Review

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Domitila Brzoskowski Chagas , Francisco Denis Souza Santos , Natasha Rodrigues de Oliveira , Thaís Larré Oliveira Bohn , Odir Antônio Dellagostin *

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Recombinant Live-Attenuated *Salmonella* Vaccine for Veterinary Use

Domitila Brzoskowski Chagas [1], Francisco Denis Souza Santos [1,2], Natasha Rodrigues de Oliveira [1], Thaís Larré Oliveira Bohn [1,†] and Odir Antônio Dellagostin [1,†,*]

[1] Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas, Pelotas 96010-610, Rio Grande do Sul, Brazil
[2] Faculdade de Medicina da Universidade Federal do Rio Grande, Rio Grande 96200-400, Rio Grande do Sul, Brazil
* Correspondence: odir@ufpel.edu.br
† These authors contributed equally to this work.

Abstract: Vaccination is essential in maintaining animal health, with a priority on safety and cost-effectiveness in veterinary vaccines. 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

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 predisposes them 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, quarantine or slaughter of contaminated animals, administration of treatment with antibiotics, antivirals, and antiparasitics, as well as 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 drugs to treat bacterial, viral, and parasitic infections that are non-toxic [8]. These challenges have become significant obstacles for 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

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Typhimurium (*S.* Typhimurium) being one of the most commonly isolated serovars from animals and humans worldwide (Ferrari et al., 2019). 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 gut-associated 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, aiming 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 *Salmonella* 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 versatile delivery vector for the prevention of various infectious diseases.

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].

The use of live-attenuated *Salmonella* vaccines, which are designed to decrease disease prevalence and confer protection against various pathogen strains, is 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

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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 and 24 days to six and 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 and piglets, and fattening pigs, resulted in control of *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 by various routes, including oral, nasal, subcutaneous and intramuscular [20]. However, for the use of live recombinant *Salmonella*, 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 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 this vector to present characteristics similar to those of the wild type, enabling its 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 *Δ*pmi or *Δ*galE 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 use the deletion or mutation of genes necessary for the biosynthesis of metabolically essential elements, such as aromatic amino acids and vitamins, including the *Δ*aroA, *Δ*aroC and *Δ*aroD deletions [47]. An example of this type of attenuation used in the study was the *S.* Typhimurium, LVR01 strain, which was constructed by introducing a null deletion in the *Δ*aroC gene of the canine parental isolate of *S.* Typhimurium, P228067 [48].

Likewise, to regulate expression at the promoters of the chromosomal repressor gene *lacI*, or through the promoter, in regulatory pathways with pleiotropic effects (*cya*, *crp*, *phoP*), including activating or repressor protein binding sequences, for genes of iron acquisition (*Δfur*), encoding the regulatory system of virulence components (*ΔphoP* and *ΔphoQ*), or containing mutations in DNA recombination and repair genes (*Δcya/crpF*), in the *cAMP* receptor protein (*Δcrp*) regulated through an *ara*C P BAD cassette [47]. Thus, the expression of these genes is regulated by arabinose or mannose supplementation, occurring only during *in vitro* growth [49]. After colonization of lymphoid tissues, the associated proteins stop being synthesized, because there is no presence of mannose or arabinose *in vivo* [50]. Therefore, attenuation manifests gradually *in vivo*, preventing the induction of disease symptoms and promoting the desired antigen-specific immune response.

Furthermore, programed bacterial cell lysis prevents the release of non-secreted protein antigens and DNA, contributing to biocontainment by ensuring the death of the candidate strain after colonization of immune tissues [17,47]. The system controls the expression of enzymes necessary for the synthesis of two components of the peptidoglycan layer of the cell wall, diaminopimelic acid (DAP) and muramic acid. DAP synthesis is regulated by aspartate semialdehyde dehydrogenase (encoded by *asd*), and UDP-N-acetylglucosamine enolpyruvyl transferase (encoded by *murA*) [44]. This expression was also engineered to be controlled by exogenous arabinose, and its absence resulted in lysis due to the inability to synthesize the cell wall [51].

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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 integration of the foreign gene into the bacterial chromosome, optimization of heterologous antigen codons [52], the use of inducible promoters *in vivo* [51], and systems 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 [53]. 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*

The use of live-attenuated *Salmonella* strains as vaccine delivery vehicles for heterologous antigens should efficiently cross the epithelial barrier, reach underlying antigen-presenting cells of the MALT, and elicit a robust response [53]. These mutants are capable of establishing limited infection in the host, and during the natural course of this harmless infection, the bacteria deliver a range of in vivo synthesized antigens directly to B and T lymphocytes present in the GALT [54,55].

