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Tese

Vacina candidata contra leptospirose utilizando *Salmonella* Typhimurium como vetor

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“Passei a minha vida tentando corrigir erros que cometi na minha ânsia de acertar. Ao tentar corrigir um erro, eu cometia outro. Sou uma culpada inocente.”

Clarice Lispector

Resumo

CHAGAS, Domitila Brzoskowski. **Vacina candidata contra leptospirose utilizando *Salmonella* Typhimurium como vetor**. 2024. 106 f. Tese (Doutorado em Biotecnologia) - Programa de Pós-Graduação em Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas, 2024.

A leptospirose é uma doença zoonótica causada por bactérias do gênero *Leptospira*, que afeta animais domésticos e selvagens em escala global. Atualmente, não há uma vacina comercial universal disponível para prevenir a doença, o que impulsiona o desenvolvimento de vacinas recombinantes como uma alternativa promissora para combater a leptospirose de maneira mais eficaz. Neste contexto, o presente estudo teve como objetivo desenvolver e avaliar protótipos vacinais contra a leptospirose. Na primeira etapa, investigou-se a imunogenicidade e o potencial protetor de vacinas bacterinas de *Escherichia coli* expressando uma proteína quimérica composta pelos antígenos de *Leptospira,* LipL32, LemA e LigAni (rQ1). A eficácia dessas formulações foi testada em hamsters, divididos em grupos experimentais que receberam diferentes formulações: bacterina recombinante (com e sem adjuvante de hidróxido de alumínio, $Al(OH)_3$), vacinas de subunidade e controles negativos. A vacinação foi administrada por via intramuscular nos dias 0 e 14. Os resultados obtidos por ELISA indireto demonstraram que o grupo que recebeu a vacina de subunidade apresentou níveis significativamente elevados de imunoglobulinas ($P < 0,05$), diferenciando-se de todos os outros grupos. O grupo da vacina de subunidade apresentou 50% de sobrevivência, enquanto os grupos vacinados com bacterinas recombinantes mostraram apenas 0-10% de proteção. Nenhum grupo vacinal foi capaz de conferir imunidade protetora significativa contra o desafio letal com a cepa de *L. interrogans*. Apesar da formulação de subunidade ter demonstrado respostas imunológicas moderadas, sua eficácia ainda é limitada, ressaltando a necessidade de otimizações adicionais para o desenvolvimento de uma vacina eficaz contra a leptospirose. Na segunda etapa do estudo, foi utilizado o antígeno quimérico de *Leptospira* (rQ1), clonado no vetor pTECH2, para expressão do antígeno em *Salmonella* Typhimurium LVR01. Hamsters foram vacinados por via oral (OR) e intramuscular (IM) com 2×10^7 unidades formadoras de colônia (UFC) de *S.* Typhimurium LVR01 carregando pTECH2/rQ1, pTECH2 sozinho ou PBS como controle. As vacinações foram administradas em duas doses, com um intervalo de 14 dias. Após a vacinação, amostras de soro foram coletadas e os anticorpos IgG e sorotipos contra o rQ1 foram medidos usando ELISA indireto. Os resultados mostraram que, no dia 28, anticorpos IgG foram detectados apenas nos hamsters vacinados via IM com a vacina recombinante atenuada de *Salmonella.* Após a segunda vacinação, os níveis de anticorpos nos hamsters vacinados via IM com pTECH2/rQ1 foram significativamente maiores ($P < 0,0001$) quando comparados aos grupos controle (PBS e pTECH2). Além disso, os níveis de IgG2/3 contra o antígeno rQ1 no grupo vacinado intramuscularmente foram também significativamente elevados ($P < 0,0001$) em comparação aos controles. Em contraste, os níveis de anticorpos no grupo vacinado via OR com pTECH2/rQ1 apresentaram diferença estatisticamente significativa ($P < 0,05$) em comparação aos controles. Concluindo, a vacina atenuada baseada em *S.*

Typhimurium não demonstrou eficácia protetora contra desafio homólogo nos hamsters vacinados com pTECH2/rQ1. Isso sugere que, embora promissora, a plataforma baseada em *Salmonella* ainda precisa de mais estudos para aprimorar a resposta imune e conferir proteção efetiva.

Palavras-chave: Leptospirose; vacinas recombinantes; antígeno quimérico; animais.

Abstract

CHAGAS, Domitila Brzoskowski. **Candidate vaccine against leptospirosis using *Salmonella* Typhimurium as a vector.** 2024. 106 f. Thesis (PhD degree in Biotechnology) - Postgraduate Program in Biotechnology, Center for Technological Development, Federal University of Pelotas, Pelotas, 2024.

Leptospirosis is a zoonotic disease caused by bacteria of the genus *Leptospira*, which affects domestic and wild animals on a global scale. Currently, there is no universal commercial vaccine available to prevent the disease, driving the development of recombinant vaccines as a promising alternative to combat leptospirosis more effectively. In this context, the present study aimed to develop and evaluate vaccine prototypes against leptospirosis. In the first phase, the immunogenicity and protective potential of bacterin vaccines derived from *Escherichia coli* expressing a chimeric protein composed of *Leptospira* antigens, LipL32, LemA, and LigAni (rQ1), were investigated. The efficacy of these formulations was tested in hamsters, divided into experimental groups that received different formulations: recombinant bacterin (with and without aluminum hydroxide adjuvant, Al $(OH)_3$), subunit vaccines, and negative controls. The vaccinations were administered intramuscularly on days 0 and 14. The results obtained via indirect ELISA demonstrated that the group receiving the subunit vaccine showed significantly elevated levels of immunoglobulins ($P < 0.05$), distinguishing it from all other groups. The subunit vaccine group showed a 50% survival rate, whereas groups vaccinated with recombinant bacterins exhibited only 0–10% protection. No vaccine group provided significant protective immunity against the lethal challenge with the *L. interrogans* strain. Although the subunit formulation elicited moderate immune responses, its efficacy remains limited, highlighting the need for further optimizations to develop an effective leptospirosis vaccine. In the second phase of the study, the *Leptospira* chimeric antigen (rQ1), cloned into the pTECH2 vector, was used to express the antigen in *Salmonella Typhimurium* LVR01. Hamsters were vaccinated orally (OR) and intramuscularly (IM) with 2×10^7 colony-forming units (CFU) of *S. Typhimurium* LVR01 carrying pTECH2/rQ1, pTECH2 alone, or PBS as a control. The vaccinations were administered in two doses, 14 days apart. Following vaccination, serum samples were collected, and IgG antibodies and serotypes against rQ1 were measured using indirect ELISA. The results showed that on day 28, IgG antibodies were detected only in hamsters vaccinated via IM with the attenuated recombinant *Salmonella* vaccine. After the second vaccination, antibody levels in hamsters vaccinated IM with pTECH2/rQ1 were significantly higher ($P < 0.0001$) compared to the control groups (PBS and pTECH2). Furthermore, IgG2/3 levels against the rQ1 antigen in the intramuscularly vaccinated group were also significantly elevated ($P < 0.0001$) compared to the controls. In contrast, antibody levels in the group vaccinated orally with pTECH2/rQ1 showed a statistically significant difference ($P < 0.05$) compared to the controls. In conclusion, the attenuated vaccine based on *S. Typhimurium* did not demonstrate protective efficacy against homologous challenge in hamsters vaccinated with pTECH2/rQ1. These findings suggest that, although promising, the *Salmonella*-based platform requires further studies to enhance immune response and provide effective protection.

Keywords: Leptospirosis; recombinant vaccines; chimeric antigen; animals.

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Lista de Abreviaturas e Siglas

DO	Densidade óptica (OD - *Optical density*)
DMSO	Dimetil sulfóxido
ED50	Dose letal à 50% dos animais
EMJH	Meio Ellinghausen-McCullough-Johnson-Harris
IgG	Imunoglobulina G (*Immunoglobulin G*)
LB	Meio Luria-Bertani
LPS	Lipopolissacarídeo
OPD	Dihidrocloreto de o-phenilenediamina
PBS	Tampão Salina de Fosfato (*Phosphate Buffer Saline*)
PCR	Reação em Cadeia da Polimerase (*Polymerase chain reaction*)
PMSF	Fluoreto de fenilmetanosulfonil (*Phenylmethylsulfonyl fluoride*)
RASV	Vacina recombinante de *Salmonella* atenuada *(Recombinant attenuated Salmonella vaccine)*
RPM	Rotações por minuto
SDS-PAGE	Eletroforese em gel de acrilaminda – dodecil sulfato de sódio
TetC	Fragmento C da toxina do tétano (N*on-toxic fragment C of tetanus toxin*)
UFC	Unidades formadoras de colônias (CFU - *Colony Forming Units*)
VIG	Via intragástrica (*Intragastric*)
VIM	Via intramuscular (*Intramuscularly*)
VIN	Via intranasal
VO	Via oral (*Orally*)
VIP	Via intraperitoneal
VR	Vacinologia reversa (RV - *reverse vaccinology*)
VSC	Via subcutânea (*Subcutaneous*)

Lista de Símbolos

<	Menor
>	Maior
®	Marca registrada
+	Mais
±	Mais ou menos
=	Igual
°C	Grau Celsius
µL	Microlitro
µM	Micrometro
%	Porcentagem
h	Hora
× *g*	Força centrífuga (*times gravity*)

Sumário

1 Introdução

A leptospirose é uma doença sistêmica que afeta humanos e animais domésticos, com notável impacto social e econômico, cujo agente etiológico são bactérias do gênero *Leptospira* (Browne *et al.*, 2022). A doença permanece endêmica na maioria das regiões tropicais e subtropicais do mundo, incluindo América Latina, Oriente Médio, África e Ásia (Srivastava, 2006). Atualmente, mais de 260 sorovares infectantes de *Leptospira* são descritos e agrupados em 25 sorogrupos (Adler; de la Peña Moctezuma, 2010). Essa diversidade de sorovares infecciosos representa um obstáculo para o controle e prevenção de doenças.

A maioria dos sorovares circulantes residem em roedores e outros reservatórios de animais domésticos, desempenhando um papel crítico na interface de transmissão de humano-animal-ambiente (Bashiru; Bahaman, 2018; Costa *et al*., 2015). A leptospirose possui uma ampla gama de padrões epidemiológicos, relacionados às atividades ocupacionais em sistemas agropecuários de subsistência, ou fatores de risco habitacionais ou climáticos, como alagamentos ou exposições recorrentes de saneamento precário especialmente em países em desenvolvimento com clima tropical, como o Brasil (Goarant, 2016). E em países de clima temperado, a contaminação zoonótica ocorre através de atividades recreativas, como esportes aquáticos (Andre-Fontaine; Aviat; Thorin, 2015).

A infecção por cepas patogênicas de *Leptospira* geralmente ocorre por meio do contato direto com a urina de um animal infectado ou indiretamente por meio de água contaminada (Adler; de la Peña Moctezuma, 2010; Davignon *et al.*, 2023). Uma vez que as leptospiras são excretadas pela urina de um mamífero reservatório, elas podem sobreviver por vários meses no solo e na água na ausência de hospedeiro animal. (Yanagihara *et al.*, 2022). A duração da sobrevivência da *Leptospira* no habitat natural é afetada por muitos fatores, incluindo fatores abióticos e bióticos (Thibeaux *et al.*, 2017).

Após a infecção no hospedeiro, as leptospiras patogênicas vivem nos túbulos renais proximais dos rins dos portadores, embora outros tecidos e órgãos também possam servir como fonte de infecção (Adler; de la Peña Moctezuma, 2010). Por conseguinte, os sinais clínicos são bastante variáveis, as manifestações clínicas que variam de infecções subclínicas a síndromes fatais (Picardeau, 2013). A maioria dos sintomas da leptospirose são inespecíficos e podem ser compartilhados com outras doenças infecciosas tropicais, representando um desafio ao diagnóstico diferencial

clínico, em humanos (Riediger *et al*., 2017). Os sintomas da leptospirose em animais, como em caninos, podem levar a múltiplas manifestações clínicas, variando de doença febril leve a doença multissistêmica com risco de vida, frequentemente caracterizada por insuficiência renal e hepática com distúrbios de coagulação e hemorragia pulmonar (Esteves *et al.*, 2022).

Em suínos, bovinos e ovinos infectados por *Leptospira* afetam principalmente o sistema reprodutivo, incluindo aborto espontâneo, mortalidade neonatal, parto prematuro e natimortos (Dib *et al*., 2014; Lilenbaum; Martins, 2014; Lucheis; Ferreira Jr., 2011). Além disso, são frequentemente relatados aumento no intervalo entre partos, redução no tamanho da ninhada, infertilidade temporária e esterilidade permanente (Ramos *et al*., 2006). A infecção por *Leptospira* é responsável por graves perdas econômicas, devido à ocorrência de abortos, natimortos, infertilidade, mastite, redução da produção de leite e morte de animais (Martins *et al*., 2012). Essas perdas incluem também custos indiretos, como gastos com tratamento, vacinas e assistência veterinária (Dib et al., 2014).

Diante disso, medidas preventivas, como a vacinação, são consideradas essenciais para o controle da doença (Lilenbaum; Martins, 2014; López-Robles *et al.*, 2021). As vacinas disponíveis para uso veterinário são baseadas em bacterinas, que são suspensões de células bacterianas polivalentes inteiras, inativadas, produzidas com os sorovares mais comuns encontrados em uma região ou país (Daroz *et al.*, 2021; Dib et al., 2014). No entanto, essas vacinas apresentam limitações justamente por terem a proteção restrita à sorovares incluídos em sua formulação e àqueles antigenicamente relacionados (Adler; de la Peña Moctozuma, 2010).

Visto que, a imunidade induzida pela vacinação é geralmente, mas não exclusivamente, mediada por anticorpos provocados por uma resposta independente de T contra moléculas de superfície de lipopolissacarídeos (LPS) da *Leptospira*, e os anticorpos induzidos pela vacina são considerados específicos do sorogrupo (Esteves *et al*., 2022). Promovendo uma proteção parcial devido à ausência de imunidade cruzada contra sorovares não incluídos na vacina e à possibilidade de circulação de outros sorovares potencialmente endêmicos na região (Fávero *et al*., 2018). Além da falta de memória imunológica e problemas de reatogenicidade (Barazzone *et al*., 2021).

Com isso, diversos fatores de virulência envolvidos na patogênese da *Leptospira* estão sendo investigados como alvos terapêuticos e potenciais candidatos

vacinais, com o objetivo de ativar mecanismos que sensibilizem o sistema imunológico (Kumar *et al*., 2021). Como as proteínas de membrana ou superfície externa (OMPs) da *Leptospira* patogênica têm se destacado, pois desempenham um papel crucial no estabelecimento da infecção (Raja; Natarajaseenivasan, 2014; Kumar *et al*., 2021). Essas proteínas são consideradas alvos promissores para vacinas, uma vez que a maioria delas é conservada entre diferentes sorovares e pode ser reconhecida pela resposta imunológica do hospedeiro nos estágios iniciais da infecção (de Oliveira *et al*., 2023; Kumar *et al*., 2021; Matsunaga *et al*., 2003).

Entre eles, LipL32 é o componente da membrana externa mais abundante encontrado na *Leptospira* patogênica, e tem sido extensivamente estudado (Riediger *et al.*, 2017). Além disso, LigA e LigB consistem em um peptídeo sinal de lipoproteína, com domínios semelhantes à imunoglobulina de leptospira, que auxiliam na evasão imunológica (Evangelista *et al.*, 2017). Assim como, a hipotética lipoproteína hipotética LemA, antígeno promissor identificado por meio de vacinologia reversa (RV), cuja modificação subsequente libera um epítopo que é apresentado na superfície dos fagócitos (Hartwing *et al.*, 2013).

A eficácia parcial de proteínas recombinantes na proteção contra a leptospirose já foi demonstrada em vacinas de subunidade, embora essas formulações ainda não tenham conseguido prevenir completamente a colonização renal em animais infectados (Hartwig *et al.*, 2013; Oliveira *et al.*, 2018; da Cunha *et al.*, 2019; Gomes *et al.*, 2020). Entretanto, um estudo realizado por Oliveira *et al.* (2019) utilizou o rBCG combinado com uma proteína multiepítopo baseada nos antígenos de *Leptospira* LipL32, LemA e LigA 11-13 (quimera 1), que proporcionou uma proteção de 80% a 100% em hamsters, sem a detecção de bactérias no tecido renal, demonstrando o potencial esterilizante dessa formulação. Ademais, Bunde et al. (2023) mostraram que rBCG-Q1 induziu uma resposta do tipo Th1 com altos níveis de IFN-γ, ligados à sua eficácia protetora e imunidade esterilizante, enquanto nenhum nível significativo de anticorpos IgG específicos para rQ1 foi detectado.

Embora alguns estudos tenham mostrado proteção promissora usando rLigBrep ou construções quiméricas, um aspecto crítico do desenvolvimento de uma vacina recombinante é garantir uma produção econômica (Conrad *et al.*, 2017; Dorneles *et al.*, 2020; Oliveira *et al.*, 2018). Assim, selecionar as plataformas de expressão e entrega mais econômicas continua sendo um desafio significativo. Um

meio eficaz é a utilização de *Salmonella* viva atenuada com vetor (Clark-Curtiss; Curtiss, 2018; Galen; Curtiss, 2014).

A inativação de genes metabólicos em vacinas vivas permite a expressão de antígenos essenciais e fatores de virulência, ao mesmo tempo em que restringe sua capacidade de proliferação (Curtiss, 2023). Essas características permitem a construção racional de vacinas recombinantes de *Salmonella* atenuadas (RASVs) com contenção biológica completa, e capazes de promove efeitos imunomoduladores (Wang; Kong; Curtiss, 2013). E ao invadir os tecidos linfoides do hospedeiro, a *Salmonella* induz continuamente uma forte resposta imune celular e de mucosa no hospedeiro (Lou *et al.*, 2019; Zhou *et al.*, 2023). Além disso, vários estudos exploraram construções de vacinas baseadas em *Salmonella* viva atenuada, carregando principalmente antígenos contra uma variedade de outros organismos, incluindo bactérias, vírus e parasitas (Ding *et al.*, 2018; Kim *et al.*, 2019; Li *et al.*, 2018; Wilde *et al.*, 2019).

Portanto, o desenvolvimento de uma vacina eficaz contra a leptospirose requer a capacidade de induzir uma resposta imunológica robusta e duradoura nos animais, aliando segurança, eficácia e viabilidade econômica. Dessa forma, no primeiro capítulo foi apresentado, o protótipo vacinal, conduzido em duas etapas, ao qual, foram testadas vacinas bacterinas baseadas em *Escherichia coli* expressando uma proteína quimérica composta pelos antígenos LipL32, LemA e LigA-Ni (rQ1). A eficácia dessas formulações foi avaliada em hamsters, mas os resultados obtidos ficaram aquém do esperado, o que motivou a busca por abordagens alternativas para explorar o potencial da quimera rQ1. O segundo capítulo consistiu em uma revisão das pesquisas que exploram o progresso das vacinas recombinantes de *Salmonella* viva atenuada (RASVs) como vetores versáteis para a prevenção de diversas doenças infecciosas. Por fim, no terceiro estudo, foi apresentado o desenvolvimento de protótipo vacinal utilizando antígeno quimérico de *Leptospira* (rQ1), clonado no vetor pTECH2 para expressão de antígeno em *Salmonella* Typhimurium LVR01.

2 Capítulos

2.1 Capítulo 1

Evaluation of the immunoprotective potential of rQ1 in a recombinant *E. coli* bacterin against leptospirosis

- Projeto experimental -

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Evaluation of the immunoprotective potential of rQ1 in a recombinant *E. coli* bacterin against leptospirosis

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Abstract: Leptospirosis is a significant zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. And the development of recombinant vaccines is a promising alternative to combat leptospirosis more effectively. **Background/Objectives:** A chimeric *Leptospira* antigen (rQ1), previously described and based on the gene sequences of *lipL32*, *lemA*, and *ligAni*, was cloned into the pAE vector for antigen expression in *Escherichia coli*. **Methods:** Hamsters were vaccinated intramuscularly (IM) with different formulations: recombinant bacterin (with and without the aluminum hydroxide adjuvant, $Al(OH)_3$), subunit vaccines, and negative controls. pAE alone or PBS as a control. Vaccinations were administered twice, with a 14-day interval. Following IM administration, serum samples were collected, and IgG antibodies against rQ1 were measured using indirect ELISA. **Results:** The results obtained by indirect ELISA demonstrated that the group with the purified Q1 vaccine + $Al(OH)_3$, exhibited significantly elevated immunoglobulin levels ($P < 0.05$) differentiating it from all other groups. Group vaccinated with the recombinant Q1 bacterin demonstrated statistically significant antibody levels compared to the control groups, despite the absence of surviving animals in this group. No vaccine group conferred significant protective immunity against the lethal challenge with the *L. interrogans* strain. **Conclusions:** Although the subunit formulation elicited immune

responses, its efficacy remains limited, underscoring the need for further optimization to develop an effective vaccine against leptospirosis.

