393	S19	3×10^9	SC	No	1.30
Nicoletti, 1979*	PP	USA	68	NI	Adults	NA	10854	S19	3×10^9	SC	No	2.01
Odeon, 1987	Ch	Argentina	1	NI	Adults	137	203	S19	3.1×10^9	SC	S19	5.50
Pinochet, 1986	Ch	Chile	8	D	Pregnant	222	213	S19	5×10^9	C	S19	18.50
Viana, 1982	PP	Brazil	1	D	Adults	NA	99	S19	4.5×10^9	C	S19	15.15
Viana, 1989	PP	Brazil	1	D	Calves/adults	NA	440	S19	3×10^9	C	S19	3.45

[a]N Herd: number of herds in vaccinated group; [b]N Control: number of animals in control group; [c]N Vac: number of animals in vaccine group; [d]Field challenge: initial prevalence on the herd (average prevalence were calculated when trials evaluated more than a herd); [e]PP: prevalence panel design; [f]D: dairy herd; [g]NA: not applicable; [h]SC: subcutaneous route; [i]B: beef herd; [j]Ch: cohort design; [k]NI: not informed; [l]C: conjunctival; [m]ID: intradermal.

*Trial from a multiple trial study, detailed information is available on Supplementary Table S4

3.2.Study designs

Two different study designs were adopted in the field trials used to assess the effectiveness of S19 and RB51 vaccine strains. Cohort was adopted in 36.36% (12/33) of the trials, in which the incidence of bovine brucellosis (infection and/or abortion) was compared between non-vaccinated (controls) and vaccinated animals. On the other hand, 63.63% (21/33) trials compared the prevalence of brucellosis (infection and/or abortion) prior and post-vaccination in a prevalence panel design (Table 1 and Figure 2).

Cattle breed was not informed in a great part of the trials [51.51% (17/33)] and, among the 16 trials (48.48%) that had breed information, Holstein was the most frequent [25.00% (4/16)], followed by Zebu breeds [31.25% (5/16)], crossbreeds (crossing of one or more breeds) [25.00% (4/16)], and Hereford [18.75% (3/16)]. The total number of animals used in the studies varied from 67 to 54,393, with an average of 2,875.89 (± 9,502.12) and median of 314 (inter quartile range – IQR = 908). The type of herd (beef or dairy) was also not informed in some trials [45.45% (15/33)] and, among those that had this information, 61.11% (11/18) used beef cattle, 33.33% (6/18) used dairy cattle, and one trial was performed in a dual-purpose herd [5.55% (1/18)]. The average number of vaccinated animals in the cohort studies was 295 (±17.06) with a median of 290 (IQR = 72.25), whereas in the control group the average number of animals was 190 (± 81.41) and the median 143 (IQR = 119.50). The average number of vaccinated animals in prevalence panel studies was 4,271.57 (± 11,941.46) with a median of 440 (IQR = 1,418.50). Detailed information about all 33 assessed trials is available in Table 1.

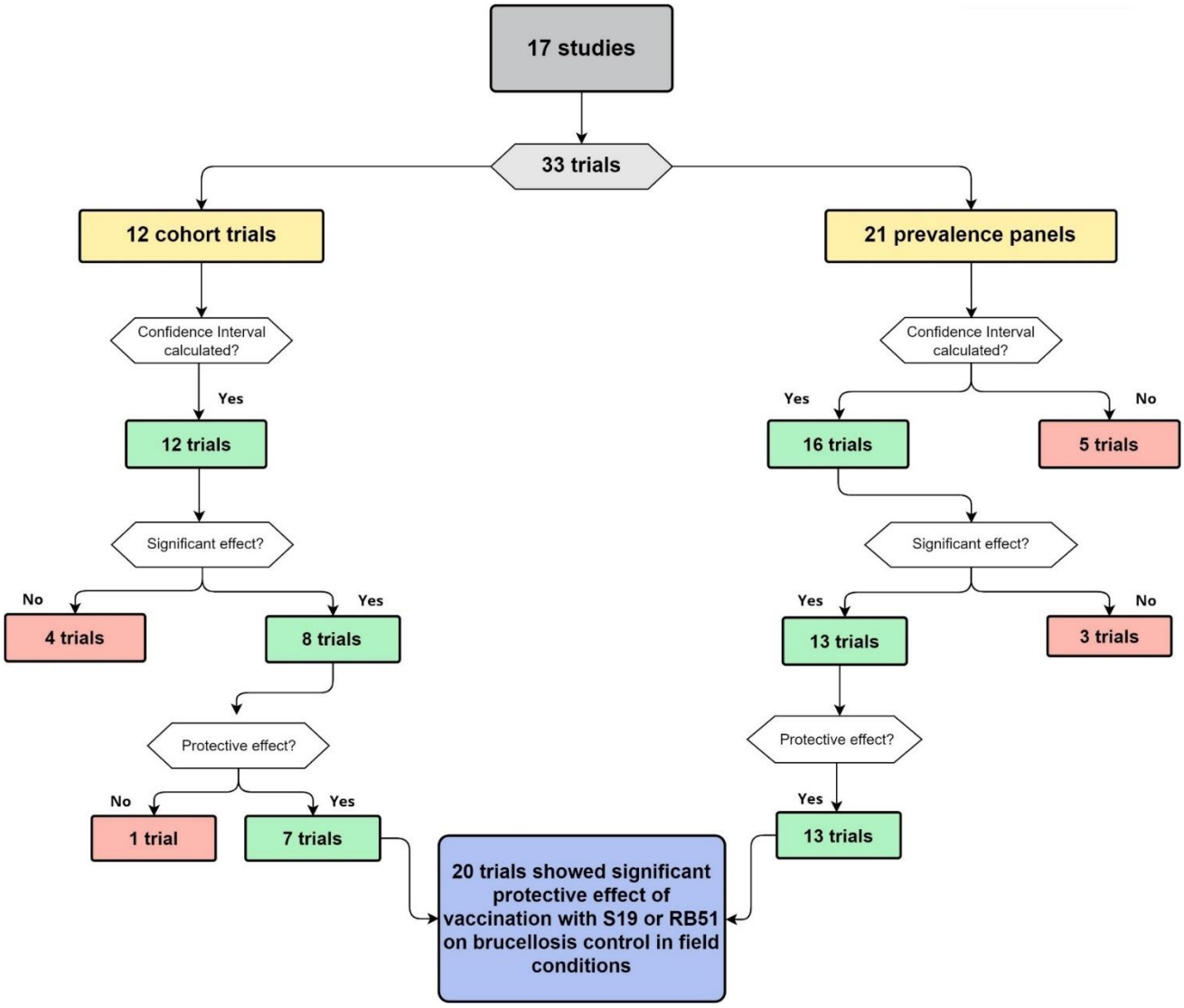
3.3. Vaccination regimens and vaccine strain, dose, and route

S19 vaccine strain was used in most of the trials [75.76% (25/33)], of which 18 [72.00% (18/25)] were prevalence panels and seven [28.00% (7/25)] were cohorts. Revaccination was adopted in only seven of these trials [28.00% (7/25)]. Most of the trials tested S19 effectiveness only in adult cattle [80.00% (20/25)] and subcutaneous was the most frequently adopted route

232 [80.00% (20/25)], followed by the conjunctival [16.00% (4/25)], and intradermal [4.00%
233 (1/25)]. The vaccine doses used vary between 0.2-0.6 x 10^9 and 4-12 x 10^{10} CFU, being the 3 x
234 10^9 CFU the most common adopted dose [28.00% (7/25)].

235 Eight trials tested RB51 [24.24% (8/33)], of which five were cohort studies [62.50%
236 (5/8)] and three used prevalence panel design [37.50% (3/8)]. The subcutaneous route was
237 adopted in all trials, whereas revaccination was performed in only four of them [50.00% (4/8)].
238 Regarding the age, only 25.00% (2/8) of the trials evaluated RB51 effectiveness in adults,
239 whereas the remaining studies used several different age categories from four months of age.
240 The vaccine doses used varied between 0.03 and 5 x 10^{10} CFU, being 5 x 10^9 CFU the most
241 frequent [37.50% (3/8)].

Figure 2. Flowchart used for describing division according to study design used effect of vaccination of the selected studies in this systematic review on effectiveness of B. abortus S19 and RB51 vaccine strains.



3.4. Outcomes assessment

Infection was assessed by serological tests and/or bacteriology in all trials and abortion were evaluated as outcome in 30.30% (10/33) of the trials. All trials adopted more than one test to assess the serological status of the herds before and after vaccination protocol, varying between two and seven different techniques per trial. The average number of different serological tests per trial was 3.75 (± 1.80) and the median was 3 (IQR = 3). Eleven different

253 serological assays were used in the assessed trials, being the complement fixation test (CFT)
254 [78.79% (26/33)] the most common, followed by the card test [75.76% (25/33)] and the rivanol
255 test [75.76% (25/33)], the standard tube agglutination test [39.39% (13/33)] and the 2-
256 mercaptoethanol [39.39% (13/33)], the Rose Bengal test (RBT) [21.21% (7/33)], the agar gel
257 immunodiffusion and the rapid plate agglutination [15.15% (5/33) each], the rapid serum
258 agglutination and the milk ring test [6.06% (2/33) each], and the indirect enzyme-linked
259 immunosorbent assay (iELISA), the competitive enzyme-linked immunosorbent assay
260 (cELISA), and the radial immunodiffusion test [3.03% (1/33) each] (Table 2). Considering only
261 the trials that used S19, the CFT was also the main adopted test [84% (21/25)], whereas, in the
262 RB51 trials, the rivanol, the card test and CFT were the most common [62.50% (5/8) each]. The
263 interval between vaccination and the first serological test was different among the trials, varying
264 between 1 and 18 months.

265 Bacteriology was performed in 66.66% (22/33) of the trials, mainly to isolate the
266 pathogen from tissues of serological positive animals or/and from cow and/or fetus tissues after
267 abortions episodes. Samples used to perform culture and isolation were mainly fetus tissues,
268 udder secretions, lymph nodes, spleen, placenta, vaginal fluids, and uterine fluids. Detailed
269 information about diagnosis tests used in each trial, as well as outcome definition approaches,
270 are depicted in Table 2.
271

272 **Table 2 –** Diagnostic tests used for diagnosis of bovine brucellosis used by 33 trials selected in this systematic review on effectiveness of bovine brucellosis
273 vaccines (S19 and RB51), 1976-2016.

First author, year	Study design	Vac dose[a]	Vac age[b]	Serological tests	Bacteriology	Outcome definition (cut-off)	Endpoint test[c]
S19							
Al-Khalaf, 1992	PP[d]	3 x 10^9	Adults	CFT[e], RBT[f], Riv[g]	Fetuses, placenta, uterine fluids	CFT (1:8) and Riv (1:100)	4 years
Bahena, 2003	PP	3 x 10^9	Adults	CFT, CT[h], Riv, iELISA[i], cELISA[j], RID[k]	Milk	RID and cELISA	9 months
Crawford, 1978	Ch[l]	3 x 10^9	Adults	CFT, CT, Riv	Milk, lymph nodes	CFT (1:80) and Riv (1:100) and Bacteriology and/or clinical signs	12 months
Crawford, 1988	PP	3 x 10^9	Adults	CT, CFT, Riv	Udder secretion	CFT (1:80) and Riv (1:100)	12 months
Enright, 1984	PP	NI[m]	Adults	CFT, CT, Riv	No	Riv (1:50) or CFT (1:41)	22-23 months
Enright, 1984	PP	NI	Adults	CFT, CT, Riv	No	Riv (1:50) or CFT (1:41)	23-29 months
Enright, 1984	PP	2-3 x 10^9	> 12 months	CFT, CT, Riv	No	Riv (1:50) or CFT (1:41)	24 months
Enright, 1984	PP	2-3 x 10^9	> 12 months	CFT, CT, Riv	No	Riv (1:50) or CFT (1:41)	24 months
Herr, 1984	PP	1.9-3.8 x 10^9	Adults	RBT, CFT	No	CFT (1:220)	23 months
Herr, 1984	PP	1.9-3.8 x 10^9	Adults	RBT, CFT	No	CFT (1:220)	23-33 months
Herr, 1984	PP	1.9-3.8 x 10^9	Adults	RBT, CFT	No	CFT (1:220)	21 months
Lord, 1998	Ch	5 x 10^9	3-8 months	CFT, CT, Riv, 2ME[n], AGID[o], RPAT[p], STAT[q]	Fetuses tissue	CFT (13 IU, sample diluted 1:5)	8 months
Lord, 1998	Ch	5 x 10^9	3-12 months	CFT, CT, Riv, 2ME, AGID, RPAT, STAT	Fetuses tissue	CFT (13 IU, sample diluted 1:5)	8 months
Nicoletti, 1976	Ch	4-12 x 10^{10}	Adults	CFT, CT, Riv, 2ME, STAT	Udder secretion, lymph nodes	Serology and bacteriology	6 months
Nicoletti, 1976	Ch	5 x 10^9	Adults	CFT, CT, Riv, 2ME, STAT	Udder secretion, lymph nodes	Serology and bacteriology	6 months