In general, the antigens that will be presented by RASV colonize internal effector lymphoid tissues without compromising protective functions and integrity [8]. The interaction between *Salmonella* and its hosts begins with several virulence factors. Among these, type III secretion systems include *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) [56]. In epithelial cells, T3SS effectors are essential for the invasion and establishment of intracellular phagosomes [54]. One pathway is linked to the invasion of host cells (T3SS-1) and the other is induced only after invasion, for modulation in the intracellular environment [55]. The SPI-1 encoded system ensures that *Salmonella* initiates invasion into various lymphoid tissues associated with the mucosa of the intestine, nasopharynx and bronchi [17]. Bacterial internalization is accompanied by changes in host cell signaling pathways, affecting several vital cellular processes, including membrane trafficking, cell division, antigen presentation and cytokine production [53].

Salmonella adapted to the mucosal surface environment begins the infection process, remaining in membrane-bound vacuoles, and after reaching the mesenteric lymph nodes in antigen-presenting cells, they produce recombinant proteins [53]. 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 [57]. Furthermore, *Salmonella* spp. can be taken up by phagocytic cells and cross the reticulo-endothelial system, thereby stimulating systemic immune responses [58]. *Salmonella* efficiently targets MALT and induces local and systemic immunity [12,59]. Dendritic cells, neutrophils, and macrophages are stimulated in response to antigens presented in MALT, and recognize pathogen-associated molecular patterns (PAMP) and endogenous danger-associated molecular pattern molecules (DAMPs), such as T3SS-1 and fimbriae, among other adhesins surface bacteria [58].

Protein antigens are processed and presented through the major histocompatibility complex (MHC), stimulating T cell responses [57]. The signaling and activation of phagocytic cells triggers a fundamental immune response to establish connections between the innate and adaptive immune systems [60]. *Salmonella* preferentially resides in macrophages, and activation of these cells by interferon gamma (IFN-γ) produced by Th1 cells plays a prominent role in bacterial killing [56]. IFN-γ, also known as macrophage activating factor (MAF), influences the duration of macrophage activation and plays an important role in infection. Secretion of IFN-γ depends on IL-18, also known as IFN-γ-inducing factor, and is essential for establishing an early host resistance to *Salmonella* infection [61]. During primary and secondary infection, *Salmonella* is dependent on IL-12, IFN-γ, and tumor necrosis factor α (TNF-α).

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Classical activation by bacterial LPS or IFN-γ leads to alteration of the cells' secretory profile through the production of organic nitrate compounds, such as nitric oxide (NO) [58]. The responses of mucosal DCs to inflammatory stimuli may maximize their ability to preselect antigens expressed by recombinant *Salmonella* for specific T and B cells [62]. Alternative activation by IL-4, IL-10 or IL-13 leads to the production of polyamines and proline, inducing proliferation. The presence of *Salmonella* in these cells leads to the secretion of cytokines and an inflammatory reaction or programed cell death through apoptosis [58].

After exposure to specific cytokines, dendritic cells undergo classical (Th1) or alternative (Th2) activation [54]. Cytokines regulate the host's innate and adaptive immune responses. They have various effects on host cells during infection [58]. Furthermore, RASV regularly produces recombinant protein for a suitable period under the control of SPI-2 conditions and is then translocated into the cytosol via SPI-2 T3SS [63]. Secreted peptides are processed and present to major histocompatibility complex (MHC) class I and II for stimulation of T cell responses [58]. Immunization with attenuated vaccines is an effective and safe method to stimulate the production of both serum and mucosal antibodies against the *Salmonella* carrier and the foreign [12,64].

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

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 were compiled in tables 1, 2 and 3 and presented in a detailed list of 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* Typhimurium vaccine (RASV) as a vaccine vector. The potential of RASV is evident, and it requires further exploration.

5.1. Recombinant Salmonella Expressing Bacterial Antigens

Several studies were reviewed and summarized in Table 1, which presents the different *Salmonella* strains, routes, doses, animal models and the type of immune response stimulated. RASV, which contains chromosomal fusion genes encoding the secretion signal for the SPI-2 effector protein, SspH2, and the pathogenic outer membrane lipoprotein from *Leptospira*, LipL32, was administered orally to mice. 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 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 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 RASV compared to the control group treated with PBS ($P < 0.05$) [57].