Keywords: Leptospirosis; bacterin; recombinant vaccine; hamster.

1. Introduction

Leptospirosis is a significant zoonotic disease caused by pathogenic spirochetes of the *Leptospira* genus. These bacteria multiply in the renal tubules of chronically infected mammals and are excreted into the environment through urine(Abe et al., 2020; Zarantonelli et al., 2018). Transmission occurs either directly or indirectly, typically through percutaneous contact with contaminated soil or water, with rodents being the primary hosts and reservoirs for *Leptospira* (Adler and de la Peña Moctezuma, 2010).

The disease is associated with occupational exposure in subsistence agricultural systems and with environmental and climatic risk factors, such as flooding or inadequate sanitation, particularly in tropical developing countries like Brazil (Goarant, 2016). In temperate regions, zoonotic contamination is more commonly linked to recreational activities, such as water sports (Andre-Fontaine et al., 2015).

In humans, leptospirosis ranges from an asymptomatic febrile infection in its initial stages to severe forms that are often mistaken for other diseases, such as malaria and dengue. This misdiagnosis complicates clinical identification and frequently results in neglect (Chacko et al., 2021). Traditional immunization strategies rely on inactivated *Leptospira* vaccines, which provide serovar-specific protection (Guglielmini et al., 2019).

Recent research, however, has focused on overcoming these limitations by developing recombinant vaccines based on conserved and immunogenic antigens capable of inducing effective cross-protection (Grassmann et al., 2017; Jorge and Dellagostin, 2017). Among these antigens, the lipoprotein LipL32, the most abundant and immunogenic in pathogenic *Leptospira*

species, has been extensively studied (Chirathaworn et al., 2016). Additionally, the Leptospiral Immunoglobulin-like proteins A and B (LigA and LigB) interact with various host proteins, including those involved in evading innate immune mechanisms, making them promising targets for vaccine development (Grassmann et al., 2017; Haake and Matsunaga, 2021).

Another lipoprotein, LemA, identified through reverse vaccinology as a potential vaccine antigen, has an unknown biological function but holds promise in leptospirosis control (Hartwig et al., 2013; Oliveira et al., 2018). LigA, LemA, and LipL32 proteins are relatively conserved among pathogenic *Leptospira* species, are surface-exposed, and are expressed during infection (Haake, 2000; Riediger et al., 2017).

The partial efficacy of these proteins in providing protection against leptospirosis has been demonstrated in recombinant subunit vaccines, although they have not completely prevented renal colonization in infected animals (da Cunha et al., 2019; Hartwig et al., 2013; Oliveira et al., 2018). However, a study by Oliveira et al. (2019) utilizing rBCG combined with a multiepitope protein based on the *Leptospira* antigens LipL32, LemA, and LigA 11-13 (chimera 1) provided 80% to 100% protection in hamsters, with no bacteria detected in renal tissues, demonstrating the sterilizing potential of this formulation.

Thus, developing vaccines based on recombinant chimeric antigens appears promising. Inactivated vaccines vectored by *Escherichia coli* expressing recombinant antigens offer advantages such as shorter production times compared to rBCG and the elimination of purification steps, reducing costs compared to recombinant subunit vaccines (Walker and Bacon, 2023). Recombinant *E. coli* bacterins expressing antigens have shown promising results in developing effective and safe vaccines against clostridial disease in sheep (Ferreira, 2019), botulism in cattle (Moreira, 2018), and lymphadenitis in goats (Pinho, 2021).

In this context, this study aims to develop and evaluate vaccine prototypes using recombinant *E. coli* bacterins expressing the chimeric antigen rQ1, with the objective of

exploring its potential to stimulate the immune system and provide protection against *Leptospira* in homologous challenge models.

2. Materials and Methods

2.1. Production and characterization of E. coli-vectored recombinant bacterin

The coding sequence of the recombinant chimera (rQ1), comprising a fusion of the antigens lipL32, lemA, and ligA 11-13, cloned into the pAE vector and previously constructed by our research group, as well as the pAE plasmid without insert, were used to transform *E. coli* BL21 Star via heat shock with 100 µL of calcium chloride ($CaCl_2$). Transformed clones were cultured in a 10 mL overnight pre-inoculum and subsequently transferred to a 100 mL inoculum in LB broth supplemented with ampicillin (50 µg/mL) and 1 M glucose. The cultures were incubated at 37 °C with agitation at 180 rpm until the logarithmic growth phase.

Protein expression was induced with 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) under the same cultivation conditions. The culture was induced for 3 hours and centrifuged at 7,000 × g for 15 minutes at 4 °C. Aliquots were collected before and after induction for protein expression analysis using 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Each pellet was resuspended in 10 mL of phosphate-buffered saline (PBS), adjusted to a concentration of 10^7 CFU/mL, and aliquoted into microtubes containing 1 mL each.

The aliquots were subjected to thermal inactivation at 80 °C for 30 minutes using a heat block. The inactivated cultures were analyzed by 12% SDS-PAGE and Western blot using an anti-histidine antibody. Additionally, plasmid extraction was performed post-inactivation to verify its integrity via 0.8% agarose gel electrophoresis. Post-inactivation aliquots were inoculated into LB broth and incubated for 18 hours at 37 °C to confirm inactivation. The samples were stored at 4 °C until vaccination.

2.2. Recombinant subunit vaccines

The rQ1 chimera was previously expressed and purified by our research group for the production of the recombinant subunit vaccine to be used as an experimental group in animal trials. For this purpose, the *Escherichia coli* BL21 Star strain was transformed, incubated, induced, and centrifuged as described in the previous subsection. The cells were resuspended in lysis buffer (20 mM NaH_2PO_4, 0.5 M NaCl, and 20 mM imidazole, pH 8) and sonicated.

After centrifugation for 1 hour at 7,000 × g, the pellet was resuspended in solubilization buffer (8 M urea, 0.1 M NaH_2PO_4, 0.5 M NaCl, and 20 mM imidazole, pH 8) and agitated overnight at 4 °C. Following solubilization of the sonicated extract and centrifugation to separate the lysed cells from the protein content, the supernatant was filtered, and the protein was purified using affinity chromatography on a sepharose column. This was followed by dialysis in 1X PBS + 0.05% Triton X-100 to reduce the urea concentration.

Each step was verified using 12% SDS-PAGE. Recombinant proteins used in immunoenzymatic assays were obtained under the same conditions.

2.3. Adsorption test with aluminum hydroxide adjuvant

Aluminum hydroxide ($Al(OH)_3$) was used as an adjuvant in the subunit vaccines for both experiments and, in Experiment 2, also in the recombinant bacterin vaccine. For protein adsorption onto aluminum, 100 µg of recombinant protein or bacterin containing recombinant protein were incubated overnight at 4 °C under agitation with 15% v/v $Al(OH)_3$. The adsorption process was verified via electrophoretic analysis using 12% SDS-PAGE.

2.4. Leptospira Cultivation for Challenge

The challenge was carried out using *Leptospira interrogans* serovar Copenhageni strain L1-130. After thawing, the bacteria were cultured at 30 °C in Ellinghausen McCullough Johnson Harris (EMJH) liquid medium enriched with EMJH supplement. The cultures were

maintained until the day of each challenge, where bacterial counts were performed in a Petroff-Hausser chamber to adjust the challenge dose.

2.5. Vaccination and challenge of hamsters

All animal procedures were conducted in an animal facility under conditions defined according to the guidelines approved by the Ethics Committee on Animal Experimentation (CEEA) of UFPel, under protocol number 036707/2021-45. Male and female Syrian hamsters, aged 4 to 6 weeks, were divided into groups of 10 animals. In Experiment 1, the animals were assigned to 4 experimental groups: subunit vaccine with rQ1 adsorbed onto the Al(OH)3 adjuvant (Group A), heat-inactivated recombinant bacterin (rQ1) (Group B), and two negative controls, PBS (Group C) and a bacterin from *E. coli* transformed with the pAE vector alone, without insert (Group D), as described in Table 1. In Experiment 2, the animals were divided into six experimental groups, four of which were identical to the groups described above. The remaining two groups received either the recombinant bacterin with Al(OH)3 adjuvant (Group E) or a heterologous prime-boost regimen, with the first dose of recombinant bacterin followed by a booster with the recombinant protein (Group F) (Table 2). In both experiments, all vaccine groups were subjected to a homologous challenge with *L. interrogans* serovar Copenhageni.

Table 1. Distribution of experimental groups for evaluation of vaccines in experiment 1.

Group	First dose	Second dose
A (n=10)	Purified Q1 + Alum	Purified Q1 + Alum
B (n=10)	Recombinant bacterin Q1	Recombinant bacterin Q1
C (n=10)	PBS + Alum	PBS + Alum
D (n=10)	Bacterin pAE	Bacterin pAE

Table 2. Distribution of experimental groups for evaluation of vaccines in experiment 2.

Group	First dose	Second dose
A (n=10)	Purified Q1 + Alum	Purified Q1 + Alum
B (n=10)	Recombinant bacterin Q1	Recombinant bacterin Q1

C (n=10)	PBS + Alum	PBS + Alum
D (n=10)	Bacterin pAE	Bacterin pAE
E (n=10)	Recombinant bacterin Q1 + Alum	Recombinant bacterin Q1 + Alum
F (n=10)	Recombinant bacterin Q1	Purified Q1 + Alum

Alum= Aluminum hydroxide ($Al(OH)_3$)

In both experiments, two doses of the vaccine formulations were administered on days 0 and 14, each containing 10^7 colony-forming units (CFU)/ml for the bacterins and 100 µg of recombinant chimera for the subunit vaccine, delivered intramuscularly. Serum samples were collected on days 0, 14, and 28 via gingival vein puncture under isoflurane anesthesia. The sera were obtained by centrifugation and stored at -20 °C until processing.

The challenge was performed on day 28 using ten times the 50% lethal dose (LD50) of *L. interrogans* serovar Copenhageni strain L1-130, administered intraperitoneally. All animals were monitored and weighed daily after the challenge for 28 days to observe and record potential clinical signs of leptospirosis. According to the guidelines and regulations of the Ethics Committee on Animal Experimentation (CEEA), moribund animals were euthanized via CO_2 inhalation. The criteria for euthanasia included a 10% weight loss from maximum weight, hematuria, nasal bleeding, prostration, ruffled fur, lethargy, and lack of appetite. Surviving animals at 28 days post-challenge were also euthanized.

2.6. Sterilizing immunity assessment

To evaluate the presence of *Leptospira* in tissues, samples of renal, pulmonary, and hepatic tissues were collected and stored in 10% formalin. These samples will be subsequently sent for histopathological analysis and stained with hematoxylin-eosin to assess tissue damage.

The presence of *Leptospira* in the kidneys will also be investigated to assess protection against renal colonization through reisolation in EMJH medium. One kidney from each animal was aseptically collected, homogenized in unsupplemented EMJH medium, and incubated for

1 hour at 28 °C. Subsequently, 500 µL of each culture was transferred to a new tube containing EMJH medium supplemented with 10% enrichment (Difco, BD, Brazil) and 60 µL of 5-fluorouracil. The cultures are being maintained at 28 °C for 8–12 weeks and monitored weekly by dark-field microscopy.

Genomic DNA from renal tissue will be extracted using a DNA purification kit, and the presence of *Leptospira* load will be determined by quantitative real-time PCR (qPCR). The bacterial count will be estimated based on the number of gene copies amplified by qPCR, using a standard curve and targeting the *lipL32* gene.

2.7. Evaluation of humoral response in hamsters

Antibody induction was evaluated using indirect ELISA with the recombinant chimera as the antigen. Plates were coated with purified chimera diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated for 16–18 hours at 4 °C. The plates were blocked with 5% skim milk diluted in PBS-T for 1 hour at 37 °C. Sera obtained from centrifuged animal blood samples were diluted in PBS-T with 2.5% skim milk and incubated for 1 hour at 37 °C. Following this incubation, hamster anti-IgG secondary antibody conjugated to peroxidase (1:5000) was added and incubated for 1 hour at 37 °C. After each step, the plates were washed three times with PBS-T. The reaction was developed using o-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide, and stopped by adding 25 µL of H2SO4. Absorbance was measured using a plate reader at a wavelength of 492 nm.

2.8. Statistical analysis

Survival and mortality analyses were conducted using the Log-Rank test and Fisher's exact test, respectively. One-way analysis of variance (ANOVA) was used to assess differences in the serological results for humoral immune response analyses. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using the GraphPad Prism 7 and Statistix 8 software packages.

3. Results

3.1. Caracterization of rQ1 integrity before and after cell inactivation

The rQ1 protein was previously expressed and purified by our group. Protein expression was confirmed through SDS-PAGE and Western blot using a monoclonal anti-histidine antibody conjugated with peroxidase (Figures 1A and B). The analysis confirmed the integrity of the chimeric rQ1 protein even after the cellular inactivation process, as it retained the expected molecular weight, consistent with the purified rQ1 used as a positive control (Figure 1). To quantify the recombinant protein present in the inactivated bacterins, a comparative method was employed using the bands observed in the Western blot (Figure 1B), where the protein concentration was inferred by visual comparison with a previously calculated concentration curve of a known protein (LigBrep). For the preparation of bacterin vaccines, a concentration of 30 µg/mL was used.

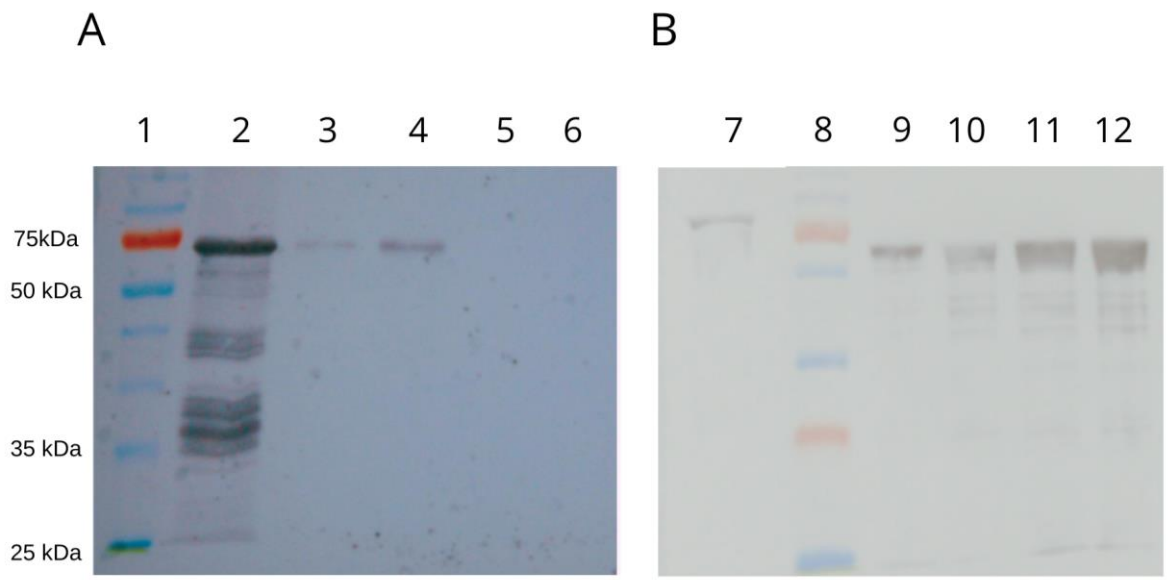

Figure 1. Characterization of rQ1 integrity after cell inactivation using Western blot. A, 1, Marker (molecular weight is described on the left of the image); 2, Positive control (purified rQ1); 3, *E. coli* transformed with pAE/Q1 vector before inactivation; 4, *E. coli* transformed with pAE/Q1 vector after inactivation; 5, *E. coli* transformed with pAE vector before inactivation;

6, *E. coli* transformed with pAE vector after inactivation; B, 7, *E. coli* transformed with pAE/Q1 vector after inactivation; 8, Marker; 9 to 12, Curve of previously calculated concentrations of a known protein (LigBrep).

The quantification of the inactivated *E. coli* extract containing only the pAE vector was performed by comparing the band densities in a 12% SDS-PAGE gel (Figure 2). The similarity of the bands to the rQ1 bacterin extract (lanes 1 and 2) was observed, leading to the assignment of the same approximate concentration of 30 µg/mL attributed to the rQ1 protein, which was quantified through band comparison with the LigBrep concentration curve.

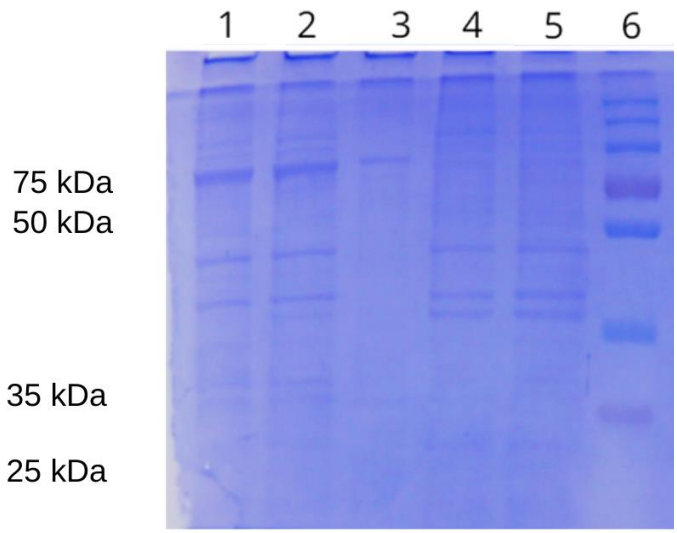

Figure 2. SDS-PAGE for the quantification of bacterins. 1, *E. coli* extract expressing the inactivated rQ1 chimera; 2, *E. coli* extract expressing the pre-inactivation rQ1 chimera; 3, Purified rQ1 chimera; 4, *E. coli* extract containing the pre-inactivation pAE plasmid; 5, *E. coli* extract containing the inactivated pAE plasmid; 6, Molecular weight marker (molecular weight is described on the left of the image).

It was possible to observe the degradation of plasmids extracted from both the *E. coli* bacterin expressing rQ1 and the *E. coli* bacterin transformed with the pAE vector in a 0.8% agarose gel. These plasmids were neither viable nor intact compared to the positive control,

which consisted of the plasmid extracted from *E. coli* transformed with the pAE vector before inactivation (Figure 3).

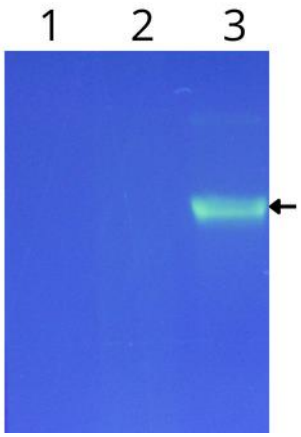

Figure 3. 0.8% agarose gel electrophoresis demonstrating plasmid DNA degradation after cell inactivation. 1, Plasmid extracted from inactivated culture of *E. coli* BL21 Star expressing the recombinant chimera; 2, Plasmid extracted from inactivated culture of E. coli transformed only with the pAE vector without insert; 3, pAE vector extracted from pre-inactivation E. coli used as a positive control indicated by the arrow.

3.2. Adsorption test

After the vaccine preparations, the adsorption of proteins to the Aluminum Hydroxide vaccine adjuvant was evaluated by SDS-PAGE. In this step, it was possible to observe (Figure 4), as expected, the absence of bands related to the proteins present in the analyzed formulations, indicating their complete adsorption to the adjuvant.