Nicoletti, 1976	PP	4-12 x 10^{10}	Adults	CFT, CT, Riv, 2ME, STAT	Udder secretion, lymph nodes	Serology and bacteriology	13-15 months
Nicoletti, 1976	PP	4-12 x 10^{10}	Adults	CFT, CT, Riv, 2ME, STAT	Udder secretion, lymph nodes	Serology and bacteriology	13-15 months
Nicoletti, 1976	PP	4-12 x 10^{10}	Adults	CFT, CT, Riv, 2ME, STAT	Udder secretion, lymph nodes	Serology and bacteriology	7-9 months
Nicoletti, 1976	PP	0.2-0.6 x 10^{9}	Adults	CFT, CT, Riv, 2ME, STAT	Udder secretion, lymph nodes	Serology and bacteriology	7-9 months
Nicoletti, 1979	PP	3 x 10^{9}	Adults	CFT, CT, Riv	Udder secretion	CFT (1:40)	13 months
Nicoletti, 1979	PP	3 x 10^{9}	Adults	CFT, CT, Riv	Udder secretion	CFT (1:40)	13 months
Odeon, 1987	Ch	3.1 x 10^{9}	Adults	2ME, STAT	No	2ME (1:25)	3-12 months
Pinochet, 1986	Ch	5 x 10^{9}	Pregnant	RBT, STAT	No	STAT (1:100)	8 months
Viana, 1982	PP	4.5 x 10^{9}	Adults	CT, Riv, RSA[s], MR[t]	No	RSA (1:100)	4 months after 2nd dose
Viana, 1989	PP	3 x 10^{9}	Calves and adults	CT, 2ME, RSA, MR	No	RSA (1:100)	24 months after 2nd dose
RB51							
Caetano, 2016	PP	1-3.4 x 10^{10}	> 4 months	CFT, RBT	Fetuses, lymph nodes, spleen	RBT or CFT (NI)	6 years
Caetano, 2016	PP	1-3.4 x 10^{10}	> 4 months	CFT, RBT	Fetuses, lymph nodes, spleen	RBT or CFT (NI)	5 months
Cantú, 2007	Ch	4 x 10^{9}	Adults	CT, Riv	Milk, vaginal exudates	CT and Riv (1:100)	12 months
Cardeña, 2009	Ch	0.03-5 x 10^{10}	> 6 months	CT, Riv	Milk	Riv (1:25)	18 months
López, 2007	PP	3 x 10^{9}	Adults	CT, Riv	Milk, vaginal exudates	Riv (1:25)	13-15 months
Lord, 1998	Ch	5 x 10^{9}	3-8 months	CFT, CT, Riv, 2ME, AGID, RPAT, STAT	Fetuses tissue	CFT (13 IU, sample diluted 1:5)	8 months
Lord, 1998	Ch	5 x 10^{9}	3-12 months	CFT, CT, Riv, 2ME, AGID, RPAT, STAT	Fetuses tissue	CFT (13 IU, sample diluted 1:5)	8 months
Lord, 1998	Ch	5 x 10^{9}	3-12 months	CFT, CT, Riv, 2ME, AGID, RPAT, STAT	Fetuses tissue	CFT (13 IU, sample diluted 1:5)	8 months

274 [a]Vac dose: vaccine dose; [b]Vac age: animal age at vaccination; [c]Endpoint test: interval between vaccination and outcome evaluation; [d]PP: prevalence
275 panel design; [e]CFT: complement fixation test; [f]RBT: rose bengal test; [g]Riv: rivanol; [h]CT: card test; [i]iELISA: indirect enzyme linked immunosorbent
276 assay; [j]C-ELISA: competitive enzyme linked immunosorbent assay; [k]RID: radial immunodiffusion; [l]Ch: cohort design; [m]NI: not informed; [n]2-ME:
277 2-mercaptoethanol, [o]AGID: agar gel immunodiffusion, [p]RPAT: rapid plate agglutination test, [q]STAT: standard tube agglutination test, [r]IU:
278 internacional unit; [s]RSA: rapid serum agglutination test; [t]MR: milk ring test.
279

3.5.Effectiveness of S19 and RB51 vaccine strains

Among the 33 trials, only 14 [42.42% (14/33)] evaluated brucellosis vaccination effectiveness without adoption of other control polices (e. g. test-and-slaughter or isolation of positive animals), being eight cohorts [66.67% (8/12)] and six prevalence panels trials [28.57% (6/21)] (Tables 3 and 4). In general, 57.14% (8/14) showed significant effect of the vaccination to control infection (RR < 1 and CI not including 1), regardless of design study or vaccine strain.

Regarding these cohort trials, four [50.00% (4/8)] used S19 strain and only one of them showed effect of the vaccination to control infection comparing vaccinated and control groups (VE = 100%). Two other S19 cohorts trials showed lower incidence of infected animals in the vaccinated group but without statistical significance (CI including 1) and the last one (Pinochet et al. [19]) did not show the vaccine effectiveness in the field to control infection. Also, two of these cohorts, both conducted by Lord et al. [30], evaluated abortion as outcome and one demonstrated significant effect of the vaccination (VE = 38.89%) while the other did not. The other four trials used RB51 and three of them showed significant effect of vaccination to control both infection and abortion, with 100% of VE, while the last one did not show significant effect. The interval between vaccination and evaluation of the outcomes in all cohort trials that did not adopt other control measures was, in average, 9.75 months (±3.38), varying between 8 and 18 months. Detailed information about outcomes and VE in the cohort trials is shown in Table 3 and Figure 3.

All six prevalence panels that evaluated brucellosis vaccination effectiveness without adoption of other control polices used S19; however, for one of them [28], it was not possible to calculated CI for PR since only prevalence value after vaccination was informed and not the number of animal at risk at this moment. Four of the five remaining trials showed significant

304 VE against infection, varying between 34.18 and 100%. One of these trials evaluated abortion
305 [14] and observed also a significant VE of 88%. Intervals between vaccination and evaluation
306 of the outcomes in these prevalence panels were, in average, 23 months (±12.60), varying from
307 9 to 48 months. Detailed information about outcomes in prevalence panels is shown in Table 4
308 and Figure 3.

309 **Table 3** – Outcomes of cohort trials selected in this systematic review on effectiveness of bovine brucellosis vaccines (S19 and RB51), 1976-2009.

First author, year	Field challenge[a] %	Vaccination				Infection incidence %			RR[c] infection (95% CI**)	VE[d] infection %	Abortion incidence %		RR abortion (95% CI**)	VE abortion %	Other policies
		Age	Dose	Route	Booster	vaccinated	control	endpoint test[b]			vaccinated	control			
S19															
Crawford, 1978	14.27	Adults	3 x 10^9	SC[e]	No	8.54 (24/281)	10.92 (31/284)	12 months	0.78 (0.47-1.30)	21.75	NI[f]	NI	NA	NA[g]	No
Lord, 1998*	2.00	3-8 months	5 x 10^9	SC	No	0.00 (0/90)	25.00 (10/40)	8 months	0.00 (0-0.33)	100	30.00 (3/10)	40.00 (8/20)	0.75 (0.25-2.23)	25.00	No
Lord, 1998*	39.00	3-12 months	5 x 10^9	SC	No	14.67 (11/75)	25.00 (10/40)	8 months	0.59 (0.27-1.26)	41.33	55.00 (11/20)	90.00 (18/20)	0.61 (0.40-0.93)	38.89	No
Nicoletti, 1976	2.13	Adults	4-12 x 10^{10}	SC	No	10.51 (33/314)	13.29 (19/143)	6 months	0.43 (0.23-0.80)	56.86	NI	NI	NA	NA	Yes
Nicoletti, 1976	2.13	Adults	5 x 10^9	C[h]	S19	9.66 (28/290)	13.29 (19/143)	6 months	0.62 (0.35-1.10)	37.71	NI	NI	NA	NA	Yes
Odeon, 1987	5.50	Adults	3.1 x 10^9	SC	S19	3.45 (7/203)	8.76 (12/137)	24 months	0.39 (0.16-0.97)	60.63	0.98 (2/203)	1.46 (2/137)	0.68 (0.10-4.73)	32.51	Yes
Pinochet, 1986	18.50	Pregnant	5 x 10^9	C	S19	4.69 (10/213)	0.90 (2/222)	8 months	5.21 (1.16-23.51)	≤0	0.47 (1/213)	0.00 (0/222)	NC[i]	NC	No
RB51															
Cantú, 2007	8.70	Adults	4 x 10^9	SC	No	2.55 (10/392)	28.57 (10/35)	12 months	0.09 (0.04-0.20)	91.07	1.53 (6/392)	11.42 (4/35)	0.13 (0.04-0.45)	86.61	Yes
Cardeña, 2009	5.00	> 6 months	0.03-5 x 10^{10}	SC	No	0.00 (0/88)	3.4 (3/88)	18 months	0.00 (0-3.07)	100	NI	NI	NA	NA	No
Lord, 1998*	2.00	3-8 months	5 x 10^9	SC	No	0.00 (0/150)	25.00 (10/40)	8 months	0.00 (0-0.20)	100	0.00 (0/30)	40.00 (8/20)	0.00 (0.00-0.87)	100	No
Lord, 1998*	39.00	3-8 months	5 x 10^9	SC	No	0.00 (0/75)	25.00 (10/40)	8 months	0.00 (0-0.39)	100	0.00 (0/24)	90.00 (18/20)	0.00 (0.00-0.62)	100	No
Lord, 1998*	39.00	3-12 months	5 x 10^9	SC	RB51	0.00 (0/60)	25.00 (10/40)	8 months	0.00 (0-0.48)	100	0.00 (0/20)	90.00 (18/20)	0.00 (0.00-0.73)	100	No

310 [a]Field challenge: interval between vaccination and evaluation of the outcomes; [b]Endpoint test: interval between vaccination and outcome
311 evaluation; [c]VE: vaccine effectiveness; [d]RR: relative risk; [e]SC: subcutaneous; [f]NI: not informed; [g]NA: not applicable; [h]C: conjunctival, [i]NC: not
312 calculated.
313 *Abortion was recorded only among animals bred with infected bulls.
314 **Confidence interval (CI) values were not calculated when the absolute number of cases or population was not provided.

315 **Table 4** – Outcomes of prevalence panels trials selected in this systematic review on effectiveness of bovine brucellosis vaccines (S19 and RB51), 1992-2016.