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	[57]
	S. Typhimurium	*ΔaroA*	O/10^9	Mice	HI, MI	[65]

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	SL3261 *S.* Typhimurium SL3261	*ΔaroA*	O/1 × 10^9	Swine	HI, MI, CI	[66]
P97R1 (*M. hyopneumoniae*)	*S.* Typhimurium CS332	*ΔaroA*	O/2 × 10^8; 2^a dose 3 × 10^8	Mice	HI, MI, CI	[67]
	S. Typhimurium CS332	*ΔaroA*	O/2 × 10^8; 2^a dose 3 × 10^8	Mice	HI, MI, CI	[68]
K88ab (*Escherichia coli*)	*S.* Typhimurium G30/pFM205	*galE*	O, IP/1 × 10^8	Mice	HI	[69]
	S. Typhimurium	*Δlon ΔcpxR Δasd*	O/1 × 10^8	Mice	HI, MI	[70]
	S. Typhimurium	*Δlon ΔcpxR Δasd*	O/2 × 10^{10}	Pregnant sows and piglets	HI, MI	[71]
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	[63]
	S. Typhimurium JOL912	*Δlon*, *ΔcpxR*, *Δasd*	IM/1 × 10^8	Mice	HI, CI, MI	[72]
Stx2eB, FedF, FedA F18+ Shiga toxin (*E. coli*)	*S.* Typhimurium JOL1311 and JOL912	*Δasd*, *Δlon*, *ΔcpxR*	IM/9 × 10^7	Mice	HI, CI, MI	[73]
fliC F18+ Shiga toxin (*E. coli*)	*S.* Typhimurium JOL1454, JOL1460, JOL1464	*Δlon*, *ΔcpxR*, *Δasd*	SC/3 × 10^7	Mice	HI, CI, MI	[27]
APEC papA, papG, iutA, and clpG (*E. coli*)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *ΔasdA16*	O/1 x 10^7	Chicken	HI, CI, MI	[74]
APEC papA, papG, iutA, and	*S.* Typhimurium	*Δlon*, *ΔcpxR*, *ΔasdA16*	O/1 x 10^7	Chicken	HI, CI, MI	[75]

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clpG (*E. coli*)	JOL912					
APEC O-antigen (*E. coli*)	*S.* Typhimurium S100	*Δasd*, *Δcrp*, *Δcya*, *ΔrfbP*	O/ × 10^9, IM/ 5.0 × 10^7	Chicken	HI, MI	[76]
APEC PapA, CTB and LTB (*E. coli*)	*S.* Typhimurium χ8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/2 × 10^9	Mice	HI, MI	[77]
APEC (*E. coli*)	*S.* Typhimurium χ8025	*Δasd*	O/1 × 10^8	Chicken	MI	[78]
tHP (*Clostridium perfringens*)	*S.* Typhimurium	*Δasd*	O/1 × 10^9	Chicken	Intestinal colonization, BSG	[79]
tHP (*C. perfringens*)	*S.* Typhimurium χ9352	*Δasd*, *lacI*	O/1.2 × 10^9	Chicken	MI	[80]
α-toxin, NetB toxin, Fba (*C. perfringens*)	*S.* Typhimurium χ11802	*Δasd*, *lacI*	O/1 x 10^8 or 1 × 10^9	Chicken	CI, MI	[81]
PLcC, GST-NetB (*C. perfringens*)	*Salmonella* vaccine *(PIESV)* χ11802 and χ12341	*asdA*, *murA*	O/~5 × 10^8	Chicken	NE Intestinal Lesion Scoring	[33]
O antigen (*Burkholderia mallei*)	*S.* Typhimurium SL326	*ΔaroA*	IN/1 × 10^7	Mice	HI, MI	[82]
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	[83]
optA, optB, LfliC, Lhly (*Lawsonia intracellularis*)	*S.* Typhimurium JOL912	*Δasd*	O/1 × 10^7	Mice	HI, MI	[84]
Sip (*Streptococcus*	*S.* Typhimurium	*ΔaroA*	IG/10^7, 10^8 and 10^9	Fish	HI	[32]