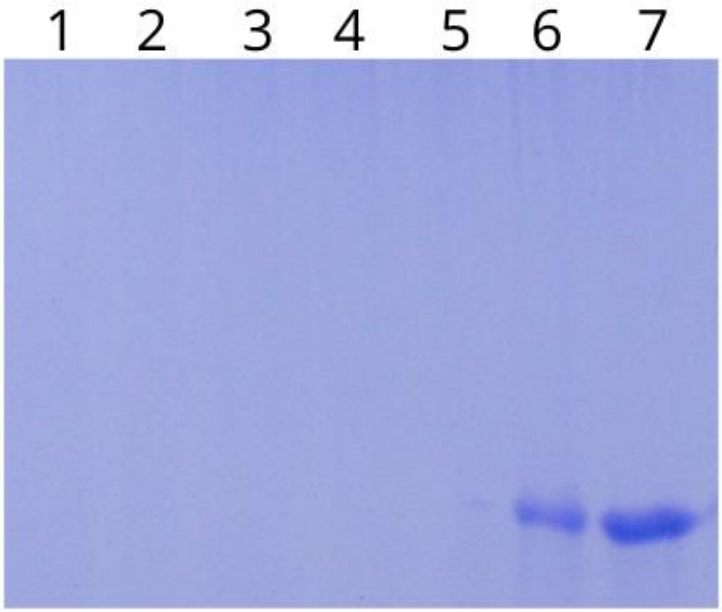

Figure 4. Adsorption test of vaccines containing Al (OH)3 in the composition, experiment 2. 1, Group A first dose; 2, Group A second dose; 3, Group E first dose; 4, Group E second dose; 5, Group F second dose; 6, Control rTBDRchi + PBS; 7, purified rTBDRchi. were evaluated at 1:25 dilution, with anti-hamster IgG, in triplicate, as a secondary antibody.

3.3. Survival experiment 1

Survival and mortality were assessed in both independent experiments. In experiment 1, 50% (5/10) of the animals vaccinated with the subunit vaccine (group A) survived the challenge, replicating this result in experiment 2 (Tables 3 and 4). The vaccine group B (recombinant bacterin Q1) had a single surviving animal (10%, 1/10) (Table 4). It is important to note that no animals survived in group D (pAE bacterin), considered one of the negative controls, in both experiments (Tables 3 and 4).

Table 3. Survival assessment Experiment 1.

Group	Immunogen		% Protection (n survival/total)	*P*-value
	First dose	Second dose		
A	Purified Q1 + Alum	Purified Q1 + Alum	50% (5/10)	0.1409

B	Recombinant bacterin Q1	Recombinant bacterin Q1	10% (1/10)	1.0
C	PBS + Alum	PBS + Alum	10% (1/10)	-
D	Bacterin pAE	Bacterin pAE	0% (0/10)	1.0

Alum= Aluminium hydroxide ($Al(OH)_3$)

The animals that did not survive the challenge showed clinical signs warranting euthanasia between days 6 and 14, both in the vaccinated groups and the control groups (Figure 5). None of the vaccinated groups showed a significant *P*-value in the Fisher's Exact Test.

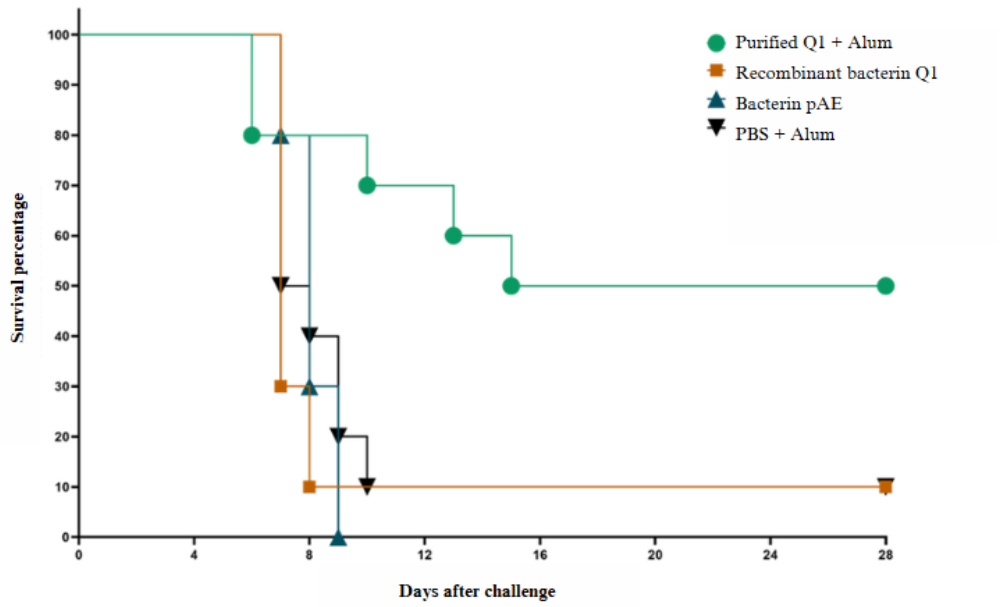

Figure 5: Survival of animals immunized in experiment 1. Survival curve of animals immunized with different vaccine formulations based on the chimeric antigen rQ1 against homologous challenge with *L. interrogans*. Analyses were performed by LogRank test using Graphpad Prism 8 software.

3.4. Survival experiment 2

Regarding the survival of the groups tested in the third experiment, in group B (recombinant bacterin Q1) and group E (recombinant bacterin Q1 + Al (OH)3), none of the

animals survived after the challenge. Group F, vaccinated with the first dose of bacterin Q1 and a second dose of purified Q1 + Al (OH)3, showed 30% protection against mortality; however, the result was not significant in the Log-rank test when compared to the PBS group (Table 4).

Table 4. Survival assessment Experiment 2.

Group	Immunogen		% Protection (n survival/total)	*P*-value
	First dose	Second dose		
A	Purified Q1 + Alum	Purified Q1 + Alum	50% (5/10)	0.1409
B	Recombinant bacterin Q1	Recombinant bacterin Q1	50% (5/10)	0.1409
C	PBS + Alum	PBS + Alum	0% (0/10)	1.0
D	Bacterin pAE	Bacterin pAE	10% (1/10)	-
E (n=10)	Recombinant bacterin Q1 + Alum	Recombinant bacterin Q1 + Alum	0% (0/10)	1.0
F (n=10)	Recombinant bacterin Q1	Purified Q1 + Alum	0% (0/10)	1.0

Alum= Aluminium hydroxide ($Al(OH)_3$)

In experiment 2, animals reached euthanasia endpoints within days 7–15 after challenge in both the vaccinated and control groups (Figure 6).

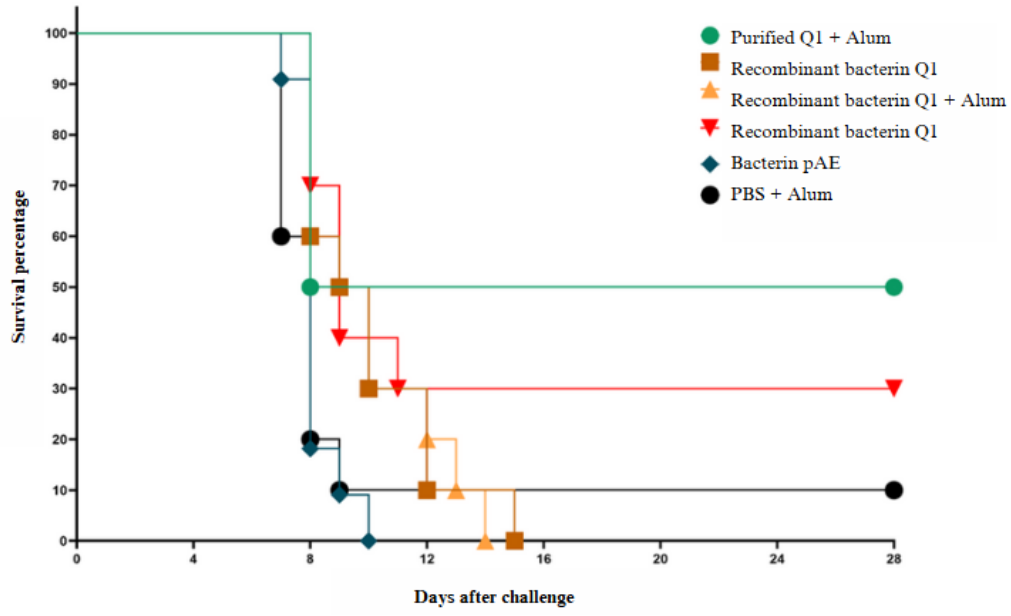

Figure 6. Survival of animals immunized in experiment 2. Survival curve of animals immunized with different vaccine formulations based on the chimeric antigen rQ1 against homologous challenge with *L. interrogans*. Analyses were performed by LogRank test using Graphpad Prism 8 software.

The survival analysis using the Log-rank test was performed, an increase in the survival of animals in group A of experiments 1 and 2 (P=0.0290 and P=0.0191) was observed when compared with the PBS group.

3.4. Evaluation of humoral response in hamsters

Regarding the analysis of the humoral response, an indirect ELISA was performed with sera from the vaccinated groups (pool) on day 28. Figure 7 shows the antibody levels post-vaccination observed in experiments 1 and 2, respectively.

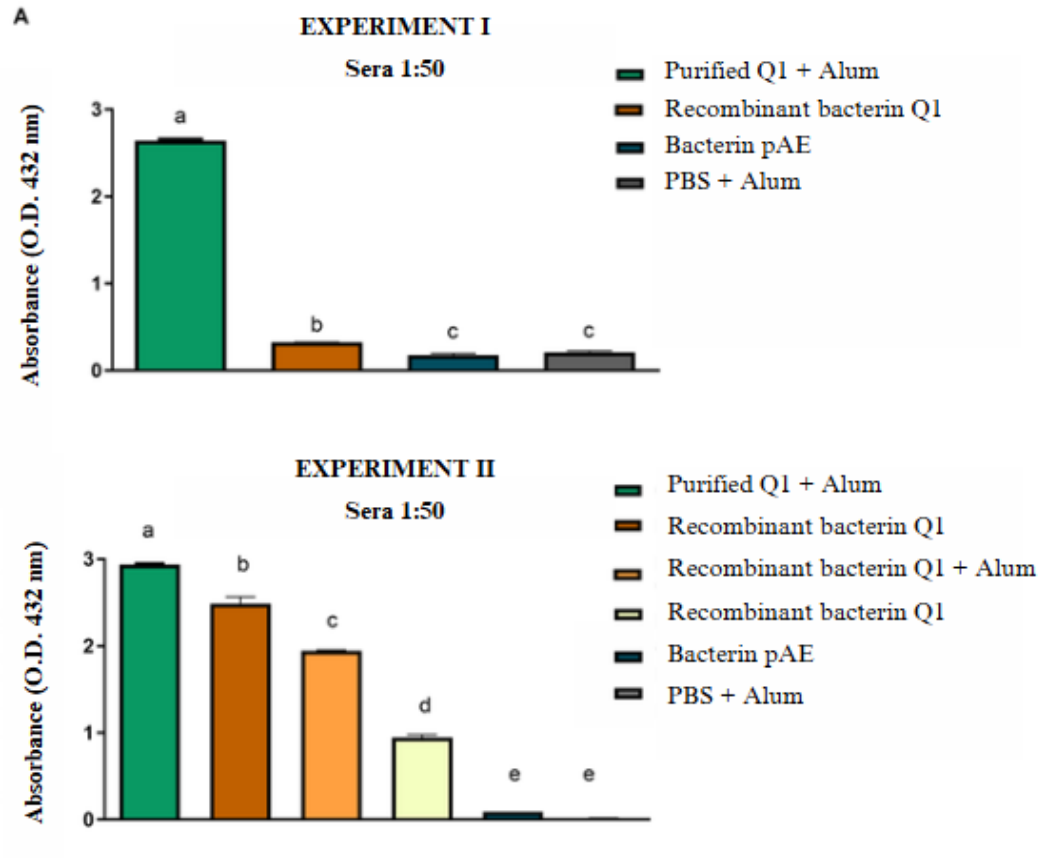

Figure 7. Humoral immune response induced by the immunization of hamsters. Evaluated by indirect ELISA. A, Evaluation of pooled sera from the vaccine groups compared to the control group, experiment I; B, Evaluation of pooled sera from the vaccine groups compared to the control group, experiment II. The columns represent the mean of the triplicates and the bars represent the standard deviation.

The indirect ELISA demonstrated that group A, vaccinated with the purified Q1 vaccine + $Al(OH)_3$, exhibited significant levels of immunoglobulins ($P > 0.05$), differing from all other groups in both experiments. This shows that, among the 4 vaccine groups, the group that received the subunit vaccine generated strong immune responses on day 28, as well as increased survival compared to the PBS control group.

In experiments 1 and 2, the group immunized with recombinant Q1 bacterin showed statistically significant antibody levels when compared to the control groups, despite no animals

surviving in this group (Figure 7). Group F, which received a heterologous prime-boost regimen (recombinant Q1 bacterin + purified Q1), showed significant levels when compared to the group that received Q1 bacterin + $Al(OH)_3$ and the controls.

4. Discussion

Recombinant vaccines have the potential to induce robust and long-lasting immunological memory responses (Jorge and Dellagostin, 2017). The proteins LigA, LemA, and LipL32 are surface-exposed and actively expressed during host infection. Due to their critical roles and interactions with the host, these proteins have been extensively studied as key vaccine targets (Haake and Matsunaga, 2021; Hartwig et al., 2013).

The partial efficacy of recombinant proteins in providing protection against leptospirosis has been demonstrated in subunit vaccines. However, these formulations have not yet succeeded in completely preventing renal colonization in infected animals (da Cunha et al., 2019; Hartwig et al., 2013; Oliveira et al., 2018). Nevertheless, a study by Oliveira et al. (2019) utilized rBCG combined with a multi-epitope protein based on the *Leptospira* antigens LipL32, LemA, and LigA 11-13 (chimera 1), achieving 80% to 100% protection in hamsters without detecting bacteria in renal tissue, thereby demonstrating the sterilizing potential of this formulation. Furthermore, Bunde et al. (2023) showed that rBCG-Q1 induced a Th1-type response characterized by high levels of IFN-γ, which were associated with its protective efficacy and sterilizing immunity, while no significant levels of rQ1-specific IgG antibodies were detected.

Given the manufacturing and immunogenicity challenges associated with BCG as a vaccine vector, the use of recombinant bacterins offers advantages, such as eliminating purification steps and reducing production costs compared to recombinant subunit vaccines (Walker and Bacon, 2023). This study aimed to develop and evaluate the protective capacity of

a recombinant *E. coli* bacterin expressing the chimeric antigen rQ1, as well as its potential to induce sterilizing immunity, using hamsters as a susceptible animal model for leptospirosis.

In this study, the systemic humoral immune response induced by vaccination was evaluated using the rQ1 chimera as the antigen in an ELISA. However, group A, vaccinated with the purified Q1 vaccine + Al $(OH)_3$, exhibited significantly elevated immunoglobulin levels ($P < 0.05$) compared to all other groups in both experiments. Among the vaccine groups, this subunit vaccine induced the strongest immune responses by day 28, along with increased survival rates compared to the PBS control group. Teixeira et al. (2019) described the ability of the aluminum hydroxide adjuvant to enhance antigen-specific immune responses and induce long-lasting humoral immunity after primary immunization, suggesting that the observed immunoglobulin levels were a result of the adjuvant.

It is noteworthy that animals in the control groups receiving pAE bacterin and PBS demonstrated low immune responses by day 28, with no significant differences between these groups. Furthermore, in our experiments 1 and 2, the group immunized with the recombinant Q1 bacterin demonstrated statistically significant antibody levels compared to the control groups, despite the absence of surviving animals in this group. Group F which received a heterologous prime-boost regimen (recombinant Q1 bacterin followed by purified Q1, experiment 2), showed significant antibody levels when compared to the group that received Q1 bacterin with Al $(OH)_3$ and the control groups. The precise mechanisms required for protection against leptospirosis are not fully understood, humoral immunity is recognized as a critical component, while the role of cellular immunity has also been highlighted in some studies (Zuerner, 2014).

Survival analysis using the Log-rank test revealed a significant increase in survival rates among animals in group A during experiments 1 and 2 ($P = 0.0290$ and $P = 0.0191$, respectively) compared to the PBS group. Supporting these findings, Pinho et al. (2021) reported increased

survival rates, with 40% protection, in mice vaccinated with recombinant *E. coli* bacterin expressing an antigen from *C. pseudotuberculosis*. These results contrast with those of Cunha (2017), who reported 100% protection in animals vaccinated with rQ1 + Al $(OH)_3$ at a 50 µg dose of the recombinant chimera (unpublished data).

Although traditional leptospirosis vaccines (bacterins) provide protective efficacy by stimulating opsonizing antibodies targeting lipopolysaccharides (LPS), with their effectiveness restricted to the serovars included in the formulation (Verma et al., 2013). Promoting partial protection due to the absence of cross-immunity against serovars not included in the vaccine and the possibility of circulation of other potentially endemic serovars in the region (Fávero et al., 2018). The development of recombinant vaccine prototypes represents a promising approach to broadening the protective spectrum against leptospirosis, and our findings highlight the critical role of expression platforms and delivery systems in vaccine development.

5. Conclusions

In summary, the protective potential of a vaccine based on recombinant *E. coli* bacterin expressing the rQ1 antigen was evaluated. However, our results indicate that none of the recombinant vaccine prototypes tested, regardless of the vaccination regimen, were able to provide significant protective immunity against the lethal challenge with the *L. interrogans* strain. Nevertheless, there was a significant increase in survival in the groups that received the subunit vaccine. Additionally, the vaccine groups were able to generate significant antibody levels when compared to the control groups, although this was not a parameter correlated with protection.

Author Contributions: Domitila Brzoskowski Chagas: Writing – original draft, Visualization, Validation, Methodology, Investigation. **Vitória Adrielly Catschor dos Santos:** Validation, Methodology, Investigation. **Mara Andrade Colares Maia:** Writing – review & editing, Methodology, Investigation, Conceptualization.

Natasha Rodrigues de Oliveira: Writing – review & editing, Methodology, Data curation. **Thaís Larré Oliveira Bohn:** Writing – review & editing, Supervision**. Odir Antônio Dellagostin:** Writing – review & editing, Supervision, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflict of interest.

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2.2 Capítulo 2

Recombinant live-attenuated *Salmonella* vaccine for veterinary use

- Revisão bibliográfica -

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Review

Recombinant live-attenuated *Salmonella* vaccine for veterinary use

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Abstract: Vaccination is essential for maintaining animal health, with priority placed on safety and cost effectiveness in veterinary use. The development of recombinant live-attenuated *Salmonella* vaccines (RASVs) has enabled the construction of balanced lethal systems, ensuring the stability of plasmid vectors encoding protective antigens post-immunization. These vaccines are particularly suitable for production animals, providing long-term immunity against a range of bacterial, viral, and parasitic pathogens. This review summarizes the progress made in this field, with a focus on clinical trials demonstrating the efficacy and commercial potential of RASVs in veterinary medicine.