First author, year	Vaccination				Prevalence infection (%)			PR[b] infection (CI 95%*)	VE[c] infection %	Prevalence abortion (%)		PR abortion (CI 95%*)	VE abortion %	Other policies
	Age	Dose	Route	Booster	Prior	Post	Endpoint test[a]			Prior	Post			
S19														
Al-Khalaf, 1992	Adults	3 x 10^9	SC[d]	S19	7.90 (948/12,000)	5.20 (624/11,052)	4 years	0.66 (0.60-0.73)	34.18	12.63 (12/95)	1.50 (2/130)	0.12 (0.03-0.53)	88.00	No
Bahena, 2003	Adults	3 x 10^9	SC	S19	8.22 (6/73)	0.00 (0/67)	9 months	0.00 (0-1.43)	100	NI[e]	NI	NA[f]	NA	No
Crawford, 1988	Adults	3 x 10^9	SC	No	34.72 (217/625)	2.5 (9/360)	12 months	0.07 (0.04-0.14)	92.80	NI	NI	NA	NA	No
Enright, 1984	Adults	2-3.0 x 10^9	SC	No	14.90 (48/322)	0.00 (0/248)	22-23 months	0.00 (0-0.19)	100	NI	NI	NA	NA	Yes
Enright, 1984	Adults	2-3.0 x 10^9	SC	No	3.54 (69/1664)	0.22 (3/1399)	23-29 months	0.06 (0.02-0.19)	93.79	NI	NI	NA	NA	Yes
Enright, 1984	> 12 months	2-3.0 x 10^9	SC	No	36.77 (82/223)	0.00 (0/136)	24 months	0.00 (0-0.14)	100	NI	NI	NA	NA	No
Enright, 1984	> 12 months	2-3.0 x 10^9	SC	No	6.54 (47/719)	0.34 (2/582)	24 months	0.05 (0-0.22)	94.75	NI	NI	NA	NA	No
Herr, 1984	Adults	1.9-3.8 x 10^9	SC	No	10.00 (106/1058)	0.90 (7/792)	23 months	0.09 (0.04-0.19)	91.00	NI	NI	NA	NA	Yes
Herr, 1984	Adults	1.9-3.8 x 10^9	SC	No	9.09 (127/1282)	0.00 (0/1044)	23-33 months	0.00 (0-0.07)	100	NI	NI	NA	NA	Yes
Herr, 1984	Adults	1.9-3.8 x 10^9	SC	No	7.70 (11/140)	4.30 (NI)	21 months	0.56 (NC[g])	44.16	NI	NI	NA	NA	No
Nicoletti, 1976	Adults	4-12 x 10^{10}	SC	No	2.46 (NI)	0.38 (NI)	13-15 months	0.15 (NC)	84.55	NI	NI	NA	NA	Yes
Nicoletti, 1976	Adults	4-12 x 10^{10}	ID[h]	No	2.75 (NI)	0.00 (NI)	13-15 months	0.00 (NC)	100	NI	NI	NA	NA	Yes
Nicoletti, 1976	Adults	4-12 x 10^{10}	SC	No	13.75 (NI)	0.74 (NI)	7-9 months	0.05 (NC)	94.62	NI	NI	NA	NA	Yes
Nicoletti, 1976	Adults	0.2-0.6 x 10^9	SC	No	13.75 (NI)	0.76 (NI)	7-9 months	0.05 (NC)	94.47	NI	NI	NA	NA	Yes
Nicoletti, 1979	Adults	3 x 10^9	SC	No	1.30 (707/54393)	00.18 (95/53699)	13 months	0.00 (0.11-0.17)	86.15	NI	NI	NA	NA	Yes

Nicoletti, 1979	Adults	3 x 10^9	SC	No	2.01 (218/10854)	0.09 (10/10633)	13 months	0.05 (0.02-0.09)	95.32	NI	NI	NA	NA	Yes
Viana, 1982	Adults	4.5 x 10^9	C[i]	S19	15.15 (15/99)	12.07 (7/58)	4 months after 2nd dose	0.80 (0.35-1.84)	20.00	27.60 (21/99)	0.00 (0/58)	0.00 (0-0.57)	100	Yes
Viana, 1989	Calves and adults	3 x 10^9	C	S19	3.45 (15/440)	0.00 (0/355)	24 months after 2nd dose	0.00 (0-0.62)	100	NI	NI	NA	NA	Yes
RB51														
Caetano, 2016	> 4 months	1-3.4 x 10^{10}	SC	RB51	39.31 (693/1763)	0.18 (2/1090)	5 years	0.00 (0.01-0.02)	100	NI	NI	NA	NA	Yes
Caetano, 2016	> 4 months	1-3.4 x 10^{10}	SC	RB51	0.49 (8/1637)	0.00 (1242)	5 years	0.00 (0-1.31)	100	NI	NI	NA	NA	Yes
López, 2007	Adults	3 x 10^9	SC	RB51	15.35	0.74	6 years	0.05 (0.00-0.12)	98.00	NI	NI	NA	NA	Yes

[a]Endpoint test: interval between vaccination and outcome evaluation; [b]PR: prevalence ratio; [c]VE: vaccine effectiveness; [d]SC: subcutaneous route; [e]NI: not informed; [f]NA: not applicable; [g]NC: not calculated; [h]ID: intradermal route; [i]C: conjunctival route.

*Confidence interval values were not calculated when the absolute number of cases or population was not provided.

320 *3.1. Effectiveness of S19 and RB51 vaccine strains along others control policies*

321 Besides vaccination, most of trials [57.57% (19/33)] also adopted other policies to
322 control brucellosis, such as the elimination of the positive animals from the herds, being four
323 [21.05% (4/19)] cohorts and 15 [78.95% (15/19)] prevalence panels. In general, 68.42% (13/19)
324 obtained significant effect of the vaccination to control infection, regardless of design study or
325 vaccine strain.

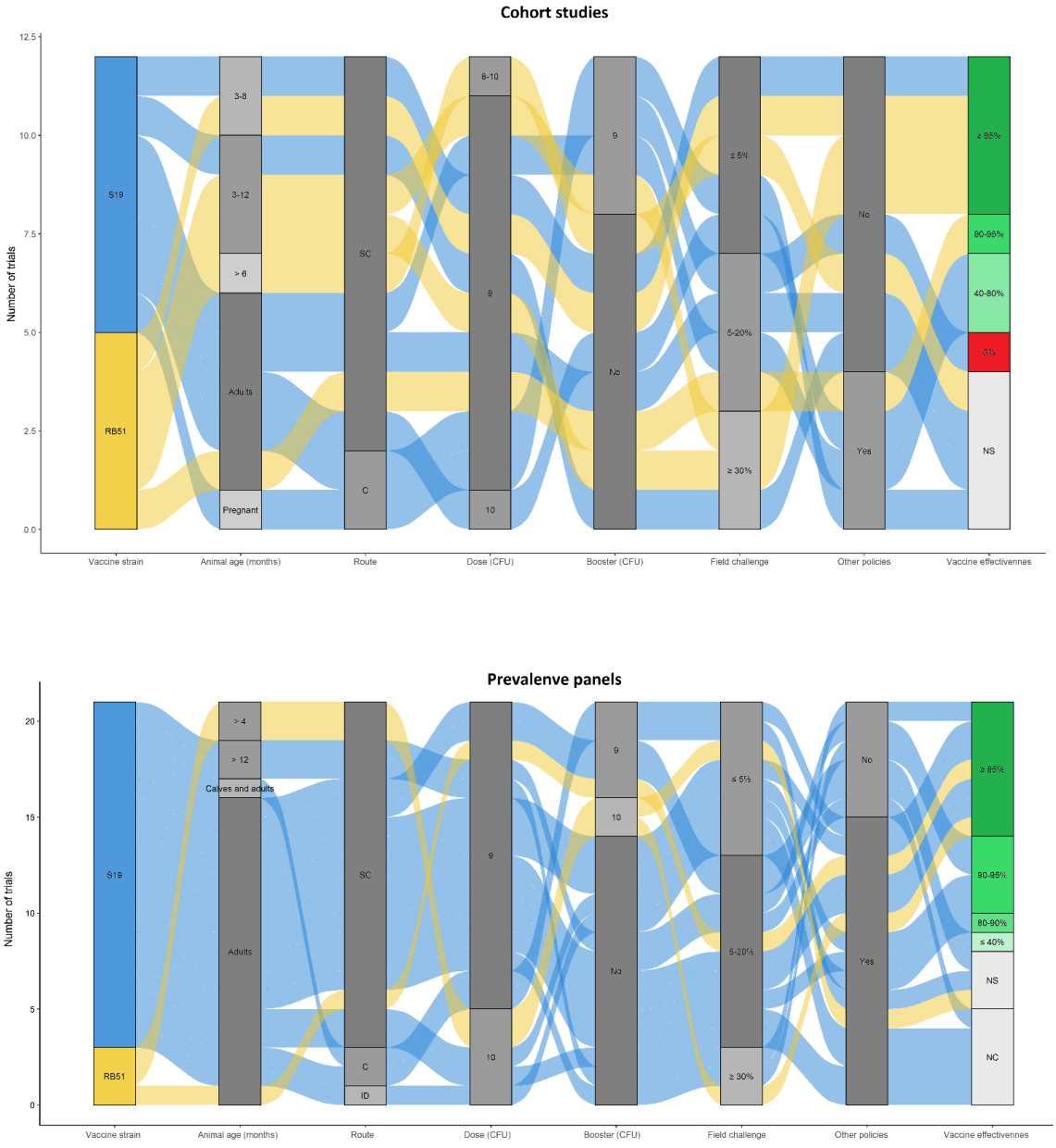
326 Regarding specifically the four cohort trials, two adopted the test-and-slaughter policy
327 of culture-positive animals [16.66% (2/12)], one performed test-and-slaughter of serological-
328 positives animals [8.33% (1/12)] and one eliminated the animals that have aborted during the
329 study period [8.33% (1/12)] (Table 3). Among these four trials [33.33% (4/12)], one used RB51
330 strain [24] and obtained 91.07% and 86.61% of significant VE for infection and abortion,
331 respectively. On the other hand, three trials used S19 strain and two demonstrated significant
332 VE for infection (56.86% and 60.63%), while one [18] also evaluated abortion, but it did not
333 find significative effect of vaccination for this outcome. The interval between vaccination and
334 outcomes evaluation in these cohort trials was, in average, 12 months (±7.35), varying between
335 6 and 24 months. Detailed information about outcomes and VE in the cohort trials is shown in
336 Table 3 and Figure 3, and the complete description of the other adopted control policies are
337 available in Supplementary Table S6.

338 Among all prevalence panel trials (21), 71.43% (15/21) used other control policies, the
339 most common being test-and-slaughter after bacteriology confirmation [28.57% (6/21)],
340 followed by slaughter of serological positive animals immediately [23.81% (5/21)] or not
341 immediately after diagnosis [14.29% (3/21)], and isolation of positive animals [4.76% (1/21)]
342 (Table 4). From these 15 trials, twelve [80% (12/15)] used S19, but it was not possible to
343 calculate CI for four of them [33.33% (4/12)] (Nicoletti, 1976), as the number of animals at risk
344 for each trial was not informed, only prevalence values before and after vaccination were

345 mentioned. Seven of the eight remaining trials [87.5% (7/8)] showed significant effect of
346 vaccination to control brucellosis in the field, with VE varying between 86.15 and 100%. Viana
347 et al. [20] did not demonstrate significant effect of vaccination on infection, although abortion
348 was significantly reduced by vaccination (VE = 100%). Among the trials that used RB51
349 [20.00% (3/15)], two showed significant effect of vaccination, with VE values of 98 and 100%.
350 Abortion was not evaluated as an outcome in none of RB51 prevalence panels. The average
351 interval between vaccination and outcomes evaluation was 25.87 months (±20.38), varying
352 between 4 and 72 months. Detailed information about outcomes in prevalence panels is shown
353 in Table 4 and Figure 3, and the complete description of the other adopted control policies is
354 available in Supplementary Table S6.

355 **Figure 3.** Alluvial diagrams showing the main experimental design characteristics and
356 vaccine effectiveness results of the 33 trials from 17 studies selected by this systematic review

357 on effectiveness of bovine brucellosis vaccines.



358 CFU: colony forming units; SC: subcutaneous; C: conjuntival; ID: intradermal; NS: not statistically significant; NC: not possible to obtain Confidence Interval.

359
360

4. Discussion

Vaccine effectiveness trials are needed to assess the balance between the benefits and side effects of a vaccination program, taking into account several heterogeneities that would not be possible to contemplate in controlled trials with an experimental challenge [31–33]. However, despite vaccination being a crucial measure for the control and eradication of brucellosis in cattle [2], the effectiveness of bovine brucellosis vaccines has not been robustly assessed so far. In this context, this present systematic review demonstrated that both S19 and RB51, mainly when associated with other control policies, are effective in field conditions, being able to reduce the infection caused by B. abortus.

Since the assessment of effectiveness is a "real world" view of vaccination programs, different populations (young and adult animals), field challenges (prevalence), vaccination strategies, and diagnostic methods were observed in the trials included in this present review. This heterogeneity precluded the performance of a meta-analysis from the data and thereby reached an average estimate of the S19 and RB51 field efficacy. Another important aspect was that most of them trials [57.57% (19/33)] adopted other control measures in addition to vaccination (Tables 3 and 4, and Supplementary Table S6), mainly the test-and-slaughter policy, an approach very common in brucellosis control programs worldwide [1]. This combination of policies is very justifiable, since it helps to accelerate disease control and brucellosis has great relevance in public health and economics [34], nevertheless, it hampers the evaluation of the isolated effect of the vaccination on the outcomes (infection and abortion). Even so, despite these issues that have prevented the performance of a meta-analysis, it was still possible to draw some important conclusions and to discuss other very relevant aspects about the effectiveness of the most adopted B. abortus vaccines, as well as about the conduction of trials for evaluating these vaccines in the field conditions.