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agalactiae)	SL7207					
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	[85]
CP39, FimA, PtfA,ToxA (*Pasteurella multocida*) F1P2 (*Bordetella bronchiseptica*)	*S.* Typhimurium JOL912	*Δlon, ΔcpxR, Δasd*	IN/1×10^5	Mice	HI, MI	[86]
CjaA (*Campylobacter jejuni*)	*S.* Typhimurium LB5010	*ΔaroA, fliM, spaS, ssaU*	O/1×10^8	Chicken	HI, MI	[87]
CjaA (*C. jejuni*)	*S.* Typhimurium *χ*9718	*Δasd*	O/1×10^8	Chicken	MI	[88]
BCSP31 (*Brucella abortus*)	*S.* Typhimurium chi 4064	*Δcya, Δcrp*	O/2×10^8 to $4x10^8$	Mice	HI, MI, Blatogenisis	[89]
BCSP31 (*B. abortus*)	*S.* Typhimurium chi 4064	*Δcya, Δcrp*	O/1×10^{10} to 2×10^{10}	Crossbred swine	HI, MI, Blatogenisis	[90]
L7/L12, BLS (*B. abortus*)	*S.* Typhimurium X4072	*Δasd*	O/1×10^9	Mice	HI, CI, MI	[91]
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 $\times 10^7$	Mice	CI, MI	[92]
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	[93]
BCSP31, Omp3b, and SOD (*B. abortus*)	*S.* Typhimurium pMMP65	*Δlon, ΔcpxR, Δasd*	SC/3×10^9	Dog	HI, CI	[94]
PrpA (*B. abortus*)	*S.* Typhimurium JOL1818 and JOL1881	*Δlon, ΔcpxR, Δasd, ΔrfaL*	IP/1×10^7	Mice	HI, CI	[92]

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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	[95]
L7/L12 (*B. abortus*)	*S.* Typhimurium JOL1800	*Δlon, ΔcpxR, Δasd, ΔrfaL*	IM/10^7	Mice	HI, MI	[96]
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,	[97]
6-PGD (*Streptococcus suis*)	*S.* Choleraesuis rSC0011	*Δasd*	O/1 ± 0.3 × 10^9	Mice	HI, MI	[98]
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	[99]
Serotypes 1/2, 2, 3, 7, 9 (*S. suis*)	*S.* Choleraesuis rSC0016	*ΔsopB, Δasd, lacI*	O/1 ± 0.2 × 10^9	Mice	HI, CI	[100]
P42, P97 (*M. hyopneumoniae*)	*S.* Choleraesuis rSC0016	*Δasd*	O/10^9	Mice	HI, MI, CI	[101]
F18+ Shiga toxin (*E. coli*)	*S.* Choleraesuis C520	*crp, Δasd*	O/2 × 10^9	Swine	HI, MI, CI	[102]

* 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. .

Other RASV using *Salmonella* Typhimurium that expresses the R2 antigen of NrdF from *Mycoplasma hyopneumoniae* was orally inoculated into mice at a dose of 10^9 CFU, with two more boosters at the same dose. In this study, a mucosal IgA-type immune response was elicited in lung washings, but not a significant level of NrdF-specific serum IgG [65]. Interestingly, Chen et al. [68] demonstrated that a DNA vaccine with a eukaryotic expression plasmid, encoding the *M. hyopneumoniae* NrdF antigen administered orally to mice with a dose of 2 × 10^8 CFU and a booster dose of 3 × 10^8 CFU, through an *S.* Typhimurium live-attenuated *aroA* induced significant NrdF-specific IFN-γ production. Although mice orally vaccinated with *S.* Typhimurium expressing NrdF encoded by a prokaryotic expression plasmid failed to induce a serum or secretory antibody response

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specific to NrdF, and IFN-γ was not produced [68]. In another study conducted by Chen and colleagues [67], used a RASV of *S.* Typhimurium, 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 in 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 [71]. In another study, Hur, Stein & Lee [70] expressed other recombinant *E. coli* fimbrial antigens K88ab, K88ac, FedA and FedF also in live-attenuated *S.* Typhimurium. 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 [63] 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 in pigs. These bacterial ghosts carried enterotoxigenic *E. coli* fimbrial antigens (ETEC) to protect against colibacillosis of piglets. All groups were orally immunized with doses of 2×10^9, 2×10^{10} and 2×10^{11} CFU in 10 mL PBS and boosted at weeks 11 and 14 of pregnancy. Serum levels of immunoglobulin Ig G and IgG and IgA in the colostrum of sows and the serum levels of the groups that received 2×10^{10} and 2×10^{11} CFU were significantly higher than those of sows in the control group. Notably, after challenge with wild-type ETEC, piglet diarrhea and mortality were not observed.