Keywords: vaccine development; *Salmonella*; attenuation; RASV; animal

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1. Introduction

The growing human population has led to an increase in the demand for foods of animal origin, resulting in consequent changes in agricultural production [1]. Animals are often produced in confinement, which makes them vulnerable to the spread of various diseases [2]. Many infectious microorganisms can be zoonotic and are transmitted from animals—whether they are food animals, companion animals, or wildlife—to humans [3,4]. Furthermore, these diseases affect the well-being and productivity of animals, causing economic losses and impacting the food supply [5]. There are solutions that can help control diseases in production animal farms, including effective sanitation in facilities; the quarantine or slaughter of contaminated animals; the administration of treatment with antibiotics, antivirals, and antiparasitics; and vaccination [2]. However, there are growing concerns related to antibiotic resistance associated with their extensive use [6,7]. This is coupled with the high cost of developing new, effective, non-toxic drugs to treat bacterial, viral, and parasitic infections [8]. These challenges have become significant obstacles for the treating of infectious diseases. Therefore, vaccination has emerged as an alternative that can reduce the reliance on antibiotics for treating infections [1]. While much of the current research has focused on recombinant subunit vaccines, the cost of veterinary vaccines is primarily due to the purification process required for these proteins, which makes them more expensive compared to traditional vaccines [9]. Despite their lower production costs, bacterins have been associated with variable efficacy and potential side effects, as observed in leptospirosis [10]. While

each vaccination strategy has its own set of advantages and disadvantages, veterinary vaccines must prioritize safety, cost effectiveness, and the ability to provide long-lasting protective immunity against pathogenic microorganisms [8]. Among the various vaccine formulations using infectious bacterial agents as carriers of immunoprotective antigens, *Salmonella* stands out. This enteropathogenic bacterium infects both humans and animals, causing a variety of illnesses from gastroenteritis to systemic typhoid fever [11,12]. The prevalence of *Salmonella* varies geographically, with *Salmonella* enterica serovar Typhimurium (S. Typhimurium) being one of the most commonly isolated serovars from animals and humans worldwide. Some serovars exhibit host specificity, such as *Salmonella* enterica serovar Choleraesuis (*S.* Cholerasuis) in pigs, *Salmonella* enterica serovar Abortusovis (*S*. Abortusovis) in sheep, and *Salmonella* enterica serovar Dublin (*S*. Dublin) in cattle [13–15]. Despite its potential pathogenicity, *Salmonella* is easily managed and genetically manipulated, making it an ideal candidate for antigen delivery systems for several reasons [16]. The inactivation of metabolic genes in live vaccines enables the expression of essential antigens and virulence factors while constraining their ability to proliferate. Consequently, vaccine strains engineered and deleted via techniques such as site-directed mutagenesis demonstrate complete biological containment [17,18]. These attenuated strains replicate the natural infection process, possessing the ability to invade and replicate within mucosa-associated lymphatic tissues (MALT) and gutassociated lymphatic tissues (GALT), such as Peyer's patches, before spreading systemically via mesenteric lymph nodes [18,19]. This characteristic dissemination pattern enables *Salmonella* to evoke robust protective immunity, particularly when administered orally, which stimulates both mucosal and systemic immune responses [20,21]. Additionally, several studies have shown that administering live-attenuated *Salmonella* vaccines via intramuscular or subcutaneous routes in animals (such as mice, dogs, and goats) effectively stimulates an immune response [22–24]. The adoption of rationally live-attenuated *Salmonella* vaccines is already underway in farm animal vaccination programs, which aim to control infections and prevent disease spread [25,26]. Additionally, numerous studies have explored vaccine constructs based on live-attenuated *Salmonella*, primarily carrying antigens against a variety of other organisms, including bacteria, viruses, and parasites [27–31]. These vaccines are preferably administered orally and nasally because of their ease of delivery, allowing for needle-free administration in a straightforward and painless manner. They can be administered via spray or mixed into water, making them more suitable for widespread use in commercial animals such as poultry, swine, and fish [31,32]. However, oral administration in ruminants still poses challenges due to the process of rumination and regurgitation of food, necessitating techniques like microparticle encapsulation to enhance efficacy [33]. The significance of using live-attenuated S*almonella* vaccines in veterinary practices is reinforced by their storage convenience [16]. They can be easily freeze-dried and maintained at room temperature, offering a notable advantage for their implementation in livestock, especially in regions lacking vaccine refrigeration facilities [33,34]. Emphasizing these diverse benefits, this review examines compelling findings from past and ongoing studies that explore the progress of recombinant live-attenuated *Salmonella* vaccines

(RASVs) as a versatile delivery vector (Figure 1) for the prevention of various infectious diseases.

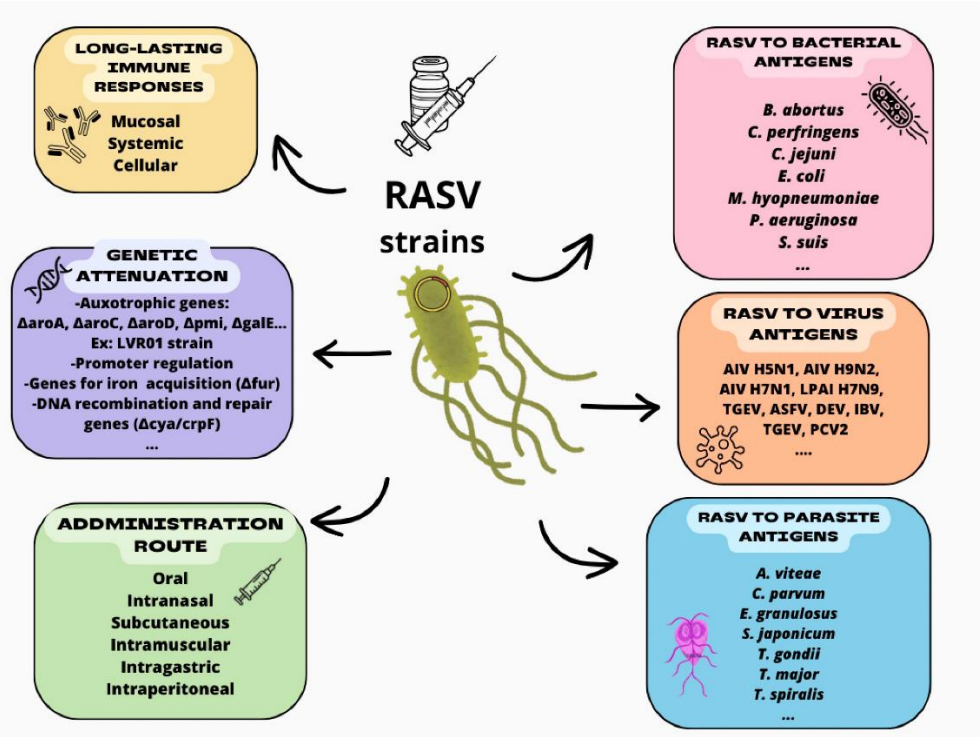

Figure 1. Recombinant live-attenuated *Salmonella* vaccines (RASV): main characteristics and applications as veterinary vaccines for bacterial, viral, and parasitic antigens. AIV, avian influenza viruses; LPAI, low pathogenic avian influenza; TGEV, transmissible gastroenteritis virus; ASFV, African swine fever virus; DEV, duck enteritis virus; IBV, infectious bronchitis virus; PCV2, porcine circovirus type 2.

2. Commercial Veterinary Vaccines Against *Salmonella* in Farm Animals

The use of vaccines to control infections caused by *Salmonella* spp. has been widely adopted in various countries [35]. However, the use of commercial bacterins poses a risk of heightened vaccine reactions at the injection site, typically following intramuscular administration, because of the presence of toxic components in bacterial cells, particularly lipopolysaccharides (LPS) and oil emulsion adjuvants [36]. Additionally, many killed whole-cell vaccines offer limited cross-protection against other antigenically related serotypes [11,14].

Live-attenuated *Salmonella* vaccines, which are designed to decrease disease prevalence and confer protection against various pathogen strains, are becoming increasingly commercially available [37,38]. These vaccines are being licensed for use in production animals across several countries [39]. Among the live-attenuated vaccines against *Salmonella* available in Europe and Australia for chicken producers are those targeting *S.* Typhimurium, *S.* Enteritidis, and *S.* Gallinarum. In a field study, Lyimu et al. [25] evaluated the effect of three commercial live-attenuated vaccine strains on cecal immune genes and compared with cytokine expression. The vaccine induced more anti-inflammatory cecal environment and Th1 responses, crucial to

limiting *Salmonella* contamination in chickens. Furthermore, they reported an increase in serum IgG in the vaccine group that received the commercial vaccine against *S.* Typhimurium when compared to the control. However, they also reported that the live vaccine can modify the shape of different microbiota profiles.

Another example of commercially available vaccines for veterinary use is the live-attenuated vaccine against *S.* Typhimurium in pigs [**26**]. The *Salmonella* vaccine has already been evaluated in sows and piglets weaned from four, three, or twenty-four days to six or seven weeks of age [**40**,**41**] and in sows and gilts [**42**]. Recently, in a study using different swine production cycles, the use of the vaccine in sows, piglets, and fattening pigs resulted in control over *S.* Typhimurium infections and decreased prevalence of positive lymph nodes at slaughter [**26**].

3. Role of Attenuation in the Recombinant *Salmonella* Vaccine

Salmonella has been used as a vector for heterologous antigens, mainly because of its ability to elicit long-lasting mucosal, systemic, and cellular immune responses [**17**]. It can be administered via various routes, including oral, nasal, subcutaneous, and intramuscular administration [**20**]. However, for the use of live recombinant *Salmonella*, the attenuation of virulence factors is mandatory in order to prevent unwanted side effects such as fever and diarrhea [**43**].

Several approaches have been studied for the development of live-attenuated recombinant *Salmonella* vaccines, including different mutations that guarantee the attenuation of these strains through regulated delayed attenuation, delayed antigen synthesis, and/or delayed lysis [**8**,**17**]. These strategies have enabled the construction of a balanced lethal system and the stability of plasmid vectors encoding protective antigens in vivo after immunization [**17**,**44**].

Regulation allows these vectors to present characteristics similar to those of the wild type, enabling their survival and transit through the gastrointestinal tract and the execution of the initial stages of infection before exhibiting attenuation [**45**]. These studies have led to advances and discoveries in biological containment and antigen delivery systems using *Salmonella* [**20**]. The deletion of Δpmi or ΔgalE genes makes the strains dependent on exogenous mannose and galactose, respectively [**46**]. These genes encode surface antigens, such as O antigen side chains, and cause phenotypic changes in lipopolysaccharides, which are crucial factors for host colonization [**18**]. Furthermore, it is possible to utilize the deletion or mutation of genes necessary for the biosynthesis of metabolically essential elements, such as aromatic amino acids and vitamins, including ΔaroA, ΔaroC, and ΔaroD deletions [**47**]. An example of this type of attenuation that has been used in a study is the *S.* Typhimurium LVR01 strain, which was constructed by introducing a null deletion in the ΔaroC gene of the canine parental isolate of *S.* Typhimurium, P228067 [**48**].

Likewise, it is possible to regulate expression at or through the promoters of the chromosomal repressor gene *lacI* in regulatory pathways with pleiotropic effects (*cya, crp, phoP*). This can include using activating or repressor protein binding sequences for genes of iron acquisition (Δ*fur*), encoding the regulatory system of virulence components (Δ*phoP* and Δ*phoQ*), or including mutations in DNA recombination and repair genes (Δ*cya*/*crpF*) or in the *cAMP* receptor protein (Δ*crp*), which is regulated via an *araC* P BAD cassette [**47**]. Thus, the expression of these genes is

regulated by arabinose or mannose supplementation, occurring only during in vitro growth [17]. Following the colonization of lymphoid tissues, synthesis of the associated proteins ceases due to the absence of mannose or arabinose in vivo [28]. Consequently, attenuation develops progressively in vivo, inhibiting the onset of disease symptoms and inducing the desired antigen-specific immune response.

Moreover, regulated delayed lysis in vivo prevents RASV persistence, aiding biocontainment by ensuring the death of the bacteria following the colonization of immune tissues [17,47]. The system regulates the expression of enzymes required for synthesizing two key components of the cell wall's peptidoglycan layer: diaminopimelic acid (DAP) and muramic acid. DAP synthesis is controlled by aspartate semialdehyde dehydrogenase (encoded by *asd*), while UDP-N-acetylglucosamine enolpyruvyl transferase (encoded by *murA*) regulates muramic acid production [44]. The expression of these enzymes is engineered to be regulated by exogenous arabinose; in its absence, cell lysis occurs due to an inability to synthesize the cell wall [49].

Another crucial aspect of a vaccine vector containing heterologous antigens is the stability of the plasmid [8]. Regulation of the levels and location of expression of these antigens can have a significant impact on the immunogenicity of the vaccine, potentially reducing colonization capacity and, consequently, immunological efficacy [18]. To address the problem of instability in the chromosomal integration of foreign genes, systems have been developed that include the integration of the foreign gene into the bacterial chromosome, the optimization of heterologous antigen codons [50], the use of inducible promoters in vivo [49], and those by which DNA encodes the foreign gene in a suicide vector [44].

Thus, vaccine strains generally incorporate more than one mutation or deletion in genetic constructs, making it possible to eliminate essential genes involved in virulence regulatory systems [45]. This ensures attenuation, prevents virulence reversal, and eliminates potential side effects [51]. Furthermore, these deletions increase the colonization capacity, survival in the mucosal environment, and immunogenicity of RASV vaccine constructs [20,47].

4. Molecular Mechanisms of Immune Stimulation by Recombinant *Salmonella*

Live-attenuated *Salmonella* strains used as vaccine delivery vehicles for heterologous antigens should effectively cross the epithelial barrier, reach the underlying antigen-presenting cells in the MALT, and trigger a strong immune response [51]. These mutants establish a limited infection in the host, and during this harmless infection, they deliver a variety of in vivo synthesized antigens directly to B and T lymphocytes in the GALT [52,53].

Typically, the antigens presented by RASV colonize internal effector lymphoid tissues without compromising protective functions or integrity. The interaction between *Salmonella* and its host is initiated by various virulence factors, including type III secretion systems associated with *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) [8,52,54]. The SPI-1-encoded system enables *Salmonella* to invade various lymphoid tissues associated with the intestinal, nasopharyngeal, and bronchial mucosa. Bacterial internalization triggers alterations in host cell signaling pathways, impacting essential cellular processes such as membrane

trafficking, cell division, antigen presentation, and cytokine production [51].

Salmonella, having adapted to the mucosal surface environment, begins the infection process, remaining in membrane-bound vacuoles, and once it reaches the mesenteric lymph nodes in antigen-presenting cells, they produce recombinant proteins [51]. Antigen delivery results in a generalized immune response that targets intestinal sensory cells, known as Peyer's patch M cells [44]. These cells play a key role in stimulating mucosal immune responses [55]. Furthermore, *Salmonella* spp. can be taken up by phagocytic cells and cross the reticulo-endothelial system, thereby stimulating systemic immune responses [56]. *Salmonella* efficiently targets MALT and induces local and systemic immunity [12,57]. Dendritic cells, neutrophils, and macrophages are activated in response to antigens being present in the MALT, having recognized pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs), such as T3SS-1, fimbriae, and other bacterial surface adhesins [56].

Protein antigens are processed and presented through the major histocompatibility complex (MHC), stimulating T cell responses [55]. The signaling and activation of phagocytic cells initiate a critical immune response that establishes links between the innate and adaptive immune systems [58]. *Salmonella* has a preference for residing within macrophages, where the activation of these cells by interferon gamma (IFN-γ) produced by Th1 cells significantly contributes to bacterial elimination [54]. IFN-γ, commonly referred to as macrophage activating factor (MAF), affects the duration of macrophage activation and is crucial during infection. The secretion of IFN-γ is dependent on IL-18, also known as the IFN-γ-inducing factor, and is vital for establishing early host resistance to *Salmonella* infection [59]. During primary and secondary infection, *Salmonella* is dependent on IL-12, IFN-γ, and tumor necrosis factor α (TNF-α).

Classical activation by bacterial LPS or IFN-γ modifies the cells' secretory profile by promoting the production of organic nitrate compounds, including nitric oxide (NO) [56]. Mucosal DC responses to inflammatory stimuli may enhance their capacity to preselect antigens expressed by recombinant *Salmonella* for targeting specific T and B cells [60]. Alternative activation by IL-4, IL-10, or IL-13 promotes the production of polyamines and proline, stimulating cell proliferation. The presence of *Salmonella* in these cells triggers cytokine secretion, leading to an inflammatory response or programmed cell death via apoptosis [56].

The RASV consistently produces recombinant protein for an appropriate duration under SPI-2-regulated conditions, after which it is translocated into the cytosol via the SPI-2 T3SS [61]. Secreted peptides are processed and presented to major histocompatibility complex (MHC) class I and II molecules to stimulate T cell responses [56]. Immunization with attenuated vaccines is a safe and effective method to induce the production of both serum and mucosal antibodies against the *Salmonella* carrier, as well as foreign antigens [12,62].

5. *Salmonella* as a Vaccine Vector Against Different Pathogens

Attenuated vaccines are produced using live, whole bacterial cells or viral particles that have been subjected to in vitro passages, chemical treatments, or genetic manipulation to obtain strains lacking virulence in

the host. Despite the loss of virulence, these attenuated strains retain their ability to provoke a strong immune response, closely mimicking natural infection [**63**]. Although several conventional attenuated vaccines are available for veterinary use, concerns about the risk of residual virulence, particularly in immunocompromised hosts, and higher sensitivity to cold chain disruptions have driven the search for safer options [**9**,**63**].

Studies in mice vaccinated with a live attenuated *Salmonella* delivering *E. coli* antigens [**64**,**65**] to control post-weaning diarrhea in swine indicated that vaccine constructs were not detected in the feces of immunized animals for up to three weeks post-oral inoculation, suggesting minimal or no environmental excretion. Another study using attenuated *S. Typhimurium* carrying a DNA vaccine against *Streptococcus agalactiae* in fish showed that the bacteria were eliminated from the tissues four weeks post-immunization [**32**]. Conversely, Jiang et al. [**66**] observed that oral vaccination with strains χ9241-tHP and χ9352-tHP resulted in high (10^4 to 10^7 CFU/g) and moderate (10^2 to 10^4 CFU/g) levels of recombinant *Salmonella* shedding in fecal samples from chickens at 14 and 11 days of age, respectively. Similarly, rectal swabs from pigs orally vaccinated with C500 variants of *S. Choleraesuis* showed variable levels of fecal shedding [**67**]. These results highlight the need to test different vectors to achieve an optimal balance among immunogenicity, stability, and biocontainment.

The effective immune response and protection induced by live-attenuated recombinant *Salmonella* has been described in several studies using parasitic, bacterial, and viral antigens. The reviewed articles are compiled and presented in a detailed list including the heterologous bacterial, parasitic and viral antigens, respectively expressed in different strains of *Salmonella*, as well as the model of attenuation used, route and dose administered, immune response induced, and animal model used. This is a summary of information published to date regarding the use of recombinant attenuated *Salmonella* vaccine (RASV) as a vaccine vector. The potential of RASV is evident, and it requires further exploration.

5.1. RASV Administration Routes

In our review, we found that mucosal administration—primarily via the oral route—is more frequently explored for RASV than parenteral routes, such as intramuscular, subcutaneous, or intraperitoneal injection. A limited number of studies have used intranasal [**31**,**68**,**69**,**70**,**71**] and intragastric routes [**32**,**72**,**73**,**74**]. The choice of administration route can impact antigen immunogenicity, influencing immune cell priming (especially antigen-presenting cells) and promoting both local and systemic immunity [**75**]. Mucosal routes may be more effective, as most infectious agents enter through mucosal surfaces. Vaccination through mucosal routes can stimulate protective responses at these surfaces and systemically [**76**]. Conversely, subcutaneous and intramuscular vaccines form antigen depots at the injection site, attracting APCs to migrate to nearby lymph nodes, where they activate the immune response [**76**].

Several studies have evaluated RASV administration routes in animal models. Lalsiamthara et al. [**77**] examined the efficacy of an RASV containing four *Brucella* antigens administered via parenteral routes (intraperitoneal, intramuscular, and subcutaneous) in mice. The parenteral administration showed higher protection rates against *B. abortus* compared to oral administration. Another study compared intraperitoneal and oral administration of an *S.* Typhimurium vaccine

expressing *B. abortus* antigens in mice. The intraperitoneal route induced stronger humoral (IgG levels) and cellular (TNF-α and IFN-γ) responses, with greater protection against *B. abortus* infection post-challenge [**29**].

In contrast, Cong et al. [**73**] demonstrated the potential of mucosal routes for an RASV expressing *T. gondii* antigens. The vaccine was administered to mice orally, nasally, or intramuscularly. Mice vaccinated via mucosal routes (oral and nasal) showed higher IgG and IgA levels than those vaccinated intramuscularly. Intranasal vaccination stimulated greater $CD4^+$ and $CD8^+$ T-cell activation, with cytokine levels (IFN-γ and IL-2) also being higher in orally and nasally vaccinated mice. Survival rates post-challenge were 60% for oral, 40% for intranasal, and 20% for intramuscular routes.

Another study by Hyoung et al. [**70**] evaluated an attenuated *S.* Typhimurium mutant expressing hemagglutinin from avian influenza viruses (H7N3, H7N7, and H7N9) in chickens via intramuscular, nasal, or oral routes. The RASV induced strong humoral (neutralizing antibodies) and cellular (IFN-γ, IL-17, and IL-10) responses across all routes, with immunization conferring significant protection against a subsequent challenge with the H7N1 virus. These findings suggest that RASVs can effectively elicit immune responses across multiple administration routes, providing flexibility in tailoring vaccine strategies based on the pathogen and target population.