In first place, this review allowed us to calculate the prevalence ratio (PR) and the relative risk (RR) for most of the trials analyzed (prevalence panels and cohorts, respectively), as well as their confidence intervals (CI) and the vaccine effectiveness (VE). Despite these measures being the most appropriate to evaluate efficacy of a vaccine in field conditions (effectiveness) [13], none of the studies selected by this review had previously calculated them, making inferences about the effectiveness of these vaccines only from the prevalence or incidence rates obtained before and after vaccination or comparing vaccinated and nonvaccinated groups. It is worth to mention that calculation of more robust and reliable measures associated with statistical parameters (CI values) are mandatory to assess

394 effectiveness in field trials and really understand the effect of the vaccination on reducing the
395 outcomes, providing sufficient evidence to conclude that the groups/panels were statistically
396 different.

397 Among the analyzed trials, it was possible to calculate CI for RR and PR for 28 trials
398 [84.84% (28/33)], of which 20 [71.43% (20/28)] exhibited significant difference between the
399 groups (vaccinated versus control) or between the panels (before and after vaccination) (Figure
400 2), suggesting that both S19 and RB51 are effective in protecting cattle against infection caused
401 by B. abortus in field conditions. It is also worth to mention that a higher percentage of trials
402 with significant effect was observed in those that combined vaccination with other control
403 measures [68.42% (13/19)] than in those that evaluated only vaccination [57.14% (8/14)],
404 suggesting that the combination of vaccination and other policies enhances the disease control.
405 On the other hand, seven trials [25.00% (7/28)] did not show significant protective effect of the
406 vaccination (CI including 1) and Pinochet et al. [35] observed negative effect of brucellosis
407 vaccination on the control of infection and abortion comparing the vaccinated group to control
408 animals. Although it is possible that there was not positive effect of vaccination on brucellosis
409 control in these populations, it seems more likely that these negative findings are related to
410 study design problems. One of the possible issues is the small sample size, that decreases the
411 power of the study and preclude finding statistical difference, even when it exists (Type II error)
412 [36,37]. In fact, among the seven studies with smaller samples sizes (n < 100 in each group or
413 before and after vaccination), 4 (57.14%) showed non-significant effectiveness - three using
414 S19 [15,24,29] and one using RB51 strain [18]. It is important to mention that this type of error
415 would have been partially fixed if it had been possible to perform a meta-analysis. Other
416 possible reason for these non-significant findings is the very low initial prevalence (< 0.5%)
417 observed in one of the prevalence panel trial [16], which could hinder to statistically
418 differentiate the proportion of cases before and after vaccination, since the initial rate was
419 already very low, which would have required a much large sampling to disclose any
420 significance. Indeed, it has been demonstrated that the effect of vaccination on low prevalence
421 rates is smaller than on higher prevalence rates [38,39].

422 Regarding specifically trials that used S19 strain, the interference of antibodies induced
423 by vaccination in the serological tests could also explain the negative findings, especially
424 considering that adult animals received two vaccinal stimulus, which leads to a more intense
425 and lasting humoral immune response [40] and possible false-positive results in serological
426 tests. In fact, other studies demonstrated that adults cows vaccinated with S19 take at least 6

months post-vaccination to return to negative status [41,42], with some animals remaining seropositive more than a year post vaccination [43]. In this way, the most common approach to confirm infection and refrain false-positive results is the use of one or more serological tests with higher sensitivity for screening, followed by a different and more specific diagnostic method as confirmatory test [13,44]. However, to combine tests in series or parallel, it is important to consider conditionally independent techniques or methods that have low conditional dependency [45], which is more likely when tests with different biological principles, e.g. direct and indirect tests [46], are combined. In this regard, among the analyzed trials, only Nicoletti [25] and Crawford et al. [19] used bacterial culture (direct test) as confirmatory, after screening the animals by serological tests (indirect tests) (Table 2). Bacteriology is a very specific and reliable technique, mainly when applied to tissues from aborted fetus, vaginal discharges or post-mortem diagnostic specimens, being also possible to be used with milk and udder secretion [6,47]. However, it is also important to point out that this is a time-consuming, biosafety level 3 restricted and low sensitivity technique (20 to 50%) [48], which generate a high proportion of false-negative results. Another option to have fast and reliable results in effectiveness trials is to use a polymerase chain reaction (PCR) method in association with serological tests or to choose a serological method of higher accuracy, as the fluorescence polarization assay (FPA) (98.66%) [46].

Still regarding diagnostic approaches, it is worth to mention that the diagnostic strategy adopted for each trial may influence the identification of cases and, consequently, vaccine effectiveness; since different approaches have different accuracies depending on the combination of tests and cut-offs adopted. The differences in the diagnostic tests adopted are probably related to the advance on diagnosis techniques throughout the years (such as improvements in diagnostic sensitivity and specificity, introduction of new techniques etc.) or technique specifications adopted in different countries for brucellosis diagnosis, as this review includes studies published between 1976 and 2016 and conducted in different places. Nevertheless, despite that the majority of the trials (85%) used very consolidated brucellosis-diagnostic tests for outcome definitions, such as CFT, 2ME, and STAT (Table 2), which have similar accuracy [46]; thereby, great differences in diagnostic accuracy between the trials are not expected.

As previously stated, most of the issues related to outcome definition (infection) in trials assessing the effectiveness of bovine brucellosis vaccines were associated with the use of S19, because this vaccine is not a DIVA (Differentiating Infected from Vaccinated Animals) and

460 induces antibodies that are detected by the routine serological tests. The reduced dose of S19
461 (109 CFU) was largely studied for vaccination of adult cattle in the decades of 1980 and early
462 1990, to accelerate brucellosis control in endemic areas [14,15,19,23,25,30], before the
463 emergence of the rough vaccine strain RB51 [7]. The present findings showed that the S19
464 reduced dose exhibited good results in reducing infection in field conditions (Table 2 and Figure
465 3). Similarly, this S19 reduced dose also shown to be effective in the meta-analysis conducted
466 previously from controlled clinical trials, which demonstrated that 109 CFU was the only
467 assessed dose of S19 that conferred protection against infection caused by B. abortus in cattle,
468 besides protection against abortion [5]. Currently, the use of S19 reduced dose in adult cattle is
469 not common although can still be used, since RB51 (1010 CFU) has comparable efficacy to
470 S19 [5], without the inconvenience of inducing antibodies that can be detected by routine
471 bovine brucellosis serological tests [7,49]. Indeed, the World Organisation for Animal Health
472 (WOAH) still recommend the use of the S19 reduced dose (5 x 109 CFU) for adult cattle by
473 the conjunctive route [6], as an alternative, especially useful for areas that do not have a well-
474 established calfhood vaccination program; even thought this vaccination can result in false-
475 positive serological reactions and, sporadically, in abortions. Among the trials included in this
476 review, four evaluated the occurrence of abortions after vaccination of adult animals with
477 reduced dose of S19 strain and two of them (50%) did not find good results [27,28]. Since, in
478 these cases, abortion was evaluated after a short period (4 to 8 months) and the abortion-causing
479 strain was not determined (wild or S19 strain), it is possible to speculate that some of the
480 abortions were caused by vaccination, decreasing the effectiveness for this outcome.

481 Moreover, it is important to mention that S19 was evaluated in a higher number of trials
482 [75.76% (25/33)] compared to RB51, which could be explained by the longer time that this
483 vaccine is in use. Also, most of the trials (21/33) included in the present review were prevalence
484 panels, which are epidemiological studies that are easier and less expensive to perform but
485 considerably less robust than cohort studies (adopted in 12 of the 33 analyzed trials) [13].
486 Indeed, prospective cohorts are the ideal design to assess the effectiveness of a vaccine, since
487 exposure and outcomes can be measured more accurately [13,50], even more when different
488 levels of prevalence (low, middle, and high) are evaluated in the same study. Furthermore, it is
489 critical that in an ideal study, vaccinated and non-vaccinated animals are in the same herd and
490 both direct and indirect diagnostic tests are used to determine the outcome. In addition, abortion
491 should be also evaluated as outcome, as it is the major clinical sign of brucellosis and the
492 principal source of infection and of Brucella spp. for bacteriology [47]. Additionally, in S19

trials, the age of the animals, the serological tests and the interval between vaccination and outcome assessment should also be carefully considered to avoid false-positive results. Finally, to undoubtedly evaluate the isolated effect of bovine brucellosis vaccines in field conditions, it is essential to perform field trials without the adoption of other control measures, as well as to adopt more robust measures for effectiveness evaluation, such as RR and VE.

The main limitation of the present study, as stated before, was the impossibility to perform a meta-analysis due to heterogeneity of the selected studies, which were very diverse in various aspects, such as the initial prevalence of brucellosis in the population, breed and age of the animals, strain and dose of the vaccines, route of vaccination, outcome definition, interval between vaccination and testing, and adoption of other control measures and vaccination booster.

In conclusion, this systematic review suggests that S19 and RB51 vaccine strains are effective in the field to control infection and abortion caused by B. abortus, mainly when associated to other brucellosis control policies. However, it was not possible to draw more definitive conclusions, since the analyzed trials were not comparable regarding important characteristics, which precluded a most robust analysis of the extracted data, and only a few trials were considered optimal to evaluate vaccine effectiveness. Among the major characteristics needed to properly evaluate the individual effect of vaccination in field, the non-adoption of other control measures is fundamental, in addition to utilization of prospective cohort design, combination of direct and indirect diagnostic tests for case definition, evaluation of infection and abortion as outcomes, and taking carefully into account the age at vaccination and the interval between test and vaccination for trials using S19 vaccine strain.

Acknowledgments

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig) (APQ-02726-21 and RED-00132-22) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes). MMO and RSA thank Capes for their scholarships, MSG thank Fapemig for her scholarship, and EMSD is indebted to CNPq to her fellowship.

Conflict of interests

The authors declare no competing interests.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as this is a review article with no original research data.

Data availability statement

The data that supports the findings of this study are available in the supplementary material.

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668 **Supplementary material**
669
670 **Supplementary Table S1** – Prisma checklist 2020.

Section and Topic	Item #	Checklist item	Paragraph where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	2 to 4
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	4
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	8
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	6
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	6 to 7
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	6 to 7
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	6-8
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	9 to 11
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	9 to 11

Section and Topic	Item #	Checklist item	Paragraph where item is reported
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	6 to 11
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	11
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	6 to 11
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	6 to 11
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	11
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	11
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	13
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	Not done
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Not done
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Not done
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	12 to 13
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Supplementary Table 4
Study characteristics	17	Cite each included study and present its characteristics.	Table 1

Section and Topic	Item #	Checklist item	Paragraph where item is reported
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Not done
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Tables 3 and 4
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Not done
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Tables 3 and 4
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	13
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	Not done
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Not done
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Not done
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	26
	23b	Discuss any limitations of the evidence included in the review.	27 to 32
	23c	Discuss any limitations of the review processes used.	33
	23d	Discuss implications of the results for practice, policy, and future research.	32
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Not registered
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Not prepared
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Not applied
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	35

Section and Topic	Item #	Checklist item	Paragraph where item is reported
Competing interests	26	Declare any competing interests of review authors.	36
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Not available

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

For more information, visit: http://www.prisma-statement.org/

674 **Supplementary Table S2 –** Search terms used in CABI, Cochrane, Pubmed, Scielo, Scopus and Web of Science databases, based on the PICO terms.

675

PICO	Search terms
Population	bovine* OR cattle OR calve* OR calf OR heifer* OR cow* OR herd*
Intervention	(RB51 OR SRB51 OR strain RB51 OR S19 OR strain 19 OR B19 OR vaccin*) OR (*Brucella* AND abortus)
Comparison	(field OR infect*) OR (prevalen* OR persisten* OR incidenc* OR epidemiol* OR erradicat* OR control* OR prevent* OR efficacy OR effect* OR immun* OR protect* OR safe*)
Outcomes	antibod* OR serolog* OR (normal OR calving) OR pregnan* OR abort* OR stillbirth OR (weak OR calve* OR calf) OR bacterial* OR infect*

676

677 **Supplementary Table S3 –** Inclusion and exclusion criteria for selection of articles in this systematic review.