Using live-attenuated *S.* Typhimurium JOL912, which contains the genes encoding P fimbriae, (*pap* gene cluster), the iron-regulated aerobactin receptor *iut*A, and CS31A surface antigen adhesin from avian pathogenic *E. coli* (APEC) evaluated the vaccine against APEC infection in chickens. The vaccine was administered orally, intramuscularly or subcutaneously, using different doses and divided into three groups: no vaccine, a single vaccine dose, and another that received primary and booster immunizations. The birds were challenged intra-air sac with a virulent APEC strain with 10^7 UFC, and the group that received two vaccine doses showed greater protection against the challenge (80%). Furthermore, it showed significantly increases in plasma IgG levels in the third and fourth weeks old compared to birds from other groups, reinforcing the use of two vaccine doses [74]. Lee and colleagues [75] reported that after a challenge with a virulent APEC strain, the vaccinated group had no deaths, while the control group had a mortality rate of 15%. Thus, the administration of primary and booster vaccination with the *Salmonella*-delivered APEC vaccine candidate significantly elevated by the generation antigen-specific sIgA, the production of IFN-γ, IL-6 and IL-2 and which protected chickens against colibacillosis. In another study, Oh et al. [77], used the P fimbria subunit, PapA from APEC, which was in live-attenuated *S.* Typhimurium. Furthermore, they used the non-toxic B subunits of CT (CTB) and LT (LTB) as adjuvants in the vaccine formulation. The study used mice and inoculated 20 μL containing 2×10^9 CFU. PapA-specific serum IgG and mucosal IgA titers increased significantly when mice received the recombinant *Salmonella* vaccine in the presence of LTB or CTB adjuvants. Rapid declines in immune responses throughout the experimental period were observed in mice immunized without adjuvant.

The benefits of the intracellular action of *Salmonella* also correlated with natural infection in mucous membranes and, respectively, in the intestinal tract, analogous to one of the most important infection routes of *Brucella abortus*. Some studies have developed safer experimental vaccines for use, Kim et al. [29], used live-attenuated *S.* Typhimurium expressing BCSP31, Omp3b and SOD proteins from *B. abortus*. Mice were divided equally into three groups, one group received intraperitoneally with 1.2×10^6 CFU/mL in 100 μL of the vector, containing only *Salmonella* as a control; two groups received 1.2×10^9 CFU/mL of the mixture of three strains delivered in 10 μL orally and another group with a dose of 1.2×10^6 CFU/mL of the mixture in 100 μL intramuscularly. The recombinant vaccine obtained serum concentrations of IgG, TNF-α and IFN-γ via the oral route (except Omp3b) and intramuscularly, higher than the control. A robust IFN-γ-mediated response helps eliminate *Brucella*

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infection in the host. Furthermore, they 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, Leya and colleagues [24], developed a *S.* Typhimurium vaccine expressing four (BLS, PrpA, Omp19, and SOD) heterologous *Brucella* antigens and inoculated them subcutaneously at two different inoculation levels; 5×10^9 CFU/mL (Group B) and 5×10^{10} CFU/mL (Group C). The goats were challenged the inoculations with virulent *B. abortus*, 6 weeks after immunization. Serum IgG titers against individual antigens in goats immunized with recombinant *Salmonella* (Group C) were significantly higher than those in non-immunized goats and the vector control group. After antigenic stimulation, levels of IFN-γ of peripheral blood mononuclear cells were significantly elevated in Groups B and C compared to the vector control Group. The immunized goats showed a significantly higher level of protection ($P < 0.05$) in the group with the highest dose (group C), however, the group with the lower dose was also able to reduce the lesions of microgranuloma foci in the livers of goats, induced by infection by *B. abortus*. Stable and group [97] reported the use of the *Salmonella* Cholerasuis chi 3781 (SC) strain expressing the BCSP31 protein from *B. abortus*, by oral via, in pigs and mice stimulated a strong serum IgG to both the recombinant protein and SC in mice. In contrast, orally inoculated pigs did not develop significant serum or intestinal antibody responses.