5.2. Recombinant Salmonella Expressing Bacterial Antigens

Several studies were reviewed and are summarized in **Table 1**, which presents the different *Salmonella* strains, routes, doses, animal models, and the types of immune response stimulated. In one study [**55**], an RASV containing chromosomal fusion genes that encode the secretion signal for the SPI-2 effector protein, SspH2, and the pathogenic outer membrane lipoprotein from *Leptospira*, LipL32, was administered orally to rats. The animals received a dose of 1×10^7 colony forming units (CFU) per mouse of different strains of *S.* Typhimurium (ST) or saline (PBS) alone on days 0, 14, and 28. After vaccination, the group that received the RASV exhibited significantly elevated titers of total immunoglobulin G (IgG) and immunoglobulin A (IgA) specific to the rLipL32 protein, with detectable levels persisting up to 77 days post-vaccination. Notably, following the third immunization on day 28, the anti-LipL32 antibody titers in mice immunized with the RASV were significantly higher than those in mice that received only PBS ($p < 0.05$). Additionally, to assess the cellular immune response, there was a significant increase in the production of the LipL32-specific cytokines IFN-γ and IL-4 in splenocytes from mice vaccinated with the RASV compared to the control group treated with PBS ($p < 0.05$) [**55**].

Table 1. Bacterial antigens expressed in *Salmonella*.

Antigen (Organism)	*Salmonella* Strain or plasmid	Attenuation	Route/dose (CFU)	Model	Immunity	Reference
SspH2, LipL32 (*Leptospira* spp.)	*S.* Typhimurium SL3261	*ΔaroA*	O/1×10^7	Rat	HI, MI, CI	[55]
NrdF (M.hyopneumoniae)	*S.* Typhimurium SL3261	*ΔaroA*	O/10^9	Mice	HI, MI	[78]
	S. Typhimurium	*ΔaroA*	O/1×10^9	Swine	HI, MI, CI	[79]

	SL3261					
P97R1 (*M. hyopneumoniae*)	*S.* Typhimurium CS332	*ΔaroA*	O/2 × 10^8; 2ª dose 3 × 10^8	Mice	HI, MI, CI	[80]
	S. Typhimurium CS332	*ΔaroA*	O/2 × 10^8; 2ª dose 3 × 10^8	Mice	HI, MI, CI	[81]
K88ab (*Escherichia coli*)	*S.* Typhimurium G30/pFM205	*galE*	O, IP/1 × 10^8	Mice	HI	[82]
	S. Typhimurium	*Δlon ΔcpxR Δasd*	O/1 × 10^8	Mice	HI, MI	[65]
	S. Typhimurium	*Δlon ΔcpxR Δasd*	O/1 × 10^{11}	Mice	HI, MI	[64]
	S. Typhimurium	*Δlon ΔcpxR Δasd*	O/2 × 10^{10}	Pregnant sows and piglets	HI, MI	[61]
K88ab, K88ac, K99, FasA, F41 (*E. coli*)	S. ghost controlled expression of ϕX174 lysis gene E	-	O/primed and boosted 2 × 10^9, 2 × 10^{10}, and 2 × 10^{11}	Pregnant sows and piglets	HI, MI	[83]
	S. Typhimurium JOL912	*Δlon*, *ΔcpxR*, *Δasd*	IM/1 × 10^8	Mice	HI, CI, MI	[84]
Stx2eB, FedF, FedA F18+ Shiga toxin (*E. coli*)	*S.* Typhimurium JOL1311 and JOL912	*Δasd*, *Δlon*, *ΔcpxR*	IM/9 × 10^7	Mice	HI, CI, MI	[27]
fliC F18+ Shiga toxin (*E. coli*)	*S.* Typhimurium JOL1454, JOL1460, JOL1464	*Δlon*, *ΔcpxR*, *Δasd*	SC/3 × 10^7	Mice	HI, CI, MI	[85]
APEC papA, papG, iutA, and clpG (*E. coli*)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *ΔasdA16*	O/1 x 10^7	Chicken	HI, CI, MI	[86]
APEC papA, papG, iutA, and clpG (*E. coli*)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *ΔasdA16*	O/1 x 10^7	Chicken	HI, CI, MI	[87]
APEC O-antigen (*E. coli*)	*S.* Typhimurium S100	*Δasd*, *Δcrp*, *Δcya*, *ΔrfbP*	O/ × 10^9, IM/ 5.0 × 10^7	Chicken	HI, MI	[88]
APEC PapA, CTB and LTB (*E. coli*)	*S.* Typhimurium *χ*8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/2 × 10^9	Mice	HI, MI	[89]
APEC (*E. coli*)	*S.* Typhimurium *χ*8025	*Δasd*	O/1 × 10^8	Chicken	MI	[89]
tHP (*Clostridium perfringens*)	*S.* Typhimurium	*Δasd*	O/1 × 10^9	Chicken	Intestinal colonization, BSG	[66]
tHP (*C. perfringens*)	*S.* Typhimurium *χ*9352	*Δasd*, *lacI*	O/1.2 × 10^9	Chicken	MI	[90]
α-toxin, NetB toxin, Fba (*C. perfringens*)	*S.* Typhimurium *χ*11802	*Δasd*, *lacI*	O/1 x 10^8 or 1 × 10^9	Chicken	CI, MI	[91]
PLcC, GST-NetB (*C. perfringens*)	*Salmonella* vaccine *(PIESV)*	*asdA*, *murA*	O/~5 × 10^8	Chicken	NE Intestinal Lesion	[33]

	*χ*11802 and *χ*12341				Scoring	
O antigen (*Burkholderia mallei*)	*S.* Typhimurium SL326	*ΔaroA*	IN/1 × 10^7	Mice	HI, MI	[68]
M protein (*Streptococcus pyogenes*)	*S.* Typhimurium *LB5000*	-	SC/Rabbit: 10^8 heat-killer bacteria or purified flagella; IP/ Mice: 1 × 10^6 to 2 × 10^6 live vaccine	Mice and Rabbit	HI	[92]
optA, optB, LfliC, Lhly (*Lawsonia intracellularis*)	*S.* Typhimurium JOL912	*Δasd*	O/1 × 10^7	Mice	HI, MI	[93]
Sip (*Streptococcus agalactiae*)	*S.* Typhimurium SL7207	*ΔaroA*	IG/10^7, 10^8 and 10^9	Fish	HI	[32]
F1, I2 (*Pseudomonas aeruginosa*)	*S.* Typhimurium LH430	*phoP/phoQ,Δasd*	O and SC/ 2.0 × 10^8 to 2.0 × 10^{10}	Mice	HI, CI, MI	[75]
CP39, FimA, PtfA,ToxA (*Pasteurella multocida*) F1P2 (*Bordetella bronchiseptica*)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *Δasd*	IN/1 × 10^5	Mice	HI, MI	[69]
CjaA (*Campylobacter jejuni*)	*S.* Typhimurium LB5010	*ΔaroA*, *fliM*, *spaS*, *ssaU*	O/1 × 10^8	Chicken	HI, MI	[94]
CjaA (*C. jejuni*)	*S.* Typhimurium *χ*9718	*Δasd*	O/1 × 10^8	Chicken	MI	[95]
BCSP31 (*Brucella abortus*)	*S.* Typhimurium chi 4064	*Δcya*, *Δcrp*	O/2 × 10^8 to 4x10^8	Mice	HI, MI, Blatogenisis	[96]
BCSP31 (*B. abortus*)	*S.* Typhimurium chi 4064	*Δcya*, *Δcrp*	O/1 × 10^{10} to 2 × 10^{10}	Crossbred swine	HI, MI, Blatogenisis	[97]
L7/L12, BLS (*B. abortus*)	*S.* Typhimurium X4072	*Δasd*	O/1 × 10^9	Mice	HI, CI, MI	[98]
BCSP31, Omp3b, SOD (*B. abortus*)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *Δasd*	IP/1.2 × 10^6; O/ 1.2 × 10^9	Mice	HI, CI	[29]
SOD, BLS, PrpA, Omp19 (*B. abortus*)	*S.* Typhimurium JOL912 and JOL1800	*Δlon*, *ΔcpxR*, *Δasd*	O and IP/2 × 10^7	Mice	CI, MI	[77]
BCSP31, Omp3b, SOD (*B. abortus*)	*S.* Typhimurium JOL911 and JOL912	*Δlon*, *ΔcpxR*, *Δasd*	IP/1.2 × 10^4, 1.2 × 10^5 and 1.2 × 10^6	Mice	HI, CI	[99]
BCSP31, Omp3b, and SOD (*B. abortus*)	*S.* Typhimurium pMMP65	*Δlon*, *ΔcpxR*, *Δasd*	SC/3 × 10^9	Dog	HI, CI	[100]
PrpA (*B. abortus*)	*S.* Typhimurium JOL1818 and JOL1881	*Δlon*, *ΔcpxR*, *Δasd*, *ΔrfaL*	IP/1 × 10^7	Mice	HI, CI	[77]
SOD, BLS, PrpA, Omp19 (*B. abortus*)	*S.* Typhimurium JOL1800	*Δlon*, *ΔcpxR*, *Δasd*	SC/5 × 10^9 and 5 × 10^{10}	Goat	HI, CI	[24]

BCSP31, Omp3b, and SOD (*B. abortus*)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *Δasd*	SC/3 × 10^9	Goat	HI, CI	[101]
L7/L12 (*B. abortus*)	*S.* Typhimurium JOL1800	*Δlon*, *ΔcpxR*, *Δasd*, *ΔrfaL*	IM/10^7	Mice	HI, MI	[102]
BCSP31 (*B. abortus*)	*S.* Choleraesuis chi 3781	*ΔcpxR*, *Δcya*	O/Mice: 4 ×10^{10}; Swine: 4 × 10^8 to 6 × 10^8	Mice and crossbred swine	HI, MI,	[103]
6-PGD (*Streptococcus suis*)	*S.* Choleraesuis rSC0011	*Δasd*	O/1 ± 0.3 × 10^9	Mice	HI, MI	[104]
Serotypes 2 and 7 (*S. suis*)	*S.* Choleraesuis rSC0016	*ΔsopB*	O/ Suis: 1 ± 0.3 × 10^9; Mice: 1 ± 0.3 × 10^9	Mice and Swine	HI, MI	[28]
SaoA (*S. suis*)	*S.* Choleraesuis rSC0012	*Δfur*	O/1 ± 0.2 × 10^9	Mice	HI, MI, CI	[105]
Serotypes 1/2, 2, 3, 7, 9 (*S. suis*)	*S.* Choleraesuis rSC0016	*ΔsopB*, *Δasd, lacI*	O/1 ± 0.2 × 10^9	Mice	HI, CI	[106]
P42, P97 (*M. hyopneumoniae*)	*S.* Choleraesuis rSC0016	*Δasd*	O/10^9	Mice	HI, MI, CI	[107]
F18+ Shiga toxin (*E. coli*)	*S.* Choleraesuis C520	*crp, Δasd*	O/2 × 10^9	Swine	HI, MI, CI	[67]

IG, intragastric; IM, intramuscular; IN, intranasal; IP, intraperitoneal; O, oral; SC, subcutaneous; CFU, colony-forming unit; HI, humoral immunity; CI, cellular immunity; MI, mucosal immunity.

Another RASV using *Salmonella* Typhimurium that expresses the R2 antigen of NrdF from *Mycoplasma hyopneumoniae* was used to orally inoculate mice at a dose of 10^9 CFU, followed by two more boosters at the same dose. In this study, a mucosal IgA-type immune response was elicited in lung washings, but a significant level of NrdF-specific serum IgG was not detected [**78**]. Interestingly, Chen et al. [**80**] demonstrated that a DNA vaccine with a eukaryotic expression plasmid, encoding the *M. hyopneumoniae* NrdF antigen through an *S.* Typhimurium live-attenuated *aroA*, when administered orally to mice at a dose of 2 × 10^8 CFU followed by a booster dose of 3 × 10^8 CFU, induced significant NrdF-specific IFN-γ production. However, mice orally vaccinated with *S.* Typhimurium expressing NrdF encoded by a prokaryotic expression plasmid failed to produce a serum or secretory antibody response specific to NrdF, and IFN-γ was not produced [**80**]. In another study conducted by Chen and colleagues [**81**], a RASV of *S*. Typhimurium was used, and the gene of interest was cloned into both eukaryotic and prokaryotic expression vectors. Immunogenicity was assessed in mice orally immunized with *M. hyopneumoniae* P97R1 adhesin, which induced specific Th1 cellular immune responses in a mouse model. However, no mucosal antibody responses against P97R1 were observed.

In the delivery system of *S*. Typhimurium expressing important fimbriae of *Escherichia coli* F4 (K88), F5 (K99), F6 (987Ps), and F41 and intimin adhesin, using a murine model with a single dose or double dose of 2 × 10^9 CFU in 20 μL, IgG and IgA titers for individual adhesins in all immunized groups were higher in the booster dose group than in the

single dose group [**64**]. In another study, Hur, Stein, and Lee [**65**] also used live-attenuated *S.* Typhimurium expressing other recombinant *E. coli* fimbrial antigens K88ab, K88ac, FedA, and FedF. The IgG2a titer was increased in the one-dose group, whereas both the IgG2a and IgG1 titers were increased in the two-dose group. Furthermore, vaccine strains were not detected in the feces excreted from immunized mice. Hur and Lee [**61**] evaluated the immune responses of various doses of *Salmonella* ghost (non-living, devoid of cytoplasmic content, maintaining their cellular morphology), with controlled expression of the φX174 E lysis gene being achieved in pigs. These bacterial ghosts carried enterotoxigenic *E. coli* fimbrial antigens (ETEC) to protect against colibacillosis in piglets. All groups were orally immunized with doses of 2×10^9, 2×10^{10}, or 2×10^{11} CFU in 10 mL PBS and boosted at weeks 11 and 14 of pregnancy. The serum levels of immunoglobulin IgG, IgG, and IgA in the colostrum of sows in groups that received 2×10^{10} or 2×10^{11} CFU were significantly higher than those of sows in the control group. Notably, after a challenge with wild-type ETEC, neither piglet diarrhea nor mortality were observed.

In another study [**85**], live-attenuated *S.* Typhimurium JOL912, which contains the genes encoding P fimbriae (the *pap* gene cluster), the iron-regulated aerobactin receptor *iut*A, and CS31A surface antigen adhesin from avian pathogenic *E. coli* (APEC), was evaluated as the vaccine against APEC infection in chickens. The vaccine was administered orally, intramuscularly, or subcutaneously, using different doses according to three groups: a no-vaccine group, a single-vaccine-dose group, and another that received primary and booster immunizations. The birds were exposed to an intra-air sac challenge using a virulent APEC strain at a dose of 10^7 UFC, and the group that received two vaccine doses showed greater protection against the challenge (80%). Furthermore, this group showed significant increases in plasma IgG levels at three and for weeks old compared to birds from other groups, reinforcing the use of two vaccine doses [**85**]. Lee and colleagues [**86**] reported that, following a challenge with a virulent APEC strain, there were no deaths in the vaccinated group, whereas the control group showed a mortality rate of 15%. The administration of primary and booster doses of the *Salmonella*-delivered APEC vaccine candidate significantly enhanced the generation of antigen-specific sIgA, as well as the production of IFN-γ, IL-6, and IL-2, thereby providing protection for chickens against colibacillosis [**86**]. In another study, Oh et al. [**88**] used the P fimbria subunit PapA from APEC, which was in live-attenuated *S.* Typhimurium. Furthermore, the study utilized the non-toxic B subunits of cholera toxin (CTB) and heat-labile toxin (LTB) as adjuvants within the vaccine formulation. Mice were inoculated with 20 μL containing 2×10^9 CFU. The findings demonstrated a significant increase in Pa-pA-specific serum IgG and mucosal IgA titers when the recombinant *Salmonella* vaccine was administered in conjunction with LTB or CTB adjuvants, highlighting the enhanced immunogenic response facilitated by these adjuvants. Rapid declines in immune responses throughout the experimental period were observed in mice immunized without an adjuvant.

The benefits of the intracellular action of *Salmonella* also correlate with natural infection in mucous membranes and in the intestinal tract, an analogous route to one of the most important infection routes of *Brucella abortus*. In a study by Kim et al. [**29**], live-attenuated *S.* Typhimurium was

engineered to express BCSP31, Omp3b, and SOD proteins from *B. abortus*. Mice were vaccinated with a mixture of the three strains, either orally or intramuscularly [**29**]. The recombinant vaccine induced serum concentrations of IgG, TNF-α, and IFN-γ that were higher than the control when administered via the oral route (except Omp3b) and intramuscularly. A robust IFN-γ-mediated response helps eliminate *Brucella* infection in the host. Furthermore, it was found that after a challenge with a virulent strain of *B. abortus*, the vaccine was able to limit the colonization of the bacteria in the spleen of mice.

Another study targeting brucellosis in goats, reported by Leya and colleagues [**24**], developed a *S.* Typhimurium vaccine expressing four (BLS, PrpA, Omp19, and SOD) heterologous *Brucella* antigens and inoculated them subcutaneously with two doses: 5×10^9 CFU/mL (Group B) and 5×10^{10} CFU/mL (Group C). The goats were challenged with a virulent *B. abortus* strain six weeks after immunization. Serum IgG titers against specific antigens in goats from Group C were significantly higher than those in non-immunized goats and the vector control group. Following antigenic stimulation, IFN-γ levels in peripheral blood mononuclear cells were significantly elevated in Groups B and C compared to the vector control group. The immunized goats in Group C, which received the highest dose, exhibited a significantly higher level of protection ($p < 0.05$); however, the group with the lower dose also showed a successful reduction in microgranuloma lesions in the liver induced by *B. abortus* infection. Stabel et al. [**103**] reported that the use of the *Salmonella* Cholerasuis chi 3781 (SC) strain expressing the BCSP31 protein from *B. abortus*, when administered orally to pigs and mice, stimulated a strong serum IgG response to both the recombinant protein and SC in mice. In contrast, orally inoculated pigs did not develop significant serum or intestinal antibody responses.

5.3. Recombinant Salmonella Expressing Virus Antigens

Live-attenuated strains of *Salmonella*, including *S.* Typhimurium, used in most of the studies reviewed in this article, but also *S.* Galinarium, *S.* Choleraesuis, and *S.* Pullorum, have been evaluated for use as live vaccines for the delivery of a variety of viral antigens (**Table 2**).

Table 2. Viral antigens expressed in *Salmonella*.