Inclusion criteria	Exclusion criteria
All countries	Vaccination with other vaccines strains
All years	Brucellosis caused by other species than *B. abortus*
Effectiveness against *Brucella abortus*	Experimental or no challenge
Vaccination of cattle	Other observational studies (case-control, case series, case report, etc)
Non-experimental challenge (field assays)	No information about serology, clinical signs, or bacteriology (post-vaccination assessment)
Cross-sectional or cohort studies	Vaccination of other animal species
Vaccination with S19 or RB51	No information about vaccine dose, strain or route used
Data on brucellosis prevalence (natural challenge)	No information about natural challenge (initial prevalence before vaccination)
Data on post-vaccination brucellosis assessment	Written in other languages than English, Spanish, French or Portuguese
	Full text not available

678

679 **Supplementary Table S4 –** Articles excluded and reasons of exclusion after quality assessment, in this systematic review on effectiveness of bovine
680 brucellosis vaccines (S19 and RB51), 1976-2016.

Author	Title	Exclusion reason
Adlam, G. H., 1978	The eradication of bovine brucellosis in New Zealand: History and objectives.	No information on dose or via of vaccination
Breitmeyer, R. E., et al., 1992	Serologic and bacteriologic test results after adult vaccination with strain 19 in three dairy herds infected with brucellosis	No information on disease prevalence (natural challenge)
Chand, P., et al., 2014	Vaccination of adult animals with a reduced dose of *Brucella abortus* S19 vaccine to control brucellosis on dairy farms in endemic areas of India	No control group or information on post vaccination assessments
Crawford, R. P., et al., 1988	Value of serologic reactions at 2 months following strain 19 vaccination of cattle herds with brucellosis	No information on disease prevalence (natural challenge)
Crawford, R. P., et al., 1986	*Brucella abortus* strain 19 vaccination of adult beef cattle and the effect of anthelmintics	No information on disease prevalence (natural challenge)
Crawford, R. P., et al., 1988	Early removal of cows with Brucellosis and the effect in strain 19 vaccinated cattle herds	Data published elsewhere
Denes, B., 1997	Serological findings obtained in cattle herds 110rganized with the *Brucella melitensis* Rev.1 and the B. abortus B19 vaccine in Mongolia	No control group or information on post vaccination assessments
Dias, R. A., et al., 2016	Controlling bovine brucellosis in the state of São Paulo, Brazil: Results after ten years of a vaccination program	Assessment of vaccination campaign
Díaz-Aparicio, E., et al., 2007	Characterization of the transitory immune response in cows immunized with RB51 and its implication on diagnosis within Brucellosis endemic zones	No control group or information on post vaccination assessments

Author	Title	Exclusion reason
Erasmus, J. A., 1989	Bovine brucellosis in the Highveld region: effect of calfhood vaccination	Assessment of vaccination campaign
González Tomé, J., et al., 1987	Eradication of bovine brucellosis in a highly infected dairy farm.	No information on disease prevalence (natural challenge)
González Tomé, J. S., et al., 1987	Bovine brucellosis: revaccination of serologically negative adult cows with a reduced dose of *Brucella abortus* strain 19.	No information on disease prevalence (natural challenge)
Gwida, M., et al., 2016	Use of serology and real time PCR to control an outbreak of bovine brucellosis at a dairy cattle farm in the Nile Delta region, Egypt	No information on disease prevalence (natural challenge)
Herrera-Lopez, E., et al., 2010	Epidemiological study of Brucellosis in cattle, immunized with *Brucella abortus* RB51 vaccine in endemic zones	Data published elsewhere
Johnson, D. W., et al., 1984	Brucellosis control in an infected herd by serological testing, removal of reactors, vaccination with reduced dose of strain 19 and improved hygiene	No information on disease prevalence (natural challenge)
Leal-Hernandez, M., et al., 2005	Protection of *Brucella abortus* RB51 revaccinated cows, introduced in a herd with active Brucellosis, with presence of atypical humoral response.	No control group or information on post vaccination assessments
Martínez Herrera, D. I., et al., 2011	Evaluation of *Brucella abortus* S19 strain for the control of bovine 111rganized111s in Actopan municipality, state of Veracruz, Mexico.	No information on disease prevalence (natural challenge)
Martins, H., et al., 2009	Eradication of bovine brucellosis in the Azores, Portugal – Outcome of a 5-year programme (2002-2007) based on test-and-slaughter and RB51 vaccination.	Assessment of vaccination campaign

Author	Title	Exclusion reason
Ndoci, B. and P. Muhedini, 2013	Control of brucellosis in cattle from Durres and Lushnja complexes through the application of *Brucella abortus* rb51 vaccine control of brucellosis in cattle.	No information on dose or via of vaccination
Nicoletti, P., 1981	The efficacy of strain 19 vaccination in reducing brucellosis in large dairy herds	Data published elsewhere
Nicoletti, P., 1981	Prevalence and persistence of *Brucella abortus* strain 19 infections and prevalence of other biotypes in vaccinated adult dairy cattle	Data published elsewhere
Nicoletti, P., 1984	Vaccination of cattle with *Brucella abortus* Strain 19 administered by differing routes and doses	Data published elsewhere
Nicoletti, P., et al., 1978	Adult vaccination with standard and reduced doses of *Brucella abortus* strain 19 vaccine in a dairy herd infected with brucellosis	No information on disease prevalence (natural challenge)
Nicoletti, P., et al., 1978	Comparison of the subcutaneous and conjunctival route of vaccination with *Brucella abortus* strain 19 vaccine in adult cattle	No information on disease prevalence (natural challenge)
Nicoletti, P. and F. W. Milward, 1984	A comparison of subcutaneous and oral administration of *Brucella abortus* strain 19 in a large infected dairy herd	No control group or information on post vaccination assessments
Peniche Cardeña, A., et al.	Evaluation of vaccination with *Brucella abortus* RB51 strain in herds naturally infected with brucellosis in productive systems found in tropical climate	No control group or information on post vaccination assessments

Author	Title	Exclusion reason
Peniche-Cardena, A., et al., 2012	Economic Analysis of Efficiency of RB51 Strain Vaccine of *Brucella abortus* Applied in Herds Naturally Infected with Brucellosis in Tropical Climate	Data published elsewhere
Saez, J. L., et al., 2014	Comparison of depopulation and S19-RB51 vaccination strategies for control of bovine brucellosis in high prevalence areas.	Assessment of vaccination campaign
Sandhu, K. S. and D. V. Joshi, 1990	Effect of *Brucella abortus* strain 19 vaccine on abortion rate in cattle and buffaloes on an 113rganized farm	No information on disease prevalence (natural challenge)
Sanz, C., et al., 2010	Mass vaccination as a complementary tool in the control of a severe outbreak of bovine brucellosis due to *Brucella abortus* in Extremadura, Spain	Assessment of vaccination campaign
Singh, B. B., et al., 2018	Cost-benefit analysis of intervention policies for prevention and control of brucellosis in India	Assessment of vaccination campaign
Tittarelli, M., et al., 2008	*Brucella abortus* strain RB51 vaccine: Immune response after calfhood vaccination and field investigation in Italian cattle population	No information on disease prevalence (natural challenge)

681

682 **Supplementary Table S5** – Detailed information about data used for trials in studies that conduced multiple trials, in this systematic review on effectiveness of
683 bovine brucellosis vaccines (S19 and RB51), 1976-2016.

Study	Trial*	Field challenge (%)	Strain	Source of data used for each trial
Caetano, 2016	1	39.31	RB51	Number of vaccinated animals and prevalence after and before vaccination were obtained from the herd with high prevalence of brucellosis. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	2	0.49	RB51	Number of vaccinated animals and prevalence after and before vaccination were obtained from the herd with low prevalence of brucellosis. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
Enright, 1984	1	14.90	S19	Number of vaccinated animals and data about prevalence after and before vaccination were obtained by grouping data from herd 3 and herd 5 of the Plan A program. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	2	3.54	S19	Number of vaccinated animals and data about prevalence after and before vaccination were obtained by grouping data from herd 1, herd 2 and herd 4 of Plan A program. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	3	36.77	S19	Number of vaccinated animals and data about prevalence after and before vaccination were obtained by grouping data from herd 1, herd 2 of Plan B program. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.

Study	Trial*	Field challenge (%)	Strain	Source of data used for each trial
	4	6.54	S19	Number of vaccinated animals and data about prevalence after and before vaccination were obtained by grouping data from herd 3, herd 4 of Plan B program. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
Herr, 1894	1	10.0	S19	Number of vaccinated animals and prevalence after and before vaccination were obtained from herd B. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	2	9.09	S19	Number of vaccinated animals and prevalence after and before vaccination were obtained by grouping herd C and herd E. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	3	7.70	S19	Number of vaccinated animals and data prevalence after and before vaccination were obtained by data showed for herd D. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
Lord, 1998	1	2.00	S19	Number of animals in control group were obtained from group X and number of vaccinated animals in vaccinated group were obtained from group XI, as well incidences for both groups, respectively. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	2	39.00	S19	Number of animals in control group were obtained combining groups I and V and number of vaccinated animals in vaccinated group were obtained from groups IV and VIII. Incidences for each group (vaccinated and control) were obtained by average. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.

Study	Trial*	Field challenge (%)	Strain	Source of data used for each trial
	3	2.00	RB51	Number of animals in control group were obtained from group IX and number of vaccinated animals in vaccinated group were obtained from group XI, as well incidences for both groups, respectively. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	4	39.00	RB51	Number of animals in control group were obtained from combining of groups II and VI and number of vaccinated animals in vaccinated group were obtained from groups IV and VIII. Incidences for each group (vaccinated and control) were obtained by average. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	5	39.00	RB51	Number of animals in control group were obtained from combining of groups III and VII and number of vaccinated animals in vaccinated group were obtained from groups IV and VIII. Incidences for each group (vaccinated and control) were obtained by average. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
Nicoletti, 1976	1	2.13	S19	Data for this cohort trial was obtained from animals of herd 5 that received 5 cc of S19 subcutaneously (40% of the herd). Control group was the animals that were not vaccinated in the same herd (20 % of the population). All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	2	2.13	S19	Data for this cohort trial was obtained from animals of herd 5 that received 0.1 cc of S19 by conjunctival route (40% of the herd). Control group was the animals that were not vaccinated in the same herd (20% of the population). All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.

Study	Trial*	Field challenge (%)	Strain	Source of data used for each trial
	3	2.46	S19	Data for this prevalence panel was obtained by combination of the data from herd 1 and half herd 2, which received 5 cc of S19 subcutaneously. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	4	2.75	S19	Number of vaccinated animals and prevalence before and after vaccination in this prevalence panel was obtained from the half of herd 2 that received 2 cc of S19 by intradermal route. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	5	13.75	S19	Number of vaccinated animals and prevalence before and after vaccination in this prevalence panel was obtained from the part of herd 4 that received 5 cc of S19 subcutaneously. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	6	13.75	S19	Number of vaccinated animals and prevalence before and after vaccination in this prevalence panel was obtained from the half of herd 4 that received 0,25 cc of S19 subcutaneously. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
Nicoletti, 1979	1	1.30	S19	Number of vaccinated animals and prevalence before and after vaccination in this prevalence panel was obtained from the data of the herd localized in Florida. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.
	2	2.01	S19	Number of vaccinated animals and prevalence before and after vaccination in this prevalence panel was obtained from the data of the herd localized in Puerto Rico. All data about vaccination strategy, other control measures and outcome evaluation were also obtained from the described for this population.

*Numbered according to the order of presentation in Table 1.

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686 **Supplementary Table S6** – Detailed information about other policies of control in addition to vaccination adopted in the trials analyzed in this systematic
687 review on effectiveness of bovine brucellosis vaccines (S19 and RB51), 1976-2016.