5.2. Recombinant Salmonella Expressing Virus Antigens

Live-attenuated strains of *Salmonella*, including, in most of studies reviewed in this article, *S.* Typhimurium, but also *S.* Galinarium, *S.* Choleraesuis and *S.* Pullorum, have been evaluated for use as live vaccines for delivery of a variety of viral antigens (Table 2). 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 group [103] 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 receiving 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 challenge with CQ95 (60%). The presence of antibodies that recognize the HA protein in serum and probe samples was assessed by the hemagglutination inhibition (HI) assay and collected 2 weeks after vaccination. Furthermore, Jazayeri and collaborators [104], reported the use of this glycoprotein HA, NA and NP, from AIV also being expressed in live-attenuated *S.* Typhimurium SV4089, administered orally in birds with the same dose as the previous study. With detection using fluorescence in situ hybridization (FISH), *Salmonella* was specifically identified using a genus-specific probe, Sal3, from homogenized sections of the spleen, liver, and cecum of infected chickens, where a distinct fluorescent signal of rod-shaped bacteria could be detected. They achieved 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.

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	[103]
chIFN-a, chIL-18	*S.*	*hisG*, *Δcrp-*	O/ 10^9 and	Chicken	CI,	[105]

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(AIV H9N2)	Typhimurium χ8501	*28*, *ΔasdA16*	10^{11}		Hemagglutination inhibition, PCR	
HA, NA, NP (AIV H5N1)	*S.* Typhimurium SV4089	*Dam*, *ΔPhoP*	O/10^{9}	Chicken	PCR; FISH, and culturing on XLT4	[104]
HA (AIV H5N1)	*S.* Typhimurium SV4089	*Dam*, *ΔPhoP*	O, IM/ 10^{9}	Chicken	CI, Hemagglutination inhibition, PCR	[106]
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	[107]
HA, M2, NA (LPAI H7N9)	*S.* Typhimurium JOL1800	O antigen deficient	O/10^{9}	Chicken	HI, CI, MI	[108]
H9N2 haemagglutinin, M2 (AIV H9N2)	*S.* Gallinarum JOL967	*Δlon*, *ΔcpxR*, *Δasd*	O, IM/ 10^{9}	Chicken	HI, CI, MI	[109]
swIFN-α, swIL-18 (TGEV)	*S.* Typhimurium 8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/ 10^{11}	Swine	Gross lesion; histopathological; qRT-PCR	[110]
Glycoprotein B (PrV)	*S.* Typhimurium SL7207	*ΔaroA*	O/5 to 10 10^{7}	Mice	HI, MI	[111]
swIL-18, swIFN-α (PrV)	*S.* Typhimurium χ8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/10^{11}	Swine	HI, CI	[112]
UL24 (DEV)	*S.* Typhimurium SL7207	*hisG46*, *DEL407*, *ΔaroA*	O/10^{11}, 10^{10} or 10^{9}	Duck	HI, CI, MI	[113]
tgB, UL24 (DEV)	*S.* Typhimurium *S739*	*Δasd-66*, *Δcrp-24*, *Δcya-25*	O/10^{10}; 10^{11} or 10^{12}	Duck	MI	[114]
CD2v/CTL/9GL, p54/p12/p72(ASFV)	*S.* Typhimurium JOL912	*Δlon*, *ΔcpxR*, *Δasd*	IM/ 10^{8}	Swine	HI, CI, MI	[115]
S1, N (IBV)	*S.* Typhimurium	*ΔaroA*	O, IN/ 1 × 10^{9}, 5 × 10^{9}	Chicken	HI, MI	[116]

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	m SL7207		or 1 × 10^{10}			
VP2/4/3 (IBVD)	*S.* Typhimurium	*Dam*, *Phop*	O/ 10^{9}, 10^{8} or 10^{7}	Chicken	HI	[117]
prM-E (TMUV)	*S.* Typhimurium *SL7207* + *adenovirus adjuvant with duck IL-2*	*ΔaroA*	O, IM/ 10^{7}, 10^{10}	Duck	HI, CI	[118]
N (TGEV)	*S.* Typhimurium SL7207	*ΔaroA*	IG/10^{7}, 10^{8} or 10^{9}	Mice	HI, MI	[119]
swIFN-α (TGEV)	*S.* Typhimurium χ8501	*hisG*, *Δcrp-28*, *ΔasdA16*	O/ 10^{9} or 10^{11}	Swine	qRT-PCR	[120]
S (TGEV, PEDV)	*S.* Typhimurium SL7207	*ΔaroA*	O/ 1.6 × 10^{11}	Swine	HI, CI, MI	[121]
N (TGEV)	*S.* Typhimurium SL7207	*ΔaroA*	O/10^{12}	Swine	HI, CI, MI	[122]
M (TGEV)	*S.* Typhimurium *m* SL7207	*ΔaroA*	IG/10^{9}	Mice	HI, CI, MI	[123]
Glycoprotein 5, TLR-5 (PRRSV)	*S.* Typhimurium SL7207, FljB		IP/ 50 μg	Mice	HI	[124]
VP1 (FMDV)	*S.* Typhimurium KST0666	*Irradiated*	IP/ 1 × 10^{4} to 3 × 10^{8}	Mice	HI, CI, MI, VN	[125]
p27 capsid (SIV)	*S.* Typhimurium PV4570	*ΔaroA*	IM, IG/ 10^{10}	Rhesus macaques	HI, CI, MI	[126]
Glycoprotein (RV), LTB (*E. coli*)	*S.* Typhimurium LH430	*phoP*, *phoQ*	O/5 × 10^{10}	Mice	HI, CI	[127]
siRNA expressing 3D, VP4 and 2B (FMDV)	*S.* Choleraesuis C500		IM/ Guinea pigs: 1.0 × 10^{9};	Guinea Pigs, Swine	SPB-ELISA	[128]