Antigen (Organism)	*Salmonella* Strain or plasmid	Attenuation	Route/dose (CFU)	Model	Immunity	Reference
HA (AIV H5N1)	*S.* Typhimurium BRD509	*ΔaroA*, *ΔaroD*	O/10^9	Chicken	Hemagglutination inhibition	[108]
chIFN-a, chIL-18 (AIV H9N2)	*S.* Typhimurium *χ*8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/ 10^9 and 10^{11}	Chicken	CI, Hemagglutination inhibition, PCR	[109]
HA, NA, NP (AIV H5N1)	*S.* Typhimurium SV4089	*Dam*, *ΔPhoP*	O/10^9	Chicken	PCR; FISH, and culturing on XLT4	[110]
HA (AIV H5N1)	*S.* Typhimurium SV4089	*Dam*, *ΔPhoP*	O, IM/ 10^9	Chicken	CI, Hemagglutination inhibition, PCR	[111]

HA (AIV H9N2)	*S. Typhimurium* JOL912, JOL1800	*Δlon*, *ΔcpxR*, *Δasd*	O/ 10^8	Chicken	HI, Hemagglutination inhibition	[30]
HA (AIV H7N1)	*S.* Typhimurium JOL1863	*Δlon*, *ΔcpxR*, *Δasd*	O, IN, IM/ 10^9	Chicken	HI, MI, Hemagglutination inhibition	[70]
HA, M2, NA (LPAI H7N9)	*S.* Typhimurium JOL1800	O antigen deficient	O/10^9	Chicken	HI, CI, MI	[112]
H9N2 haemagglutinin, M2 (AIV H9N2)	*S.* Gallinarum JOL967	*Δlon*, *ΔcpxR*, *Δasd*	O, IM/ 10^9	Chicken	HI, CI, MI	[113]
swIFN-α, swIL-18 (TGEV)	*S.* Typhimurium 8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/ 10^{11}	Swine	Gross lesion; histopathological; qRT-PCR	[114]
Glycoprotein B (PrV)	*S.* Typhimurium SL7207	*ΔaroA*	O/5 to 10 10^7	Mice	HI, MI	[115]
swIL-18, swIFN-α (PrV)	*S.* Typhimurium *χ*8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/10^{11}	Swine	HI, CI	[116]
UL24 (DEV)	*S.* Typhimurium SL7207	*hisG46*, *DEL407*, *ΔaroA*	O/10^{11}, 10^{10} or 10^9	Duck	HI, CI, MI	[117]
tgB, UL24 (DEV)	*S.* Typhimurium *S*739	*Δasd-66*, *Δcrp-24*, *Δcya-25*	O/10^{10}; 10^{11} or 10^{12}	Duck	MI	[118]
CD2v/CTL/9GL, p54/p12/p72(ASFV)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *Δasd*	IM/ 10^8	Swine	HI, CI, MI	[119]
S1, N (IBV)	*S.* Typhimurium SL7207	*ΔaroA*	O, IN/ 1×10^9, 5×10^9 or 1×10^{10}	Chicken	HI, MI	[71]
VP2/4/3 (IBVD)	*S.* Typhimurium	*Dam*, *Phop*	O/ 10^9, 10^8 or 10^7	Chicken	HI	[120]
prM-E (TMUV)	*S.* Typhimurium *SL7207 + adenovirus adjuvant with duck IL-2*	*ΔaroA*	O, IM/ 10^7, 10^{10}	Duck	HI, CI	[121]
N (TGEV)	*S.* Typhimurium SL7207	*ΔaroA*	IG/10^7, 10^8 or 10^9	Mice	HI, MI	[72]
swIFN-α (TGEV)	*S.* Typhimurium *χ*8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/ 10^9 or 10^{11}	Swine	qRT-PCR	[122]
S (TGEV, PEDV)	*S.* Typhimurium SL7207	*ΔaroA*	O/ 1.6×10^{11}	Swine	HI, CI, MI	[123]
N (TGEV)	*S.*	*ΔaroA*	O/10^{12}	Swine	HI, CI, MI	[124]

	Typhimurium SL7207					
M (TGEV)	*S.* Typhimurium *m* SL7207	*ΔaroA*	IG/10^9	Mice	HI, CI, MI	[125]
Glycoprotein 5, TLR-5 (PRRSV)	*S.* Typhimurium SL7207, FljB		IP/ 50 µg	Mice	HI	[126]
VP1 (FMDV)	*S.* Typhimurium KST0666	*Irradiated*	IP/ 1×10^4 to 3×10^8	Mice	HI, CI, MI, VN	[127]
p27 capsid (SIV)	*S.* Typhimurium PV4570	*ΔaroA*	IM, IG/ 10^{10}	Rhesus macaques	HI, CI, MI	[128]
Glycoprotein (RV), LTB (*E. coli*)	*S.* Typhimurium LH430	*phoP*, *phoQ*	O/5×10^{10}	Mice	HI, CI	[129]
siRNA expressing 3D, VP4 and 2B (FMDV)	*S.* Choleraesuis C500		IM/ Guinea pigs: 1.0×10^9; Swines: 5×10^9	Guinea Pigs, Swine	SPB-ELISA	[130]
Cap (PCV2)	*S.* Choleraesuis rSC0016	*ΔsopB*, *ΔasdA*	O/10^9	Mice	HI, CI, MI, qPCR, VN	[28]
HN (NDV)	*S.* Pullorum C79-13	*Δcrp*, *Δasd*	O/10^9	Chicken	HI, MI, Hemagglutination inhibition	[131])
S1 (IBV)	*S.* Gallinarum JOL2068, JOL2077	*Δlon*, *ΔcpxR*, *Δasd*	O/10^9	Chicken	HI, MI	[23]
M2e, CD154 (AIV H5N1)	*Salmonella enteritidis*	*ΔaroA*, *ΔhtrA*	O/ 10^6 to 10^8	Chicken	MI, Hemagglutination inhibition	[132]

IG, intragastric; IM, intramuscular; IN, intranasal; IP, intraperitoneal; O, oral; CFU, colony-forming unit; HI, humoral immunity; CI, cellular immunity; MI, mucosal immunity; VN, virus neutralization assays; AIV, avian influenza viruses; ASFV, african swine fever virus; DEV, duck enteritis virus; FMDV, foot-and-mouth disease virus; IBV, infectious bronchitis virus; IBVD, infectious bursal disease virus; LTB, heat-labile enterotoxin B; NDV, newcastle disease virus; PRRSV, porcine reproductive and respiratory syndrome; PrV, pseudorabies virus; p3D-NT56, siRNA directed against the polymerase gene 3D of FMDV; SIV, simian immunodeficiency virus, TGEV, porcine transmissible gastroenteritis virus; TLR-5, Toll-like receptor 5 TMUV, tembusu virus; VLP, virus-like particles;

Among the targets studied were the hemagglutinin gene (HA1) from one of the avian influenza viruses (AIV or HPAI) of the H5N1 subtype. Liljebjelke and colleagues [**108**] reported the use of *S.* Typhimurium expressing AIV HA1 as an oral vaccine carrier in birds with doses of 10^9 CFU. Animals were challenged with homologous A/whooper swan/Mongolia/3/2005-(CQ95) or heterologous A/Chicken/Queretaro/14588-19/95-(WM05) strains of the HPAI virus. Groups that received the recombinant vaccine demonstrated a statistically significant increase in survival compared with control groups (100%) for the low-dose homologous challenge with CQ95 and partial protection

against the low-dose challenge with WM05. Neither vaccine provided protection to chickens when challenged with high doses of either HPAI virus, although survival was better against the challenge with CQ95 (60%). The presence of antibodies that recognize the HA protein in serum and probe samples was assessed by hemagglutination inhibition (HI) assay and collected 2 weeks after vaccination. Furthermore, Jazayeri and collaborators [**110**] reported the use of glycoproteins HA, NA, and NP from AIV expressed in live-attenuated *S.* Typhimurium SV4089, administered orally to birds using the same dose as the previous study. Fluorescence in situ hybridization (FISH) was used for detection, and *Salmonella* was specifically identified using the genus-specific probe Sal3 from homogenized sections of the spleen, liver, and cecum of infected chickens, where the distinct fluorescent signal of rod-shaped bacteria could be detected. They achieved the successful elimination of *Salmonella* from the spleen and liver of infected birds, but it was still detectable in the cecum even 35 days after inoculation, demonstrating that live-attenuated *S.* Typhimurium provides an alternative in terms of in vitro stability of the transfected plasmid.

In a study using subtype H9N2 avian influenza virus, two attenuated *S. Typhimurium* strains were constructed: one with the O antigen of LPS intact (smooth strain) and the other without it (rough strain), as the removal of this antigen can increase the immunogenicity of surface proteins. This experiment, conducted in an avian model with recombinant *Salmonella* expressing H9N2 hemagglutinin (HA), evaluated whether *Salmonella* could act as a transporter to enhance immune responses to delivered antigens. Both the S-HA and R-HA strains elicited similar HA-specific immune responses, as indicated by serum IgG and HI titers, suggesting that the deletion of the O antigen does not impact the immunogenicity and delivery properties of the *Salmonella* system [**30**]. Additionally, Hajam et al. [**113**] used *S.* Gallinarum (SG) for expressing HA1, HA2, and/or the conserved ectodomain of matrix protein 2 (M2e) in the development of H9N2 vaccine strains, which were compared to a commercially available oil-adjuvanted inactivated H9N2 full-virus vaccine in a chicken model [**30**,**113**]. In search of a cheaper vaccine, and without the use of exogenous adjuvants, the experiment vaccinated chickens with a single dose, orally at a dose of 10^9 CFU, in groups who received either the individual H9N2 genes in SG, or as a mixture of these, while the control was vaccinated intramuscularly with the inactivated commercial H9N2 vaccine. An indirect ELISA of IgY with serum samples collected on days 14 and 28 post-vaccination showed that animals vaccinated with the RASV or a mixture of vaccine strains showed specific systemic responses to HA1 that were significantly higher ($p < 0.05$) than those of the control group. Cytokine gene expression revealed that IFN-γ increased by over four times ($p < 0.05$) in all groups inoculated with RASV constructs compared to the PBS control group. However, the commercial vaccine induced significantly greater responses for the HA1 and HA2 genes but was not as robust for M2 when compared to the RASV. Upon challenge, chickens immunized with both vaccines exhibited comparable lung inflammation and viral loads, although both were significantly lower than those in the group vaccinated with SG alone. However, immunization with the RASV managed to efficiently inhibit the infection and spread of H9N2.

Another use of orally administered vaccines has been to combat duck enteritis virus (DEV), an acute disease that affects ducks, geese, swans,

and other free-living aquatic birds, with high mortality. Yu and colleagues [**117**] used live-attenuated *S.* Typhimurium (SL7207) with *E. coli* LTB as an adjuvant, fused to the DEV UL24 gene in ducks. Birds were orally inoculated with SL7207 (pVAX-UL24) or SL7207 (pVAX-LTB-UL24) with 1×10^{10} CFU. Immunization of animals with the recombinant LTB vaccine showed superior protective efficacy (60–80%) against a lethal DEV challenge, compared to the limited survival rate (40%) of those immunized with the vaccine without the adjuvant. To support this study's results, Liu et al. [**118**] orally immunized ducks with *S.* Typhimurium S739 expressing DEV genes and adjuvants (LTB subunit and duck DuIL-2 gene). After a booster immunization, 90% of ducks immunized with recombinant *Salmonella* and the LTB adjuvant were protected during lethal challenge. IgY levels were slightly higher against the tUL24 protein in ducks vaccinated with UL24-LTB and UL24-DuIL-2 on days 10, 21, and 28 post-immunization ($p < 0.05$). Serum IgY and bile IgA levels in response to purified DEV were slightly lower than those for tUL24, but higher IgY titers against DEV were observed in ducks vaccinated with UL24 and tgB compared to those for tUL24 ($p < 0.05$). Among all groups, the highest bile IgA levels were seen in ducks receiving the attenuated *Salmonella*-DEV DNA recombinant vaccine [**118**]. Although the two adjuvants stimulated a high immune response in ducks, the vaccination with recombinant *Salmonella* and DuIL-2 was not capable of providing protection against homologous challenge.

Cytokines like IFN-α help modulate innate and adaptive immunity, providing a first line of defense against viral infections. However, their use in livestock is costly. Kim et al. [**122**] tested an oral vaccine using live-attenuated *S.* Typhimurium engineered to secrete porcine IFN-α (swIFN-α) to prevent clinical signs of transmissible gastroenteritis virus (TGEV), a significant economic threat in the swine industry. Administered at doses of 10^9 and 10^{11} CFU per pig, the vaccine effectively reduced the severity of TGEV-induced clinical signs [**122**]. To assess the virus's spread in piglets infected with TGEV, the quantity of TGEV in fecal samples collected from the infected piglets was measured. Virus shedding was detected one day after TGEV infection and reached its peak at four days post-infection. However, piglets that received the recombinant vaccine (at doses of 10^9 and 10^{11} UFC) exhibited reduced viral shedding at four days post-infection. Likewise, the amount of TGEV was lower in the intestinal tissues and mesenteric lymph nodes of piglets inoculated with the recombinant vaccine when compared to the control, helping to reduce the severity of clinical signs caused by TGEV infection.

In another study on TGEV, Zhang and collaborators [**123**] sought to evaluate an experimental vaccine delivered by live-attenuated *S.* Typhimurium expressing the structural protein of the virus, which is correlated with another virus that causes swine epidemic diarrhea (PEDV). These viruses are members of the Coronaviridae family, and both viruses can cause severe enteropathogenic diarrhea in pigs; therefore, the simultaneous induction of immune responses is promising for the food industry. Piglets were immunized orally with recombinant *Salmonella* at a dosage of 1.6×10^{11} CFU per piglet and then immunized with a booster of 2×10^{11} CFU. The RASV with two S proteins from TGEV and PEDV simultaneously stimulated immune responses against both viruses after oral immunization. Antibody levels against PEDV or TGEV in piglets immunized with the RASV of *S.* Typhimurium began to increase at 2 weeks, but the difference compared to controls was

not statistically significant until the sixth week. Serum IgG levels against PEDV and TGEV were significantly higher ($p < 0.01$) in piglets immunized with the recombinant vaccine than with PBS or empty vector from weeks 4 to 8. Significantly elevated levels of IgG and IgA antibodies against PEDV and TGEV were induced by the RASV in week 6, though these levels were slightly lower than those induced by the monogenic vaccine and empty vector. The results showed that T lymphocyte proliferation levels increased to a statistically significant level compared to the control group in weeks 4 to 6, being higher in piglets immunized with the RASV when compared to other vaccine groups, but no significant differences were observed ($p > 0.05$). The results also indicated that IFN-γ and IL-4 levels in piglets treated with the RASV were significantly higher ($p < 0.01$) than in control groups.

5.4. Recombinant Salmonella Expressing Parasite Antigens

The protective mechanisms required to combat parasites differ significantly from those required for other pathogens, and parasites can actively suppress the host's immune response [**133**]. This has made it challenging to identify an effective combination of antigens, adjuvants, and routes of administration for vaccination [**133**,**134**]. However, recent years have seen notable advances in the development of vaccines utilizing recombinant antigens from these parasites, although studies in this area remain limited [**134**].

In this review, we compiled 14 articles (**Table 3**) that investigate the use of attenuated strains of *Salmonella* engineered to express parasite antigens. These attenuated strains are particularly attractive as live vectors because they can elicit strong mucosal immunity, which is crucial for controlling certain parasites, such as *Trichinella spiralis*, in the intestinal mucosa [**134**].

Table 3. Parasitic antigens expressed in *Salmonella*.

Antigen (Organism)	*Salmonella* Strain or plasmid	Attenuation	Route/dose (CFU)	Model	Immunity	Reference
Ts87 (*Trichinella spiralis*)	*S.* Typhimurium SL7207	*ΔaroA*	O/10^8	Mice	HI, CI, MI	[135]
Ag30 (*T. spiralis*)	*S.* Typhimurium SL3261	*ΔaroA*	IN/10^9	Mice	HI, CI, MI	[31]
TsNd (*T. spiralis*)	*S.* Typhimurium SL1344	*Δcya*	O/10^8	Mice	HI, CI, MI	[136]
DNase II (*T. spiralis*)	*S.* Typhimurium SL1344	*Δcya*	O/10^8	Mice	HI, CI, MI	[137]
rTsSP1.2 (*T. spiralis*)	*S.* Typhimurium SL1344	*Δcya*	O/10^8	Mice	HI, CI, MI	[138]
FABP (*Echinococcus granulosus*)	*S.* Typhimurium SL3261	*ΔaroA*	IV/10^6, O/4 × 10^9	Mice	HI, CI, MI	[139]
FABP (*E. granulosus*)	*S.* Typhimurium LVR01	*ΔaroC*	O/ 5 × 10^{10}	Dog	HI, CI, MI	[48]
EmGAPDH (Echinococcus multilocularis)	*S.* Typhimurium		O/2 × 10^{10} or IP: 5 × 10^5	Mice	Western blotting	[140]

gp63 (*Leishmania major*)	*S.* Typhimurium BRD509	*ΔaroA*, *ΔaroD*	O/ 1 × 10^{10}	Mice	HI, CI	[141]
SAG, SAG2 (*Toxoplasma gondii*)	*S.* Typhimurium BRD509	*ΔaroA*, *ΔaroD*	IG/10^{9}	Mice	HI, CI	[73]
Tachyzoite and bradyzoite proteins (*T. gondii*)	*S.* Typhimurium BRD509	*ΔaroA*, *ΔaroD*	O, IN, IM/1 to 5 × 10^{9}	Mice	HI, CI, MI	[22]
Cp23, Cp40 (*Cryptosporidium parvum*)	*S.* Typhimurium SL3261 and LB5010	*ΔaroA*, *galE*	IG/5 × 10^{9}	Mice	HI, MI	[142]
Sj23LHD-GST (*Schistosoma japonicum*)	*S.* Typhimurium VNP20009	*purI*, *msbB*	O/10^{9}	Mice	HI, CI	[142]
EC-SOD (*Acanthocheilonema viteae*)	*S.* Typhimurium SL3261	*ΔaroA*	O/5 × 10^{8}	Jird	HI	[143]

IG, intragastric; IM, intramuscular; IN, intranasal; IP, intraperitoneal; IV, intravenous; O, oral; CFU, colony-forming unit; HI, humoral immunity; CI, cellular immunity; MI, mucosal; EmGAPDH, immunityglyceraldehyde-3-phosphate dehydrogenase.

Pompa-Mera and collaborators [**31**] used *S.* Typhimurium SL3261 and inserted a fusion glycoprotein from *T. spiralis* larvae. The vaccine was administered intranasally at a dose of 1× 10^{8} CFU to BALB/c mice. After challenge, mice immunized intranasally with recombinant *Salmonella* saw a reduction in the parasite load of adult *T. spiralis* by 61.83% on the eighth day post-infection, indicating a protective immune response. This immune response was characterized by the induction of antigen-specific IgG1 and IL-5 production. In another study using *T. spiralis*, the Ts87 gene was attenuated to strain *S.* Typhimurium SL7207, administered only orally to mice. They also reported a statistically significant 29.8% reduction in adult worm burden and a 34.2% reduction in larvae following *T. Spiralis* larvae challenge, compared with mice immunized with empty *Salmonella* or a PBS control. However, mice that received the recombinant *Salmonella* vaccine exhibited elevated levels of IgG2a and IgG1 subclass antibodies, with no significant difference ($p > 0.05$) between IgG2a and IgG1 levels, indicating a mixed Th1/Th2 immune response. Additionally, there was a notable increase ($p < 0.05$) in total intestinal IgA levels among mice immunized with the recombinant vaccine compared to those in the vector or PBS-only groups. Another important parasite for veterinary medicine is the cestode *Echinococcus granulosus* (EgDf1), which infects the intestines of dogs, in addition to having intermediate hosts such as herbivorous and omnivorous animals and, accidentally, humans. Chabalgoity and colleagues [**139**] produced a vaccine in which fatty-acid-binding proteins (FABPs) of EgDf1 fused with a C-terminal fragment of tetanus toxin (TetC) were expressed in *S.* Typhimurium LVR01. The inoculation was administered intravenously with a dose of 10^{6} CFU, as well as via an oral dose of 4 × 10^{9} CFU, in mice, eliciting an antibody response to EgDf1, the production of Th1-related antigen-specific cytokines, and significant levels of a Th2 cytokine protein in the spleen cells of orally immunized mice. Furthermore, sera from immune mice reacted strongly with fixed sections of the larval stage of the worm. Another study by the group used *S.* Typhimurium LVR01 expressing EgDf1 FABP in dogs, which were orally vaccinated at a dose of 5 ×

10^{10} CFU in 2 mL of PBS or given PBS alone. The dogs presented IgG antibody responses against EgDf1 when immunized with LVR01 (pTECH ± EgDf1). All animals developed high titers of IgG antibodies against LPS in serum by week 4 after a single dose of the recombinant vaccine [**48**].

Cong et al. [**73**] tested a vaccine using live-attenuated *S.* Typhimurium as a vector for the recombinant plasmid pSAG1-2/CTA2/B, which encodes *Toxoplasma gondii* antigens SAG1 and SAG2 linked to cholera toxin subunits (CTA2/B). Orally administered in mice, this vaccine induced anti-*T. gondii* IgG antibodies, with increased IgG levels in mice that received CTA2/B as a genetic adjuvant compared to controls ($p = 0.003$, $p = 0.004$). Although both groups generated anti-*T. gondii* antibodies, mice vaccinated with CTA2/B showed a predominant Th1 response, while those without it exhibited a Th2 response. Upon challenge with virulent *T. gondii*, mice vaccinated with CTA2/B had longer survival times and a 40% survival rate ($p = 0.003$) [**73**].