First author, year	Study design	Field Challenge (%)	Strain	Dose	Route	Other policies of control
Al-Khalaf, 1992	PP	7.90	S19	3 x 10^9	SC	No
Bahena, 2003	PP	8.22	S19	3 x 10^9	SC	No
Caetano, 2016	PP	39.31	RB51	1-3.4 x 10^{10}	SC	Test-and-slaughter of positives
Caetano, 2016	PP	0.49	RB51	1-3.4 x 10^{10}	SC	Test-and-slaughter of positives
Cantú, 2007	Ch[j]	8.70	RB51	4 x 10^9	SC	Test-and-slaughter of positives
Cardeña, 2009	Ch	5.00	RB51	0.03-5 x 10^{10}	SC	No
Crawford, 1978	Ch	14.27	S19	3 x 10^9	SC	No
Crawford, 1988	PP	34.72	S19	3 x 10^9	SC	No
Enright, 1984	PP	14.90	S19	2-3 x 10^9	SC	Test-and-slaughter of positives
Enright, 1984	PP	3.54	S19	2-3 x 10^9	SC	Test-and-slaughter of positives
Enright, 1984	PP	36.77	S19	2-3 x 10^9	SC	No
Enright, 1984	PP	6.54	S19	2-3 x 10^9	SC	No
Herr, 1984	PP	10.00	S19	1.9-3.8 x 10^9	SC	Isolation of positives
Herr, 1984	PP	9.09	S19	1.9-3.8 x 10^9	SC	Test-and-slaughter of positives
Herr, 1984	PP	7.70	S19	1.9-3.8 x 10^9	SC	No
López, 2007	PP	15.35	RB51	3 x 10^9	SC	Test-and-slaughter of positives (not immediately)
Lord, 1998	Ch	2.00	S19	5 x 10^9	SC	No

Lord, 1998	Ch	39.00	S19	5 x 10^9	SC	No
Lord, 1998	Ch	2.00	RB51	5 x 10^9	SC	No
Lord, 1998	Ch	39.00	RB51	5 x 10^9	SC	No
Lord, 1998	Ch	39.00	RB51	5 x 10^9	SC	No
Nicoletti, 1976	Ch	2.13	S19	4-12 x 10^{10}	SC	Test-and-slaughter of positives after bacteriology
Nicoletti, 1976	Ch	2.13	S19	5 x 10^9	C	Test-and-slaughter of positives after bacteriology
Nicoletti, 1976	PP	2.46	S19	4-12 x 10^{10}	SC	Test-and-slaughter of positives after bacteriology
Nicoletti, 1976	PP	2.75	S19	4-12 x 10^{10}	ID	Test-and-slaughter of positives after bacteriology
Nicoletti, 1976	PP	13.75	S19	4-12 x 10^{10}	SC	Test-and-slaughter of positives after bacteriology
Nicoletti, 1976	PP	13.75	S19	0.2-0.6 x 10^9	SC	Test-and-slaughter of positives after bacteriology
Nicoletti, 1979	PP	1.30	S19	3 x 10^9	SC	Test-and-slaughter of positives (not immediately)
Nicoletti, 1979	PP	2.01	S19	3 x 10^9	SC	Test-and-slaughter of positives (not immediately)
Odeon, 1987	Ch	5.50	S19	3.1 x 10^9	SC	Elimination of animals that have aborted
Pinochet, 1986	Ch	18.50	S19	5 x 10^9	C	No
Viana, 1982	PP	15.15	S19	4.5 x 10^9	IC	Test-and-slaughter of positives (not immediately)
Viana, 1989	PP	3.45	S19	3 x 10^9	IC	Test-and-slaughter of positives (not immediately)

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CHAPTER IV

Published at: Veterinary Immunology and Immunopathology (doi: 10.1016/j.vetimm.2025.110919)

Short communication: Effects of age on the immune response induced by *Brucella abortus* S19 or RB51 vaccination in calves

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Abstract

Vaccination of bovine calves is one of the main policies for bovine brucellosis control in endemic areas. However, the effect of animal age on vaccine immunogenicity is still unknown and could help to determine an ideal age for vaccination, in order to maximize immune response. Thus, the objective of this study was to compare the in vitro expression of IFN-γ by stimulated PBMC after vaccination with B. abortus S19 and RB51 strains in calves vaccinated at different ages, between 3 and 8 months. Cell-mediated immune response was assessed through culture of peripheral blood mononuclear cells (PBMC) and quantification of IFN-γ in the supernatant by enzyme-linked immunosorbent assay (ELISA). In addition, serological assays were performed using 2-mercaptoethanol (2-ME) and Standard Tube Agglutination (STAT) tests. Blood samples and sera were collected in the inoculation day, as well as at 28 and 56 days after vaccination. A generalized linear mixed model was used to evaluate effect of age at vaccination on in vitro production of IFN-γ and no differences were observed comparing the

different ages, for both RB51 and S19 vaccines ($p > 0.05$). A higher percentage of animals vaccinated with S19 at 3-4 months-old [77.28% (7/9)] returned to the serological negative status at day 56, when compared to 5-6-months [50% (5/10)] and 7-8 months-old animals [27.28% (3/11)]. In conclusion, our findings indicated similar levels of IFN-γ in vitro production in animals between 3 to 8 months of age, following vaccination with S19 and RB51 strains.

1. Introduction

Brucellosis is an infectious contagious disease that can affect several species, including humans. *Brucella abortus* is the main species infecting cattle and it is also a zoonotic agent, being transmitted to humans by ingestion of non-pasteurized milk and dairy products, as well as through contact with infected animals (Corbel et al., 2006; Dadar et al., 2021; WOAH, 2022). In cattle, brucellosis mainly causes reproductive signs, such as infertility, abortions in the final third of pregnancy, stillbirth and weak calves (Corbel et al., 2006), leading to high economic losses for livestock (Bernués et al., 1997). In humans, it is a chronic and debilitating disease, being considered one of the most prevalent bacterial zoonosis worldwide, especially important in low-income countries (Franc et al., 2018; Laine et al., 2023).

Vaccination of calves with the live *B. abortus* vaccines, particularly S19 or RB51, is a key tool to control brucellosis in cattle herds in many countries (Zhang et al., 2018), an approach that aims to reduce the disease prevalence in both human and animal populations. Even though these vaccines have a satisfactory efficacy, average around 60-70% (Oliveira et al., 2022), vaccination is frequently associate d with other control policies, mainly test-and-slaughter, in order to accelerate control programs and ensure the reduction of bovine brucellosis prevalence in the field (Gonçalves et al., 2024; Zhang et al., 2018).

In addition to test-and-slaughter policy, optimizing individual immune response induced by vaccination is also an option to improve herd immunity and, consequently, enhance brucellosis control. Revaccination could be a pathway to achieved this improvement (Dorneles et al., 2015a; Olsen and Boggiatto, 2022), but it is not yet widely adopted, since most of the control programs is based on single vaccination during calfhood. An alternative to maximize the immune response trigged by single vaccination would be the determination of the ideal age at vaccination to maximize the immune response triggered.

Indeed, the World Organisation for Animal Health (WOAH) recommends to vaccinate animals from 3 months to 12 months-old (S19: 3 to 8 months; RB51: 3 to 12 months) (WOAH, 2022), covering an vaccination interval of up 9 months. However, during calfhood, animals are still in development and age is a factor that can influence the establishment of immune response (Zimmermann and Curtis, 2019). As examples, vaccination of animals close to the lower age limit (3 months) may be influenced by passive immunity antibodies and the immaturity of the immune system (Cunningham, 1977; Redman et al., 1967), while vaccination of older animals

may be influenced by sexual maturity (Manthei, 1968; Nicoletti, 1990), especially in early maturing animals (e.g. *Bos taurus taurus*).

In fact, some studies observed differences in the persistence of vaccinal antibodies in animals vaccinated with S19 at different ages, suggesting that age indeed influences the establishment of the immune response trigged by brucellosis vaccination (Gilman and Wagner, 1959; King and Frank, 1961; McDiarmid, 1957). On the other hand, other studies that performed experimental challenge in cows suggested that there are no differences in the incidence of infection and abortion comparing animals vaccinated with S19 and RB51 between 3 and 10 months of age (Cheville et al., 1996; Gilman and Wagner, 1959).

Therefore, findings obtained so far are still not conclusive, especially considering that none of them have evaluated parameters of cell-mediated immune response, particularly Th1 immune response. Immune response after infection or vaccination against brucellosis is mainly characterized by a strong IFN-γ production by $CD4^+$ T cells (Dorneles et al., 2015b). This cytokine activates macrophages and increases their efficiency on killing bacteria. Consequently, *Brucella* spp. replication inside macrophages is inhibited, hampering the establishment of chronic diseases (Dorneles et al., 2015b). Thus, assessment of the *in vitro* production of IFN-γ following vaccination is a reliable parameter for evaluating immunogenicity and compare vaccination approaches, including different ages at vaccination (Dorneles et al., 2014; E. M. S. Dorneles et al., 2015a).

Hence, the objective of this study was to compare the *in vitro* expression of IFN-γ by stimulated peripheral blood mononuclear cells (PBMC) after vaccination with *B. abortus* S19 and RB51 strains in calves vaccinated at different ages, between 3 and 8 months.

2. Material and Methods

2.1. Ethics Statement

Experiments with cattle were carried out in strict accordance with Brazilian law on use of animal in research and teaching (Lei n° 11.794/2008) and approved by the Ethical Committee for Animal Use (Comitê de Ética para o Uso de Animais) of the Universidade Federal de Lavras (CEUA-UFLA) under protocol 069/2018.

2.2. Animals and experimental design

The study was conducted in a dairy farm with no history of brucellosis, localized in Ilicínea, Minas Gerais, Brazil. The herd was composed exclusively by Holstein cows. Weaned calves were maintained on pasture paddocks, grouped according to age and weight. Diet was mainly based on pasture with concentrate and mineral supplementation and water *ad libitum*. One hundred and eight (108) female calves, non-vaccinated against brucellosis were selected. Vaccination was performed at three different timeframes (three blocks), due to the limitation of personal and laboratory resources for sampling and carry out all the tests at once. The animals were randomly divided into nine experimental groups, considering vaccinal stimulus (S19 strain, RB51 strain, or control), age (3-4 months, 5-6 months, and 7-8 months) and block. Vaccination was performed at day 0 and the immune response was evaluated at the days 28 and 56 post-vaccination, considering each block. Blood samples were collected by jugular venipuncture from all calves at days 0, 28 and 56 post-vaccination in heparin tubes and tubes for plasma blood collection, for cell culture and serological tests, respectively. The experimental design and the number of animals per group are shown in Figure 1.

2.3. Vaccines and vaccination

Animals vaccinated with S19 received 0.6 - 1.2 x 10^{11} colony-forming unit (CFU) of a commercial vaccine (MSD Animal Health, Brazil), in a volume of 2 mL inoculated subcutaneously in the injection triangle region of the neck using a 25 X 0.8 mm needle. Animals vaccinated with RB51 received 2 mL (1 – 3,4 x 10^{10} CFU) of a commercial vaccine (MSD Animal Health, Brazil) (Brazil. Ministério da Agricultura Pecuária e Abastecimento, 2017) and control groups were inoculated with 2 mL of sterile saline solution (0.85%) under the same conditions. All animals of the control group were vaccinated with RB51 (1 – 3,4 x 10^{10} CFU) at the end of the study.