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			Swines: 5 × 10^9			
Cap (PCV2)	*S.* Choleraesuis rSC0016	*ΔsopB*, *ΔasdA*	O/10^9	Mice	HI, CI, MI, qPCR, VN	[129]
HN (NDV)	*S.* Pullorum C79-13	*Δcrp*, *Δasd*	O/10^9	Chicken	HI, MI, Hemagglutination inhibition	[130])
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	[131]

* 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.

Another study using the avian influenza virus, this time using subtype H9N2, two attenuated strains of *S.* Typhimurium was constructed with the O antigen of LPS intact (smooth strain), whereas in the other the O antigen was eliminated (rough strain), an important immunodominant antigen of *Salmonella*, and its removal can lead to increased immunogenicity of outer membrane proteins and other surface antigens. The objective of these constructions was to determine whether, as a transporter *Salmonella* could affect immune responses against the delivered antigens. The experiment was performed with oral therapy in an avian model using recombinant *Salmonella* expressing H9N2 hemagglutinin (HA). They evaluated the effect of preexisting anti-*Salmonella* immunity on the subsequent elicitation of HA-specific immune responses. They also found that oral immunization with the S-HA or R-HA strain elicited comparable HA-specific immune responses, as indicated by serum IgG and HI titers. These results suggest that deletion of the O antigen does not affect the immunogenicity and delivery properties of the *Salmonella* system [30]. In addition, with subtype H9N2, Hajam and group [109] used *S.* Gallinarum (SG) for the expression and delivery of HA1, HA2 and/or the conserved ectodomain of matrix protein 2 (M2e). In this study, the efficacy of these RASV of SG based H9N2 vaccine strains was compared with that of the commercially available oil-adjuvanted inactivated H9N2 full-virus vaccine in a chicken model. 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 with individual H9N2 genes in SG, or as a mixture of these, and the control was vaccinated intramuscularly with the inactivated commercial H9N2 vaccine. In the indirect ELISA for IgY with serum samples collected on days 14 and 28 post-vaccination, animals vaccinated with RASV or mixture of vaccine strains showed specific systemic responses to HA1 comparable to those significantly higher ($P < 0.05$) than those in the control group. Cytokine gene expression revealed that IFN-γ increased by over 4 times ($P < 0.05$) in all groups inoculated with RASV constructs compared to the PBS control group. However, for the HA1 and HA2 genes the commercial vaccine induced significantly greater responses but was not as robust for M2 when

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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, the groups immunized with the RASV managed to efficiently inhibit the infection and spread of H9N2.

Another focus of using orally administered vaccines was to combat duck enteritis virus (DEV), an acute disease that affects ducks, geese and swans, and other free-living aquatic birds, with high mortality. Yu and group [113] 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. Corroborating this study, Liu and colleagues [114] orally immunized ducks with *S.* Typhimurium S739 expressing the DEV genes and used adjuvants (LTB subunit and duck DuIL-2 gene). During lethal challenge, 90% of animals that were immunized with recombinant Salmonella and LTB adjuvant, were protected after an initial booster immunization. The 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 after the first immunization compared to other groups ($P < 0.05$). Serum levels of IgY and bile IgA in response to purified DEV were slightly lower than those in response to the tUL24 protein. However, higher serum IgY titers against DEV were detected in ducks vaccinated with UL24 and tgB compared to those receiving tUL24 ($P < 0.05$). Similarly, among all treated groups, the highest levels of bile IgA were found in ducks vaccinated with the attenuated *Salmonella*-DEV DNA recombinant vaccine. Although the two adjuvants stimulated a high immune response in ducks, the group vaccinated with recombinant *Salmonella* and DuIL-2 was not capable of protection against homologous challenge.