In another study, Cong et al. [**22**] developed a live-attenuated *S.* Typhimurium vaccine encoding *T. gondii* epitopes (SAG1, GRA1, ROP2, GRA4, SAG2C, SAG2X) linked to CTA2/B, which was delivered to BALB/c mice via oral, nasal, or intramuscular routes. Mice immunized orally and nasally showed higher anti-*T. gondii* antibody levels than those vaccinated intramuscularly ($p < 0.05$). Flow cytometry revealed CD4+ and CD8+ T-cell activation, with intramuscular and intranasal immunizations inducing 28.54% and 30.01% activation, respectively. IFN-γ and IL-2 levels were significantly higher in orally and nasally vaccinated groups than in the control ($p = 0.02$), while IL-4 and IL-5 remained low across groups. Antigen-specific lymphocyte proliferation was also higher in the oral and nasal groups. After a lethal *T. gondii* challenge, survival rates were 20% for intramuscular, 40% for intranasal, and 60% for oral immunization. These results highlight the potential of oral and nasal routes for delivering live-attenuated *Salmonella* vaccines in inducing stronger immune responses and improved protection against *T. gondii* [**22**].

Benitez, McNair, and Mead [**74**] utilized strains of live-attenuated *S.* Typhimurium expressing Cp23 and Cp40 from *Cryptosporidium parvum*, which are recognized as surface immunodominant antigens, as they are recognized by serum antibodies from humans and various animal species. In the study, mice received an oral immunization of 5×10^9 CFU per mouse and an intragastrical immunization of 0.2 mL of PBS for each vaccine, which included a vector with an empty plasmid, the RASV vaccine expressing the Cp23 gene, and another for the CP40 gene. Two booster doses, consisting of 100 μg of RASV, were injected subcutaneously on days 0 and 14, followed by oral immunization against *Salmonella*. The production of IgG and IgG1 subclasses was observed in vaccinated mice after 7 weeks of immunization. The specific serum levels of anti-Cp23 and anti-Cp40 IgG were significantly increased in mice immunized with the RASV vaccine compared to mice immunized with the control vector. IgA titers were detected in mice immunized with the RASV expressing Cp23 but not in animals immunized with the Cp40 construct ($p > 0.05$). Only an IgG1 antibody response was obtained, with no IgG2a response, suggesting a Th2-type response was elicited.

Chen et al. [**142**] studied a live-attenuated *S.* Typhimurium vaccine using active promoters (nirB, pagC, or pMohly) to express the *Schistosoma japonicum* antigen Sj23LHD-GST via the *Salmonella* type III secretion

system or α-hemolysin. Mice were orally immunized with 0.2 mL PBS containing 10^9 CFUs of either recombinant *S.* Typhimurium or an empty vector, with a PBS-only group as a control. After three doses, mice vaccinated with the nirB promoter showed a moderate IgG response and the highest IgG2a ratio, indicating a strong Th1-type response. CD44 expression in splenocytes was significantly elevated in mice that received the nirB-driven antigen (25 ± 2%) compared to other groups ($p < 0.01$). Following *S. japonicum* challenge, mice immunized with *S.* Typhimurium containing nirB, pagC, or pMohly1 promoters showed egg burden reductions of 57.71%, 30.07%, and 40.46%, respectively. The nirB-driven antigen delivered by type III secretion reduced parasite burden by 51.35% and egg burden by 62.59%, showing promising in vivo protective efficacy. These findings highlight the protective efficacy of antigens delivered by the *Salmonella* type III secretion system using the nirB promoter [**142**].

6. Conclusion and Future Directions

Developing attenuated vaccines requires balancing safety and immunogenicity. Although *Salmonella* shows promise as a vaccine vector, many studies have not achieved significant protective efficacy. Over-attenuation can reduce immunogenicity and tissue colonization, while insufficient attenuation may pose biosafety risks. While safety is often evaluated based on the absence of adverse reactions and strain stability, few studies assess environmental release or potential transmission through vertical or horizontal pathways.

The development and commercialization of RASVs represents a significant advancement in veterinary medicine. These vaccines offer a safe and effective means of controlling infectious diseases in livestock, reducing the reliance on antibiotics and mitigating the risks associated with antibiotic resistance. The ability of RASVs to deliver antigens from a wide range of pathogens makes them versatile tools for disease prevention.

Looking forward, future research should focus on optimizing vaccine formulations and delivery methods, particularly for ruminants and other species where oral administration remains a challenge. Additionally, there is potential to expand the use of RASVs beyond veterinary applications, with possible implications for human medicine, particularly in the context of zoonotic diseases.

The studies reviewed here highlight the promise of RASVs as part of an integrated approach to managing infectious diseases in animal populations. Continued investment in research and development will be essential to fully realize the potential of these vaccines and to address the challenges that remain.

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2.3 Capítulo 3

Attenuated *Salmonella* Typhimurium expressing chimeric antigen from *Leptospira* as a recombinant vaccine candidate against leptospirosis

- Artigo experimental -

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Artigo submetido para o periódico *Research in Veterinary Science.*

Attenuated *Salmonella* Typhimurium expressing chimeric antigen from *Leptospira* as a recombinant vaccine candidate against leptospirosis

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Abstract: Leptospirosis is a significant zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. Attenuated *Salmonella* strains represent a promising vector for the development of safe and effective vaccines capable of eliciting a specific immune response. **Background/Objectives:** A chimeric *Leptospira* antigen (rQ1), previously described and based on the gene sequences of *lipL32*, *lemA*, and *ligAni*, was cloned into the pTECH2 vector for antigen expression in *Salmonella Typhimurium* LVR01. **Methods:** Hamsters were vaccinated orally (OR) and intramuscularly (IM) with 2 × 10^7 CFU of *S. Typhimurium* LVR01 carrying pTECH2/rQ1, pTECH2 alone, or PBS as a control. Vaccinations were administered twice, with a 14-day interval. Following OR and IM administration, serum samples were collected, and IgG antibodies against rQ1 were measured using indirect ELISA. **Results:** The results showed that after the second immunization, IgG antibody levels in hamsters immunized

IM with pTECH2/rQ1 were significantly higher ($P < 0.0001$) than in the control groups (PBS and pTECH2). Isotype analyses revealed significant levels of IgG2 ($P < 0.05$) in the group vaccinated IM with pTECH2/rQ1. However, the attenuated *S. Typhimurium* vaccine did not confer protective efficacy against homologous challenge in the hamsters immunized with pTECH2/rQ1. **Conclusions:** Further studies are necessary to explore this novel recombinant attenuated *S. Typhimurium* LVR01 vaccine and to further characterize the immune mechanisms involved.

Keywords: Leptospirosis; *Salmonella* Typhimurium; attenuated recombinant vaccine.

1. Introduction

Leptospirosis is a significant zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira*. These bacteria multiply in the renal tubules of chronically infected mammals and are excreted into the environment through urine (Abe et al., 2020; Zarantonelli et al., 2018). Transmission can occur either directly or indirectly through percutaneous exposure to contaminated soil and water, with rodents serving as the primary reservoir hosts for *Leptospira* spp. (Adler and de la Peña Moctezuma, 2010). In humans, the infection may manifest as an asymptomatic febrile illness during its initial phase, which can easily be mistaken for other diseases such as malaria and dengue, making clinical diagnosis both challenging and frequently overlooked (Chacko et al., 2021).

Classic immunization strategies for disease control rely on whole-cell inactivated *Leptospira* vaccines, which provide serogroup-specific protection (Guglielmini et al., 2019). However, these vaccines have also been associated with side effects and short-term immune responses (Wunder et al., 2021). These limitations restrict their use primarily to animals, and as a result, only a few endemic countries have approved vaccination programs for high-risk human populations (Verma et al., 2013). The development of a recombinant vaccine appears to

be the most promising strategy for achieving a more effective and broadly applicable solution to combat leptospirosis.

Several recombinant vaccine candidates have been identified, focusing primarily on well-conserved and immunogenic antigens with potential for cross-protection (de Oliveira et al., 2023; Grassmann et al., 2017). LipL32, the most abundant and immunogenic lipoprotein in pathogenic *Leptospira* spp., has been extensively studied (Chirathaworn et al., 2016). Leptospiral immunoglobulin-like proteins A and B (LigA and LigB) also play key roles in host interactions, aiding in immune evasion (Grassmann et al., 2017; Haake, 2000). Another promising antigen is LemA, a hypothetical lipoprotein identified through reverse vaccinology (RV) (Hartwig et al., 2013; Oliveira et al., 2018).

The immunoprotective potential of these antigens has been evaluated across various expression platforms and vaccine formulations (da Cunha et al., 2019; Hartwig et al., 2013; Oliveira et al., 2018). Although subunit vaccines are the most explored strategy in leptospirosis recombinant vaccine development, their high purity and absence of intrinsic pathogen-associated molecular patterns (PAMPs) result in lower immunogenicity (Oliveira et al., 2018). While some studies have shown promising protection using rLigBrep or chimeric constructs, a critical aspect of recombinant vaccine development is ensuring cost-effective production (Conrad et al., 2017; Dorneles et al., 2020; Oliveira et al., 2019). Therefore, selecting the most cost-effective expression and delivery platforms remains a significant challenge.

Salmonella has emerged as a versatile platform for producing recombinant antigens (Clark-Curtiss and Curtiss, 2018). Its low production costs, combined with advancements in strain attenuation through deletions and mutations, have enabled its use in vaccines against various viruses, parasites, and bacteria (Galen and Curtiss, 2014; Wang et al., 2013). One of the key advantages of engineered *Salmonella* strains over other vaccine delivery systems is their ability to invade and colonize deep lymphoid tissues following mucosal delivery (Malik-Kale

et al., 2011; Mastroeni et al., 2001; Roland and Brenneman, 2013). This property allows them to stimulate both mucosal and systemic immune responses, promoting robust humoral and cellular immunity (Gayet et al., 2017; Lou et al., 2019).

Given the limited studies using *Salmonella* as a vaccine platform for leptospiral antigens, this work developed a recombinant attenuated *Salmonella Typhimurium* vaccine expressing a chimeric protein composed of the LipL32, LemA, and LigA antigens (rQ1). The aim was to explore its potential to elicit an immune response and provide protection against *Leptospira* in the context of a homologous challenge.

2. Materials and Methods

2.1. Ethics statement

All animal experiments were conducted in accordance with protocols approved by the Ethics Committee on Animal Experimentation (CEEA N° 003391/2023-77) at the Federal University of Pelotas (UFPel). The CEEA at UFPel is accredited by the Brazilian National Council for Animal Experimentation Control (CONCEA).

2.2. Strains and growing conditions

Salmonella enterica serovar Typhimurium LVR01, an attenuated strain constructed by introducing a null deletion into the *aro*C gene of the parental canine isolate *S.* Typhimurium P228067, was used in this study (Chabalgoity et al., 2000). *S.* Typhimurium LVR01 was grown in Luria-Bertani (LB) medium, either broth or agar, supplemented with 100 μg/mL of ampicillin at 37 °C. *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 was maintained at 30 ºC in Ellinghausen McCullough-Johnson-Harris (EMJH) (Difco, BD, SP, Brazil) liquid medium, using *Leptospira* enrichment EMJH (Difco, BD, SP, Brazil) for supplementation.

2.3. Production of the recombinant Q1 protein

The rQ1 chimera was expressed and purified as previously described by our research group (Oliveira et al., 2019). This protein was produced for use as antigen for serological analysis.

2.4. Construction of the recombinant attenuated Salmonella and protein expression analysis

A previously recombinant vector, pAE/rQ1 (Oliveira et al., 2019) was used as a DNA template to amplify the rQ1 gene by PCR with the following primers: F: 5'-GCCTCTAGAGATGCTAGCGGTGGTC-3' and R: 5'-GTCAAGCTTTTACCTAGGCGATCCTC-3'. The PCR product was digested with the restriction enzymes *Xba*I and *Hin*dIII (Invitrogen, Carlsbad, CA, USA) and cloned into the *Salmonella* expression vector pTECH2, which had been previously digested with *Xba*I e *Hin*dIII (Invitrogen), generating the pTECH2/rQ1 plasmid. The pTECH2 vector allows the expression of a foreign antigen as a C-terminal fusion to the non-toxic fragment C of tetanus toxin (TetC) (Anjam Khan et al., 1994). Cloning was confirmed by sequencing.

The recombinant pTECH2/rQ1 was then transformed into *S.* Typhimurium LVR01 (Chabalgoity et al., 2000) by electroporation Transformants were selected on LB agar plates containing ampicillin. The expression of the recombinant protein by *S.* Typhimurium LVR01 was assessed by Western blotting using anti-rQ1 and anti-TetC sera. Briefly, the *S.* Typhimurium LVR01:pTECH2/rQ1 was cultured overnight on LB agar containing 100 μg/mL of ampicillin and incubated at 37 °C. Colonies were then inoculated into 10 mL of LB with 50 μg/mL of ampicillin and incubated at 37 °C for 14-16 h. The culture was subsequently transferred to larger volumes of LB broth with ampicillin (100 μg/mL) and grown at 37 °C with continuous shaking for 4 h. Cells were collected by centrifugation (4,000 × g, 20 min, 4 ºC) and resuspended in phosphate-buffered saline (PBS) (pH 7.4), 0.5 M EDTA, and 100 mM Phenylmethylsulfonyl fluoride (PMSF) (Sigma–Aldrich, MO, USA), and subjected to

sonication with six 30-s pulses on ice, followed by centrifugation. Supernatants were separated on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-rad, Hercules, CA, USA). Membranes were incubated with rat hyperimmune anti-rQ1 sera at 1:5,000 dilution, followed by anti-rat HRP-conjugated immunoglobulin G (IgG) (Sigma Aldrich, MO, USA) diluted at 1:10,000. Additionally, membranes were incubated with rabbit anti-TetC polyclonal sera (Rockland Immunochemicals, Pottstown, PA, USA) at 1:2,000 dilution followed by anti-rabbit HRP-conjugated IgG (Sigma–Aldrich, MO, USA) diluted 1:5,000. Immunoblots were developed using Pierce ECL Western Blotting Substrate (Thermo-Fisher, IL, USA).

2.5. Formulation of the recombinant attenuated Salmonella vaccine

Salmonella Typhimurium LVR01 carrying pTECH2/rQ1 and pTECH2 (negative control) were cultured overnight on LB agar plates containing ampicillin at 37°C. The colonies were then inoculated into 10 mL of LB with 100 μg/mL ampicillin and incubated at 37°C for 14–16 hours. The next day, the overnight culture was diluted 1:20 into 10 mL of LB broth with ampicillin (100 μg/mL) and grown with shaking at 37°C for approximately 4 hours, until the optical density (OD600) reached 1.3 to 1.5. The cultures were then diluted 1:50. The final concentration of these cultures was determined by Colony Forming Units (CFU). Briefly, serial dilutions (log10) of the bacterial suspension were spread onto LB agar plates and incubated at 37°C for 16 hours. Colonies were counted, and CFU was calculated. The cultures were diluted in PBS, and the vaccine doses were adjusted to 2×10^7 CFU.

2.6. Vaccination and challenge of hamsters

Male and female Golden Syrian hamsters, aged between 4 and 6 weeks, were used and divided into groups of 10 animals each. Each animal was immunized with 2×10^7 CFU of *S.* Typhimurium strains or with PBS, with a 14-day interval between doses (the first dose on day 0 and the second dose on day 14). The animals were divided into six experimental groups, with

three groups receiving the doses orally (OR) and the other three groups receiving them intramuscularly (IM). Among these groups, two received the recombinant attenuated Salmonella (pTECH2/rQ1) either OR or IM. Two groups were vaccinated only with recombinant attenuated Salmonella carrying the pTECH2 plasmid (pTECH2), and two negative control groups received PBS. After each vaccine administration, hamsters were closely monitored for up to 72 hours to assess vaccine safety. Blood samples were collected by puncture of the gingival vein under isoflurane anesthesia on day 0 (before vaccination) and on day 28, after the first immunization. Blood samples were centrifuged at 3,000 × g for 10 minutes at 4°C. Sera were removed and stored at −20°C until use.

Challenge was performed intraperitoneally 28 days after the first immunization, using a dose of 10 × the 50% Endpoint Dose (ED50) of *L. interrogans* serovar Copenhageni strain L1-130. All animals were observed and weighed daily for 28 days post-challenge to monitor and record any clinical signs of leptospirosis. Animals meeting any of the endpoint criteria were euthanized by CO_2. Endpoints included: >10% weight loss, prostration, bristling, apathy, and lack of appetite.

2.7. Evaluation of the humoral immune response

Antibody responses against rQ1 were evaluated in sera by indirect ELISA. For this, 96-well microtiter plates (Corning, Costar) were coated with 800 ng of rQ1 per well, diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6), and incubated for 16–18 hours at 4°C. Plates were then blocked with 5% skimmed milk powder diluted in PBS-T for 1 hour at 37°C. Sera from immunized animals were diluted 1:25 in PBS-T with 2.5% skimmed milk powder and incubated for 1 hour at 37°C. After incubation, peroxidase-conjugated secondary antibodies specific for hamster immunoglobulins IgG, IgG1, IgG2/G3, or IgG3 (Sigma Aldrich, MO, USA) at a dilution of 1:5,000 were added and incubated for 1 hour at 37°C. At the end of each step, the plates were washed three times with PBS-T. The reactions were developed using o-

phenylenediamine dihydrochloride (Sigma Aldrich, MO, USA) and hydrogen peroxide, and the reaction was stopped by the addition of 25 µL of 4 N H_2SO_4. Absorbance was measured using a plate reader at a wavelength of 492 nm (plate reader DR-200BS-NM-BI, Kasuaki).

2.8. Statistical analysis

Protection against lethal leptospirosis and survival rates were evaluated using a two-tailed Fisher's exact test and Log-rank test (GraphPad Prism 8.0.1), respectively. For the analysis of the humoral immune response, two-way analysis of variance (ANOVA), followed by Tukey multiple comparisons, was used to determine the differences between the experimental groups. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using GraphPad Prism 8.0.1 and Statistix 8 software packages.

3. Results

3.1. Evaluation of the humoral immune response

The rQ1 gene was successfully cloned into the *Salmonella* expression vector, pTECH2, generating pTECH2/rQ1, as shown in Figure 1.

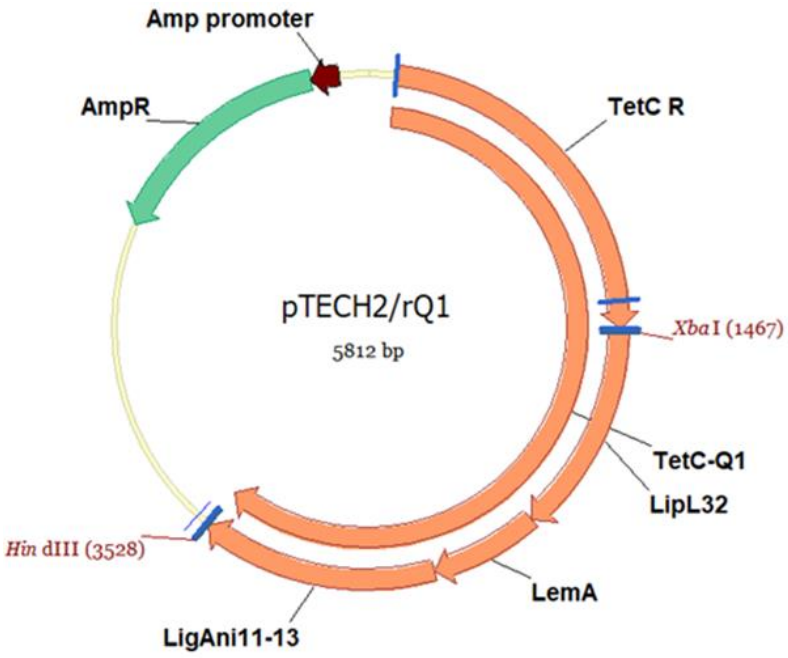

Figure 1. Diagrammatic representation of the pTECH2 plasmid for TetC-rQ1 expression in *S.* Typhimurium LVR01.

The pTECH2/rQ1 plasmid was transformed into *S.* Typhimurium LVR01, resulting in the recombinant *S.* Typhimurium LVR01 (pTECH2/rQ1). Western blot analysis of bacterial lysates, using anti-rQ1 and anti-TetC sera, demonstrated that the TetC-rQ1 fusion protein was expressed in recombinant *S.* Typhimurium LVR01 at approximately 127 kDa, which corresponds to the expected molecular weight (Figure 2).