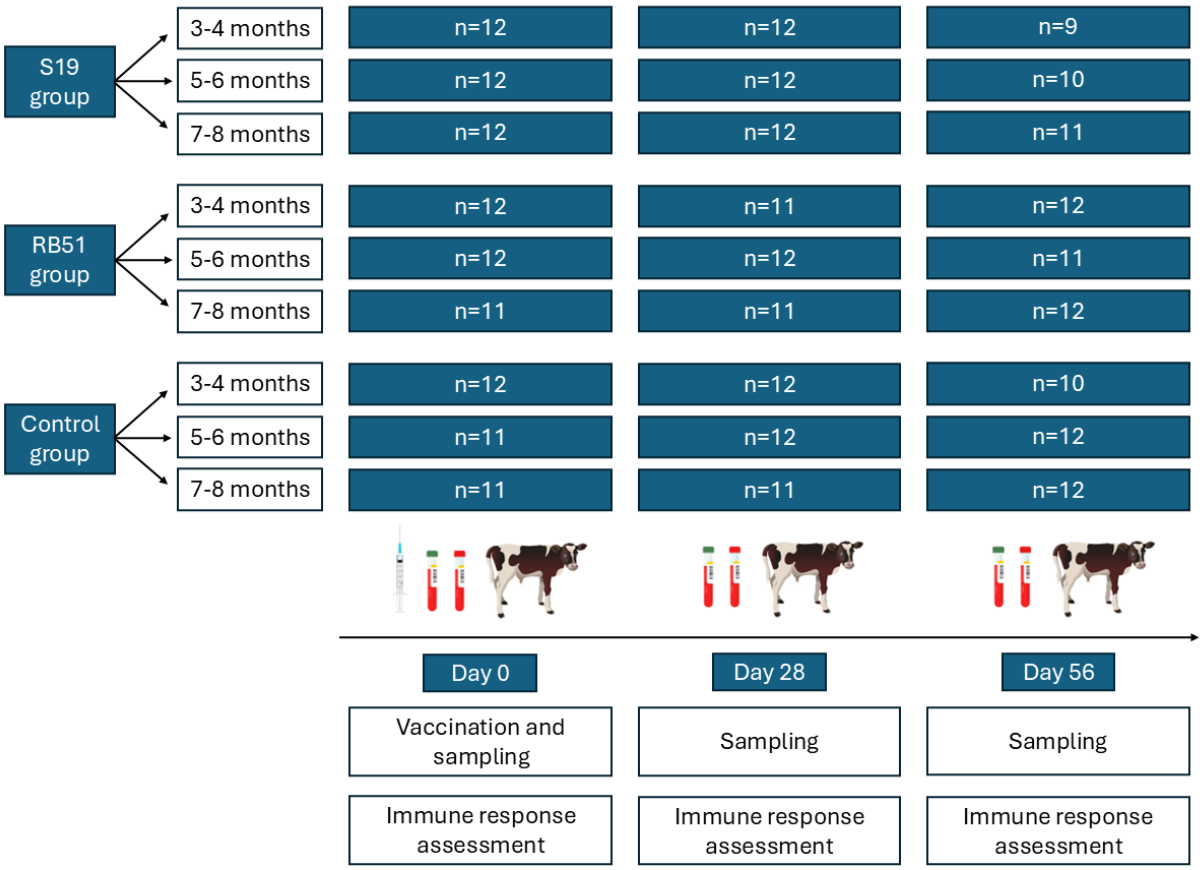

Figure 1. Experimental design for evaluation of the impact of age on the immune response induced by *Brucella abortus* s19 or RB51 vaccination in calves. One hundred and eight females calves aged between 3 to 8 months were divided in nine experimental groups: three S19 groups, vaccinated at day 0 of the experiment (3-4, 5-6 and 7-8 months old); three RB51 groups vaccinated at day 0 of the experiment (3-4, 5-6 and 7-8 months old) and three control groups, inoculated with saline solution (0.85%) at day 0 of the experiment (3-4, 5-6 and 7-8 months old). Immune response of all animals was assessed at day 0, 28 and 56 of the experiment. The number of animals per group and in each day is shown in the figure.

2.4. Peripheral blood mononuclear cells (PBMCs) isolation, culture and IFN-γ detection in the supernatant

Peripheral blood mononuclear cells (PBMCs) were extracted from peripheral heparinized blood by Ficoll® gradient centrifugation, as described by Palmer et al. [16] with modifications suggested by Dorneles et al. [17].

To evaluate the accumulation of IFN-γ in the culture supernatant, PBMC were cultured for 72 hours at 37 ºC and 5% CO_2 in 48-well flat bottom plates (Corning, USA). Antigen

stimulated cultures were incubated with γ-irradiated (1.4 x 10^6 rads) *B. abortus* strain 2308 (10^8 CFU/mL), control cultures with RPMI 1640 (Sigma-Aldrich, USA) and positive control cultures with phytohaemagglutinin-L (PHA-L) (Merck, Germany) (5 μg/mL).

Following incubation, supernatant was collected and IFN-γ amounts were quantified by enzyme-linked immunosorbent assay (ELISA). Assays were performed using the Bovine IFN-γ ELISA kit (ESS0026B - Thermo Fisher Scientific, USA) according to the manufacturer's recommendations.

2.5. Humoral immune response assays

The 2-mercaptoethanol (2-ME) and the Standard Tube Agglutination Test (STAT) test for assessment of smooth-LPS antibodies were performed according to the recommendations of the National Program of Control and Erradication of Brucellosis and Tubercullosis [Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose (PNCEBT)] [11].

2.6. Statistical analysis

As no IFN- γ production were expected for the control group at all evaluated time frames and for vaccinated groups at day 0, all zeros in the dataset were imputed with values in the lognormal distribution, truncated at the detection threshold (Figure S1 depicts an example of such imputation). To estimate the parameters of the truncated normal distribution from which the imputation would be made, the Method of Moments was used (Searle et al., 2009). In this case, the mean and sample variances of each (lognormal) distribution from an initial analysis were equated to their expectations from truncated normal distributions. With the parameters of the truncated distribution, we proceeded to impute the truncated data and analyze the resulting Gauss Markov Normal mixed linear model. The description of the model with fixed effects of time and treatment and random effect of animal follows. The results presented here are a mean of 10,000 such imputations. The main research questions regarding the vaccine treatments were organized in two orthogonal contrasts: Control vs. Vaccinated (S19 and RB51) and S19 vs. RB51. Those contrasts were also applied to possible interacting fixed effects to make the summary of the imputations directly interpretable.

R software (version 4.4.0) [24] was used for all statistical analyses and packages ‘truncnorm’ (Mersmann et al., 2023) and ‘lme4’ (Bates et al., 2024) were used for model building. Percentage of positive animals in serological tests (2-ME and STAT) were obtained according to each treatment and time post-vaccination.

3. Results

The final number of samples analyzed in each assessment is detailed in Figure 1, considering losses of animals along the study or punctual losses of samples at each post-vaccination time.

3.1. IFN-γ quantification

IFN- γ data had an excess of zeros (62.4%) and, in these cases, it was assumed that the cytokine accumulated in the culture supernatant did not reach the detection limit of the device in these cases, which was estimated as 13.75 pg/mL. All results of IFN-γ production according to age at vaccination and vaccine strain, in the different timeframes, are shown in Figure 2. Results for the average model are summarized in Tables 1 and 2. Regarding age, there were no differences in IFN-γ production according to age at vaccination, for both vaccines in any time post-vaccination ($p > 0.05$). Independently of age and time post-vaccination, contrast comparing vaccinated animals (treatment) and non-vaccinated animals showed very significative effect of treatment ($p < 0.01$), while there is no difference comparing the two different vaccines S19 and RB51 ($p > 0.05$).

3.2. Humoral immune response

Results of 2-ME, STAT, and FPA showed that all animals were seronegative at the beginning of the experiment and, at day 28, all animals vaccinated with S19 seroconverted, while those in control and RB51 groups remained negative in all evaluated timeframes.

For 2-ME, at day 28, all animals vaccinated with S19 were positive. At day 56, 50% (15/30) of the animals vaccinated with S19 were negative in the serological tests. In addition, it was observed that younger animals have become negative faster compared with older animals. Indeed, 77.78% (7/9) of negative results were observed at 56 days post-vaccination for animals vaccinated at 3-4 months of age, 50% (5/10) for 5-6 months and only 27.28% (3/11) for 7-8 months old animals.

For FPA, at day 56, 70.00% (21/30) of the animals vaccinated with S19 were positive and 23.33% (7/30) showed “suspect” results. Considering groups by age at vaccination, only 70% (7/10) of the animals vaccinated at 3-4 months of age were negative at day 56, compared to 22.22% (2/9) for the 5-6 months group and 36.36%, for animals vaccinated at 7-8 months old.

208 **Table 1.** Type III Analysis of Variance Table with Satterthwaite's method for the average model
209 for assessment of effect of age at vaccination with S19 and RB51 *Brucella abortus* strains on
210 immune response, through in vitro IFN-γ production.

	Sum Sq	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr (>F)**
Treatment	51.980	25.990	1.993	88.200	6.088	**0.0067**
Time	112.634	56.317	1.993	95.882	13.187	**0.0000**
Age	9.210	4.605	1.993	88.539	1.085	0.6842
Treatment:Time	39.837	9.959	3.987	94.876	2.334	0.1227
Treatment:Age	24.016	6.004	3.987	86.900	1.408	0.4766
Time:Age	23.054	5.764	3.987	95.272	1.345	0.5179
Treatment:Time:Age	24.461	3.058	7.973	94.124	0.715	1.3544

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213 **Table 2**. Final generalized linear mixed model for assessment of effect of age at vaccination
214 with S19 and RB51 *B abortus* strains on immune response, through in vitro IFN-γ production.

	Estimate	Std. Error	df	t value	Pr (>|t|)
(Intercept)	4.9896	0.2151	89.8228	23.1413	**0.0000**
Control vs. Vaccinated	1.2878	0.4248	92.4459	3.0216	**0.0033**
S19 vs. RB51	0.5383	0.3117	84.9905	1.7231	0.0885
Linear time	0.9075	0.3895	96.3722	2.3205	**0.0224**
Quadratic time	-1.6499	0.3504	95.3642	-4.6957	2.0000
Linear age	-0.5465	0.3861	93.4470	-1.4137	1.8392
Quadratic age	-0.1873	0.3586	85.5791	-0.5247	1.3989
Control vs. Vaccinated:Linear time	1.5207	0.7712	97.4610	1.9662	0.0521
S19 vs. RB51:Linear time	0.8437	0.5618	93.6283	1.4971	0.1377
Control vs Vaccinated:Quadratic time	0.0913	0.6915	96.3992	0.1336	0.8940
S19 vs. RB51:Quadratic time	0.7687	0.5082	93.1118	1.5088	0.1347
Control vs. Vaccinated:Linear age	1.5145	0.7824	96.6650	1.9311	0.0564
S19 vs. RB51:Linear age	0.0133	0.5313	85.0408	0.0246	0.9804
Control vs. Vaccinated:Quadratic age	-0.4135	0.6860	85.9909	-0.5980	1.4486
S19 vs. RB51:Quadratic age	0.2248	0.5484	84.9435	0.4087	0.6838
Linear time:Lineae age	-1.3804	0.6986	97.7263	-1.9686	1.9482
Quadratic time:Linear age	0.3596	0.6317	96.7059	0.5663	0.5725
Linear time:Quadratic age	-0.1797	0.6499	94.2455	-0.2755	1.2164
Quadratic time:Quadratic age	-0.5041	0.5809	93.4754	-0.8639	1.6101
Control vs. Vaccinated:Linear time:Linear age	1.3245	1.4246	98.5064	0.9269	0.3563
S19 vs. RB51:Linear time:Linear age	0.1276	0.9483	93.9772	0.1347	0.8931
Control vs. Vaccinated:Quadratic time:Linear age	-0.2776	1.2753	97.8955	-0.2166	1.1710
S19 vs. RB51:Quadratic time:Linear age	0.9511	0.8764	92.8809	1.0818	0.2821
Control vs. Vaccinated:Linear time:Quadratic age	0.6092	1.2407	94.8208	0.4911	0.6245
S19 vs. RB51:Linear time:Quadratic age	-1.0826	0.9973	93.3070	-1.0827	1.7183
Control vs. Vaccinated:Quadratic time:Quadratic age	1.0119	1.1149	93.5617	0.9018	0.3695
S19 vs. RB51:Quadratic time:Quadratic age	0.9734	0.8841	93.3364	1.0981	0.2750