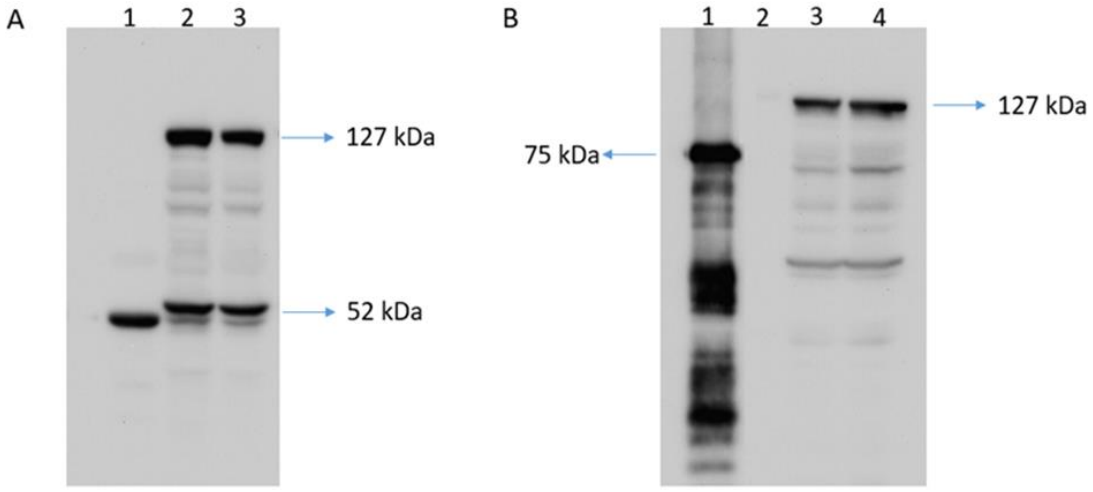

Figure 2. Expression of the TetC-rQ1 fusion in *S.* Typhimurium LVR01 was determined by Western blotting using anti-TetC (**A**) and anti-rQ1 sera (**B**). (**A**) Lane 1: TetC. Lanes 2 to 3: whole-cell lysates from two different colonies of *S.* Typhimurium LVR01 transformed with pTECH2/rQ1. (**B**) Lane 1: rQ1. Lane 2: whole-cell lysates from *S.* Typhimurium LVR01 transformed with pTECH2 alone. Lanes 3 to 4: whole-cell lysates from two different colonies of *S.* Typhimurium LVR01 transformed with pTECH2/rQ1.

3.2. Determination of hamster-specific humoral immune response and IgG isotyping after oral and intramuscular immunization with recombinant attenuated *Salmonella* LVR01

Golden Syrian hamsters were immunized orally (OR) and intramuscularly (IM) with PBS or approximately 2×10^7 CFU of each vaccine strain, with two doses administered at 14-day intervals. The antibody response induced by vaccination was evaluated by indirect ELISA. Total IgG antibody levels were detected on day 28 only in hamsters inoculated IM with the recombinant attenuated *Salmonella* vaccine. After the second immunization on day 28, the antibody levels of hamsters immunized IM with pTECH2/rQ1 were significantly higher ($P < 0.0001$) compared to the control groups (PBS and pTECH2). In contrast, the antibody levels induced in the group immunized OR with pTECH2/rQ1 were not statistically significant on day 28 ($P < 0.05$) compared to the control groups (pTECH2 and PBS) (Figure 3). To evaluate the safety of the recombinant *S.* Typhimurium LVR01 vaccine, the animals were assessed 72 hours after immunization, and no local inflammatory reactions, such as fever and edema on site of vaccine injection or gastrointestinal disorders, were observed.

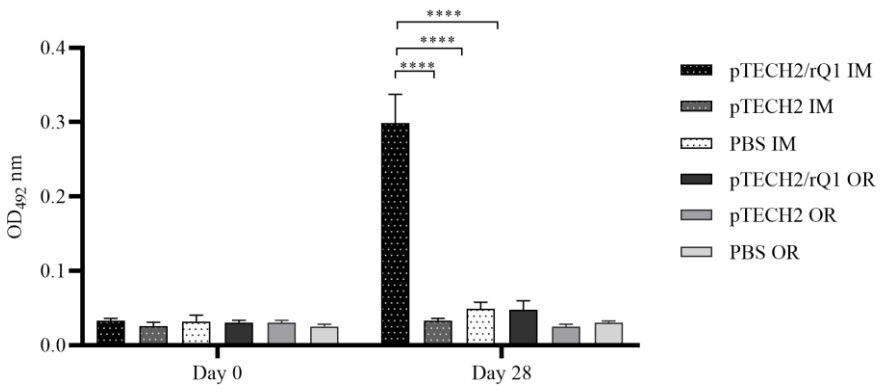

Figure 3. IgG antibody response in hamsters immunized with recombinant attenuated *Salmonella* LVR01 evaluated using indirect ELISA. ELISAs were performed to determine antibody levels in hamsters vaccinated with pTECH2/Q1 intramuscularly (IM) or pTECH2/rQ1 orally (OR). Sera from hamsters immunized with PBS and pTECH2 (controls) and pTECH2r/Q1 (both routes) collected on day 0 (pre-immune), and on day 28 (pre-challenge) were evaluated at 1:25 dilution, with anti-hamster IgG, in triplicate, as a secondary antibody.

The results are presented as mean optical density values (OD_{492} nm) ± standard error (bars). Significant differences were determined by two-way ANOVA, followed by Tukey multiple comparisons. The asterisks indicate a significant difference ($P < 0.0001$) to other groups.

Furthermore, the immune response to the rQ1-specific antigen was examined by measuring the levels of IgG isotypes, specifically IgG1, IgG2/3, and IgG3, using serum samples collected on day 28 (pre-challenge). In the group vaccinated intramuscularly with pTECH2/rQ1, IgG2/3 levels against the rQ1 antigen were significantly elevated ($P < 0.0001$) compared to the control groups (Figure 4). No significant differences were observed in the levels of IgG1 and IgG3 isotypes when compared to the control sera.

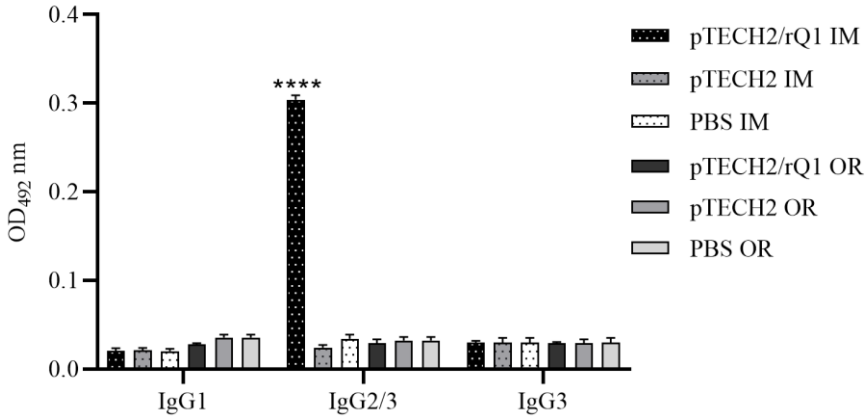

Figure 4. IgG isotype subclasses IgG1, IgG2/3, and IgG3 induced by immunization with recombinant attenuated *Salmonella* LVR01 were characterized using indirect ELISAs to determine antibody levels in hamsters immunized with pTECH2/rQ1 intramuscularly (IM) and orally (OR). Sera from hamsters immunized with PBS and pTECH2 (controls) and pTECH2/rQ1 (both routes), collected on day 28 (pre-challenge), were evaluated at a 1:25 dilution, with conjugates for anti-hamster IgG1, IgG2/3, and IgG3 in triplicate. The results are presented as mean optical density values (OD492 nm) ± standard error (bars). Significant

differences were determined by two-way ANOVA, followed by Tukey multiple comparisons. The asterisks indicate a significant difference ($P < 0.0001$) from other groups.

3.3. Immunoprotection efficacy assay

To evaluate whether recombinant *S.* Typhimurium could induce immunoprotection against leptospirosis, all groups were challenged on day 28 post-first immunization with a virulent *L. interrogans* serovar Copenhageni L1-130 strain. None of the animals survived the challenge, and the results were not statistically significant in the Log-rank test compared to the control groups. The animals reached the euthanasia endpoints between days 7 and 13 after the challenge in both the vaccinated and control groups (Figure 5).

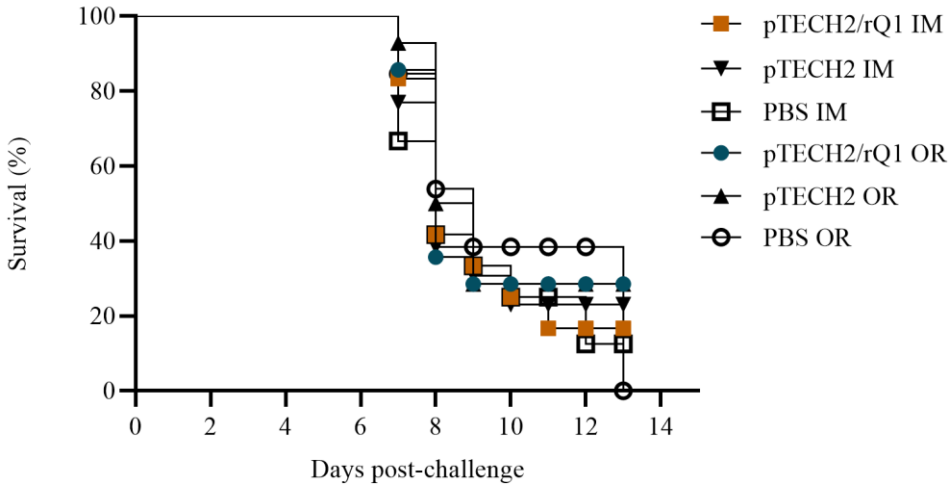

Figure 5. Survival data from vaccinated hamsters after challenge with a lethal dose of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130. Hamsters were immunized orally and intramuscularly with 2×10^7 CFU of recombinant attenuated *Salmonella*. None of the groups (n = 10) survived the challenge. Animals reached endpoint criteria for euthanasia between days 7 and 13 post-infection.

4. Discussion

Engineered *Salmonella* strains have emerged as a promising tool for expressing and delivering recombinant antigens in the ongoing development of vaccines for several pathogens. *Salmonella* vectors are cost-effective, genetically stable, and safe, and they can deliver antigens directly to antigen-presenting cells (Roland and Brenneman, 2013; Wang et al., 2013). Additionally, the characteristics of this expression system allow for persistence in the host, providing prolonged exposure and, consequently, a longer-lasting immune response (Clark-Curtiss and Curtiss, 2018).

In light of these advantages and the existing need for a new vaccine for leptospirosis, we successfully cloned and expressed rQ1, a validated chimeric *Leptospira* antigen (LipL32-LemA-LigA), fused to tetanus toxin fragment C (TetC) in *S.* Typhimurium LVR01 as a recombinant live attenuated vaccine candidate against leptospirosis. Recombinant attenuated *Salmonella* vaccines based on heterologous antigen fusion with TetC have been constructed and tested, demonstrating the ability to stimulate protection in mice against salmonellosis, tetanus, schistosomiasis, and herpes simplex virus infection (Chabalgoity et al., 1997, 1996). Here, oral and intramuscular immunizations were performed in hamsters, followed by a homologous challenge with a lethal dose of *L. interrogans* serovar Copenhageni and characterization of the induced humoral immune response. Hamsters vaccinated with the *Salmonella* formulations showed no side effects, indicating that the live attenuated *S.* Typhimurium vaccine is safe and can be administered both orally and intramuscularly without adverse effects.

Classical leptospirosis vaccines (bacterins) achieve their protective efficacy by inducing opsonizing antibodies against LPS and are specific to the serovars included in the formulation (Verma et al., 2013). However, the immune response modulation involved in the protection conferred by recombinant antigens against leptospirosis remains poorly understood. In this study, the systemic humoral immune response induced by vaccination was evaluated using the

rQ1 chimera as the antigen in an ELISA. Intramuscular vaccination induced a specific immune response with IgG antibodies, while oral administration did not significantly generate a response against the rQ1 antigen. Although oral *Salmonella* vaccines have shown effectiveness in previous studies (Chabalgoity et al., 2000; Fagan et al., 2001; Liu et al., 2023; Samakchan et al., 2021), parenteral vaccination (intraperitoneal, intramuscular, and subcutaneous) has also proven promising in rodents, goats, and dogs (Kim et al., 2018; Lalsiamthara and Lee, 2018; Leya et al., 2018; Moustafa et al., 2015; Won and John Hwa, 2017).

The use of *Salmonella* as a vector vaccine based on *S.* Typhimurium LVR01 expressing heterologous antigens, such as a fatty acid binding protein (FABP) from *Echinococcus granulosus*, a mouse prion protein cellular (PrPC), and a cervid PrP, induces a robust humoral response in dogs, mice, and deer, respectively (Chabalgoity et al., 2000; Goñi et al., 2015, 2005). However, some attenuated strains with auxotrophic mutations did not confer protection against virulent challenges (O'callaghan et al., 1988). Yu et al. (2012) used live attenuated *S.* Typhimurium expressing the *E. coli* heat-labile enterotoxin B (LTB) as an adjuvant to enhance immune responses against duck enteritis virus through oral immunization. The highest dose of adjuvant provided 60–80% protection with strong mucosal and systemic immune responses, demonstrating the importance of mucosal adjuvants in immunization. Du and Wang (2005) compared oral and intramuscular vaccination in chickens with an attenuated *S.* Typhimurium expressing *Eimeria tenella* antigens. Higher oral doses (10^8 and 10^9 CFU) and intramuscular vaccination achieved 55.0%, 57.5%, and 53.44% protection against *E. tenella*, respectively, and elicited stronger specific humoral responses. In contrast, Zhang et al. (2015) found that subcutaneous administration of a recombinant *Salmonella* vaccine against *Pseudomonas aeruginosa* provided better survival (77.78%) and stronger immune responses (serum IgG, intestinal IgA) than oral vaccination (41.18% survival). These studies demonstrate that

attenuated *S.* Typhimurium vaccines can induce strong immune responses and provide partial protection, with administration routes affecting efficacy.

The rQ1 chimeric antigen was proven previously to be effective in subunit and BCG-vectored vaccines against leptospirosis (Oliveira et al., 2019), with rBCG providing sterilizing immunity through kidney clearance. Given the manufacturing and immunogenicity limitations associated with BCG as a vaccine vector (Walker and Bacon, 2023), we explored *Salmonella* as a promising alternative for producing a recombinant live vaccine vector against leptospirosis. Samakchan et al. (2021) reported that *S.* Typhimurium SL3261 expressing the LipL32 antigen effectively stimulated mucosal and systemic immune responses against pathogenic leptospires in rats. Despite these promising characteristics and the potential of *Salmonella* vectors, our study found that vaccine formulations using recombinant attenuated *S.* Typhimurium LVR01 expressing the rQ1 antigen did not confer protection against a homologous challenge with a virulent *Leptospira* strain, despite eliciting a systemic humoral immune response.

Our results underscore the importance of expression platforms and delivery systems in vaccine development. Previous work by our group (Bunde et al., 2023) showed that rBCG-Q1 induced a Th1-type response with high IFN-γ levels, linked to its protective efficacy and sterilizing immunity, while no significant levels of specific IgG antibodies to rQ1 were detected. In contrast, *S.* Typhimurium expressing pTECH2/rQ1 primarily elicited an IgG2 response, indicating a Th1-type modulation, but did not provide protective efficacy. Our oral vaccination did not produce detectable IgG levels, possibly due to a predominant IgA response typical of oral *Salmonella* vaccines (Diard et al., 2021). A limitation of our study was the inability to analyze mucosal responses due to a lack of suitable reagents. Although hamsters are considered a better model for susceptibility to leptospirosis, there is still a lack of commercially available reagents to study the immune response in this model (Teixeira et al., 2019), such as anti-IgA antibodies.

5. Conclusions

In summary, while the recombinant *Salmonella* vaccine administered intramuscularly induced a humoral immune response characterized by IgG and IgG2 isotypes, it did not confer protection against a virulent *Leptospira* strain. Future research should assess the stability of the vaccine, dose effect, and further investigate the immune mechanisms to understand the reasons for the lack of protection.

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Considerações Finais

- O protótipo vacinal à base de bacterina recombinante de *E. coli* expressando o antígeno rQ1 não conferiu imunidade protetora significativa frente ao desafio letal com a cepa de *Leptospira interrogans*.
- Apesar da formulação de subunidade ter demonstrado respostas imunológicas moderadas, sua eficácia ainda é limitada, ressaltando a necessidade de otimizações adicionais para o desenvolvimento de uma vacina eficaz contra a leptospirose.
- Diversos ensaios clínicos e estudos demonstraram a eficácia e a segurança do uso de *Salmonella* viva atenuada recombinante como sistema de entrega de antígenos heterólogos, além de destacar sua relação custo-benefício.
- Espera-se que a pesquisa abordada sobre *Salmonella* viva atenuada contribua para as estratégias atuais e para o desenvolvimento de vacinas contra diversas bactérias, vírus e parasitas.
- Quanto a construção do protótipo de vacina recombinante de *Salmonella* expressando um antígeno quimérico (rQ1), a vacina administrada por via intramuscular, induziu uma resposta imune humoral, caracterizada pela produção dos isótipos IgG e IgG2.
- No entanto, nenhuma das formulações vacinais utilizando *S.* Typhimurium LVR01 atenuado e recombinante expressando o antígeno rQ1 conferiu proteção contra o desafio homólogo com uma cepa virulenta de *L. interrogans*.
- Portanto, são necessários mais estudos para aprimorar a indução da resposta imune contra a leptospirose, de modo a garantir proteção, além de investigar mais profundamente os mecanismos imunológicos envolvidos.

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Anexos

Anexo A – Parecer de aprovação do CEEA, experimento do capítulo 1: Vacinas bacterinas de *Escherichia coli* expressando proteína quimérica.

PARECER Nº **17/2022/CEUA/REITORIA**
PROCESSO Nº 23110.036707/2021-45

Certificado

Certificamos que a proposta intitulada **"Avaliação do potencial imunoprotetor de uma quimera composta por proteínas de membrana externa de *Leptospira* em diferentes formulações vacinais"**, registrada com o n° **23110.036707/2021-45,** sob a responsabilidade de **Odir Antônio Dellagostin** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética nu Uso de Animais da Universidade Federal de Pelotas, em reunião de 14 de fevereiro de 2022.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	Início = 01/03/2022 Término = 01/03/2024
Espécie/linhagem/raça	*Mesocricetus auratus* / Golden Syriam
N° de animais	219 (109 machos e 110 fêmeas)
Idade	4-6 semanas
Sexo	Machos e fêmeas
Origem	Biotério Central - UFPel

Código para cadastro nº **CEUA 036707/2021-45**

Priscila Marques Moura de Leon

Coordenadora da CEUA

Documento assinado eletronicamente por **PRISCILA MARQUES MOURA DE LEON, Professor do Magistério Superior/Adjunto**, em 09/03/2022, às 11:39, conforme horário oficial de Brasília, com fundamento no art. 4º, § 3º, do Decreto nº 10.543, de 13 de novembro de 2020.

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Anexo B – Parecer de aprovação do CEEA, experimento do capítulo 3: Vacina recombinante de *Salmonella* Typhimurium expressando proteína quimérica.

PARECER Nº **24/2023/CEUA/REITORIA**
PROCESSO Nº 23110.003391/2023-77

Certificado

Certificamos que a proposta intitulada **"Avaliação de *Salmonella enterica* e esporos de *Bacillus subtilis* como plataformas para o desenvolvimento de vacinas contra leptospirose"**, registrada com o n° **23110.003391/2023-77,** sob a responsabilidade de **Odir Antônio Dellagostin** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua execução pela Comissão de Ética no Uso de Animais da Universidade Federal de Pelotas em reunião do dia 16 de fevereiro de 2023.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	Início: 01/05/2023 Término: 31/12/2025
Espécie/linhagem/raça	*Mesocricetus auratus* / Golden Syriam
N° de animais	**160**
Idade	4 a 6 semanas
Sexo	Machos e fêmeas
Origem	Biotério Central da Universidade Federal de Pelotas, Campus Capão do Leão.

Código para cadastro nº **CEUA 003391/2023-77**

Priscila Marques Moura de Leon

Coordenadora da CEUA

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