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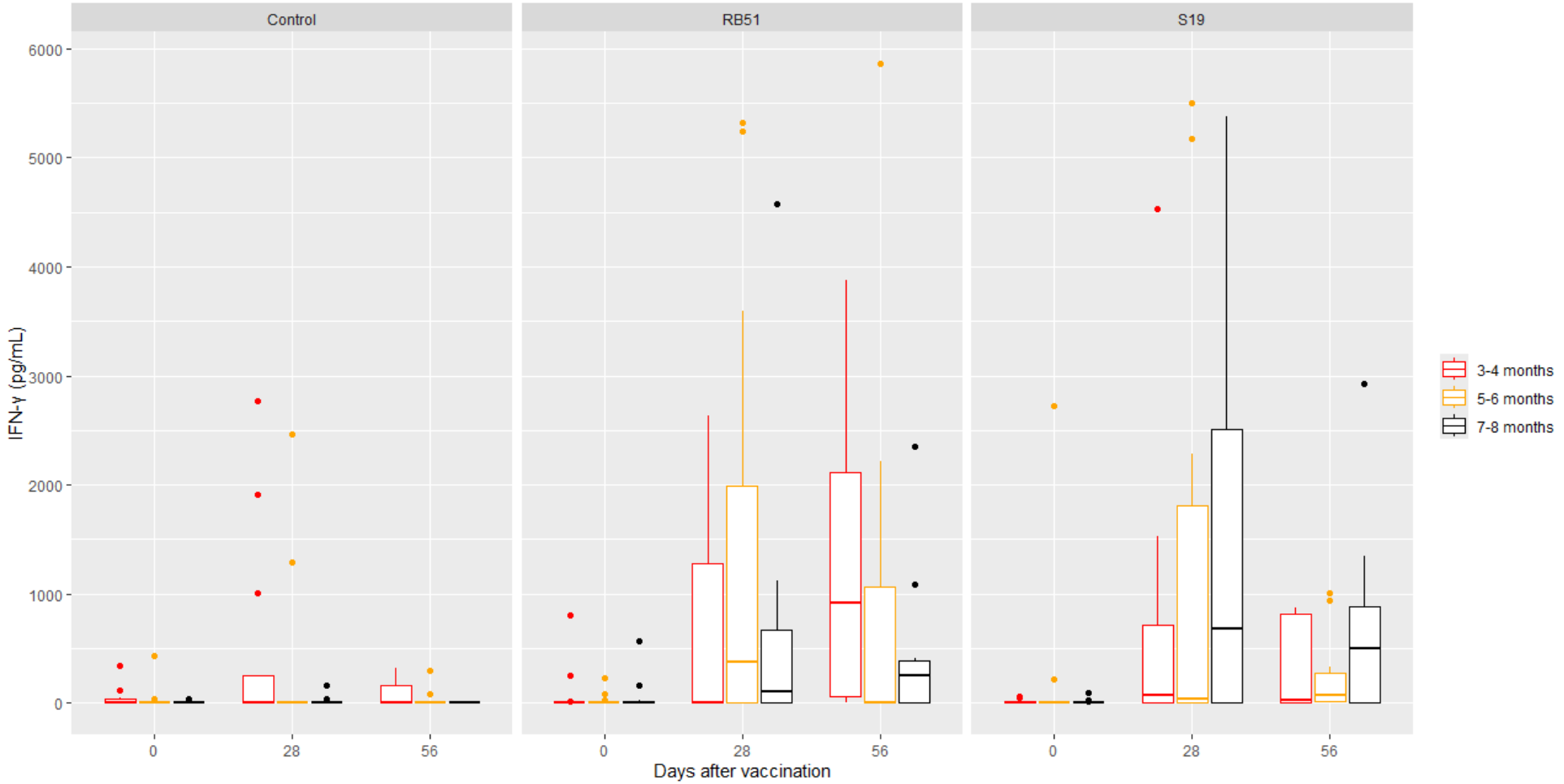

Figure 2. *In vitro* IFN-γ production of calves vaccinated at different ages following challenged with γ-irradiated *B. abortus* 2308 strain. One hundred and eight females calves aged between 3 to 8 months were divided in nine experimental groups: three S19 groups, vaccinated at day 0 of the experiment (3-4, 5-6 and 7-8 months old); three RB51 groups vaccinated at day 0 of the experiment (3-4, 5-6 and 7-8 months old) and three

220 control groups, inoculated with saline solution (0.85%) at day 0 of the experiment (3-4, 5-6 and 7-8 months old). Immune response of all animals
221 was assessed at day 0, 28 and 56 of the experiment.

4. Discussion

Vaccination of female calves between 3 and 8 months of age with the live attenuated *B. abortus* vaccines S19 or RB51 is a central and consolidated measure for controlling bovine brucellosis worldwide (Dorneles et al., 2017; Dorneles et al., 2015; Zhang et al., 2018). This approach, although it induces good and long-lasting immune response (McDiarmid, 1957; Olsen and Boggiatto, 2022), does not confer full protection against infection and abortion (Oliveira et al., 2022). Thus, the aim of this study was to suggest a more precise age at vaccination within this interval (3 to 8 months of age) in order to optimize individual immunity, and, consequently, vaccine efficacy and herd immunity. Nonetheless, our findings demonstrated that the age at vaccination does not affect the *in vitro* production of IFN-γ for either the S19 or RB51 strains, suggesting that vaccinating calves at any age between 3 and 8 months results in similar immunogenicity.

These results corroborate and complement the findings of Gilman and Wagner (Gilman and Wagner, 1959) and Cheville et al. (Cheville et al., 1996) that performed experimental challenges in pregnant cows vaccinated at different ages during calfhood. These authors did not find the effect of age in the protection against infection and abortion conferred by S19 and RB51 strains, when challenge was performed at the first pregnancy. Albeit the goal was to determine the best age for vaccination, the findings of the present study and previously cited ones are very useful from the herd management point of view. The evidence that animals can be vaccinated at any age between 3 and 8 months contributes for a better organization of the vaccinal schedule at farm level, aiming to avoid performing brucellosis vaccination at the same time or close to other vaccines. This is especially important when it comes to vaccines that induce Th2 response type (e.g. *Clostridium* spp. vaccines), since the immunity conferred by these vaccines can be negatively impacted by simultaneous administration of brucellosis vaccine (Diniz Neto et al., 2021). Furthermore, a wider interval allows vaccination of groups of calves with different ages, which is particularly important for herds in which vaccination management is performed only once or twice a year (e.g. extensive beef production systems) or small herds with only a few female calves to be vaccinated annually.

One of the main concerns about age at vaccination was that in younger animals (3 months-old) the not fully mature immune system and/or the presence of maternal antibodies could impair the establishment of the immune response induced by vaccination (Cunningham, 1977; Redman et al., 1967). Nonetheless, considering that 3 months-old animals responded similarly to brucellosis vaccination compared to older animals, farms that intent to optimize health management should vaccinate the animals as younger as possible. This is justified by the

period at risk - between the decrease of maternal antibodies and increase of self-immune response induced by vaccination - will be reduced, contributing for a better control of the disease in the herd. Also, vaccination of younger animals is especially important in dairy farms that use European breeds (*Bos taurus taurus*), which become reproductive mature earlier compared to Zebu breeds and the interval of time to carry out vaccination schedule against reproductive diseases is reduced. Additionally, even though it was not observed difference in the IFN-γ production (cellular immune response) among the assessed groups of age, it has been demonstrated that the younger the animal is vaccinated, the faster the titers of vaccinal antibodies (humoral response) induced by S19 strain decrease (King and Frank, 1961; McDiarmid, 1957). This effect was also observed in our study, with 70% of the animals vaccinated at 3-4 months of age negative in the 2-ME and FPA tests 56 days after vaccination, while the other groups showed lower percentages, especially in FPA. Therefore, together the similar immunogenicity among calves vaccinated between 3 and 8 months and the faster decrease of vaccinal antibodies, point to the possibility to reduce the current age for testing (usually after 24 months old) animals vaccinated at this age with S19, without risk of failures in the immune response or false positive results in the serological tests, as previously proposed by other authors (Gilman and Wagner, 1959; King and Frank, 1961). This is particularly important because S19 is a well-established vaccine for brucellosis control and is still widely used worldwide (Zhang et al., 2018). The broader use of S19 compared to RB51 in bovine brucellosis control programs is mainly due to its lower cost compared to RB51, as well as the lack of patent rights, which allows a greater number of companies to manufacture the vaccine.

As expected, it was observed an effect of time in the IFN-γ production, with both vaccinated groups being significantly different from the control groups in post-vaccination times assessed (28 and 56 days post-vaccination), which, once again, highlights IFN-γ as a key cytokine for immune response against brucellosis, as demonstrated elsewhere (Dorneles et al., 2015a; Lowry et al., 2011; Pasquali et al., 2001). It is also worth to mention that, at all evaluated times, no significative differences between S19 and RB51 groups regarding IFN- γ accumulated in the culture supernatant was observed, which suggests that both vaccines induce equivalent immune response and thereby protection against bovine brucellosis as previously reported (Dorneles et al., 2015a; Oliveira et al., 2022).

Finally, although it brings great contribution for the brucellosis vaccination knowledge, this study has some limitations, as the absence of assessment of the cellular subsets of the Th1 immune response by flow cytometry. This assessment could help to understand whether there are or not differences in the subpopulations involved in immune response after vaccination, as

the development of memory cells, according to different ages at vaccination. In addition, an assessment of the immune response after an experimental challenge would be ideal to the determine the correlates of protection triggered by vaccination (Dorneles et al., 2015b). However, this type of study is costly and requires more severe ethical issues regarding animal use, considering that animals must be slaughtered at the end of the study, in addition to the need of a biosafety level 3 facility for large animals (BSL3), making its execution even difficult. In this sense, the assessment of the immune response after vaccination, as performed in this present study, even though not being the optimal design, allows making inferences about the establishment of immune response and memory triggered by vaccination.

5. Conclusions

In conclusion, our findings indicated no significant difference in the *in vitro* production of IFN-γ after brucellosis vaccination regardless of the vaccine used (S19 or RB51) and across the studied age range (3-8 months). This suggests that the immune response triggered by both *B. abortus* S19 or RB51 strains is similar for animals vaccinated at any time between 3 and 8 months of age.

Acknowledgments

This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig) (APQ-02726-21 and RED-00132-22), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes). MSG thanks Fapemig for her scholarship, and EMSD is indebted to CNPq to her fellowship.

Conflict of interests

The authors declare no competing interests.

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Supplementary material:

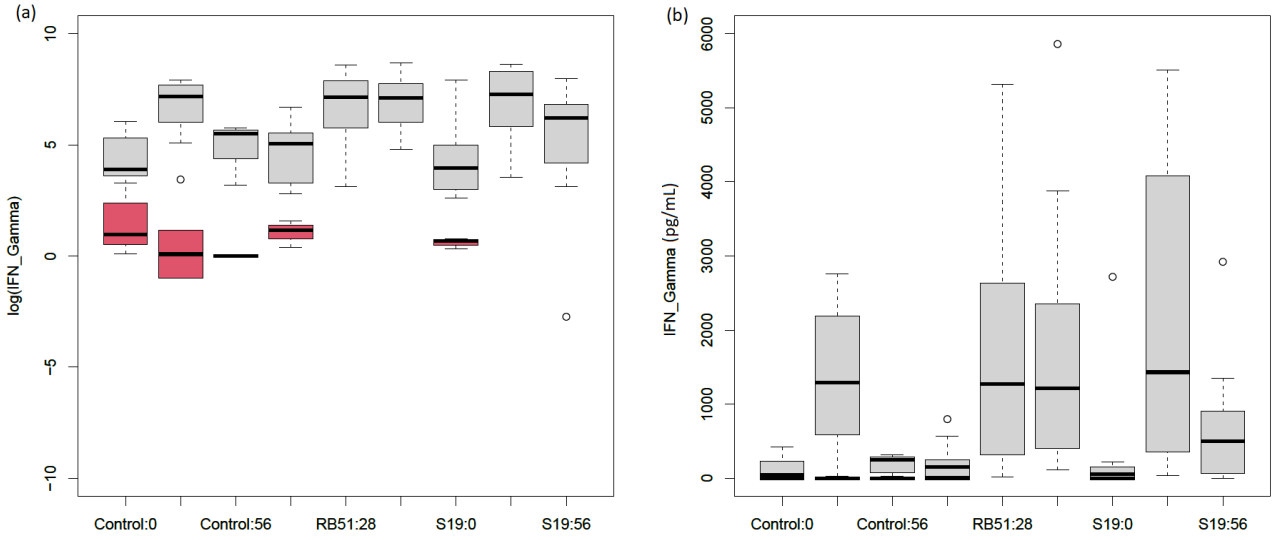

Figure S1. Example of data imputation using values of the lognormal distribution in log scale (a) and variable original scale (b), truncated at the detection threshold of the IFN-γ ELISA for evaluation of the impact of age on the immune response induced by *Brucella abortus* s19 or RB51 vaccination in calves.

FINAL CONSIDERATIONS

Vaccination is a crucial factor in controlling brucellosis in animals. Given that brucellosis is a complex disease affecting many hosts, including humans, numerous efforts have been made to improve vaccination efficacy, however, many aspects still need clarification. This manuscript sheds light on some aspects of this complex disease, contributing to the improvement of bovine brucellosis control in Brazil and worldwide. The principal findings include: (1) clarification of various aspects about dissociation of smooth Brucella strains and its use for bovine vaccination; (2) confirmation of the effectiveness of *B. abortus* S19 and RB51 vaccines in the field, particularly when combined with other control policies; and (3) observation of similar immunogenicity of the S19 and RB51 strains in calves vaccinated at different ages within the 3 to 8-month range.