The modulation in innate and adaptative immunity by cytokines such as IFN-α appears to be useful as a first line of defense against viral infections, but the use of cytokines in livestock farming has a high cost for mass production and administration. Kim and group [120](tested the efficacy of live-attenuated *S.* Typhimurium engineered to secrete porcine IFN-α (swIFN-α) protein to prevent clinical signs caused by transmissible gastroenteritis virus (TGEV) infection, one of the causes of economic losses in the swine industry. The vaccine was administered orally at a dose of 10^9 and 10^{11} CFU/pig, reducing the severit tested the efficacy of live-attenuated *S.* Typhimurium engineered to secrete porcine IFN-α (swIFN-α) protein to prevent clinical signs caused by transmissible gastroenteritis virus (TGEV) infection, one of the causes of economic losses in the swine industry. The vaccine was administered orally at a dose of 10^9 and 10^{11} CFU/pig, reducing the severity of clinical signs caused by TGEV infection. 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 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, with TGEV, Zhang and collaborators [121] sought to evaluate experimental vaccine delivered by live-attenuated *S.* Typhimurium expressing the structural protein of the virus, which is correlated with another virus, which 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 group that received the RASV with two S proteins from TGEV and PEDV simultaneously stimulated immune responses against both viruses after oral immunization. The 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

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PEDV or 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 in the RASV week 6, albeit 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 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 RASV were significantly higher ($P < 0.01$) than in control groups.

5.3. Recombinant Salmonella Expressing Parasite Antigen

The protective mechanisms required to combat parasites differ significantly from those for other pathogens, and parasites can actively suppress the host's immune response [132]. This has made it challenging to identify an effective combination of antigens, adjuvants, and routes of administration for vaccination [132,133]. 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 [133].

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*, at the intestinal mucosa [133]. 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 in BALB/c mice. Mice immunized intranasally with recombinant *Salmonella*, after challenge reduced the parasite load of adult T. spiralis by 61.83% on the eighth day post-infection, inducing 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 in 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-alone 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 group [134], produced a vaccine with fatty acid binding proteins (FABPs) of EgDf1 fusion with a C-terminal fragment of tetanus toxin (TetC) expressed in *S.* Typhimurium LVR01. The inoculation was intravenous with a dose of 10^6 CFU, as well as an oral dose with 4×10^9 CFU in mice eliciting an antibody response to EgDf1 and 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 using in *S.* Typhimurium LVR01 expressing EgDf1 FABP, in dogs, orally vaccinated at a dose of 5×10^{10} CFU in 2 mL of PBS, or PBS alone. The dogs presented IgG antibodies responses against EgDf1 in the 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].

Table 3. Parasitic antigens expressed in *Salmonella*.

Antigen (Organism)	*Salmonella* Strain or plasmid	Attenuation	Route/dose (CFU)	Model	Immunity	Reference

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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	[134]
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	[139]
gp63 (*Leishmania major*)	*S.* Typhimurium BRD509	*ΔaroA*, *ΔaroD*	O/ 1 × 10^{10}	Mice	HI, CI	[140]
SAG, SAG2 (*Toxoplasma gondii*)	*S.* Typhimurium BRD509	*ΔaroA*, *ΔaroD*	IG/10^9	Mice	HI, CI	[141]
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	[143]
EC-SOD (*Acanthocheilonema viteae*)	*S.* Typhimurium SL3261	*ΔaroA*	O/5 × 10^8	Jird	HI	[144]

* 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.

Cong and colleagues [141] evaluated a vaccine using live-attenuated *S.* Typhimurium as a delivery vector for the recombinant eukaryotic plasmid pSAG1-2/CTA2/B, which expresses a multiantigenic gene encoding SAG1 and SAG2 from *Toxoplasma gondii* linked to the A2/B subunits of cholera toxin (CTA2/B) via oral administration in mice. Levels of anti-*T. gondii* IgG antibodies were detected in animals that received *S.* Typhimurium delivering the recombinant plasmid expressing the SAG1/SAG2 surface antigen. IgG anti-*T. gondii* values increased in the group immunized with the CTA2/B subunits, compared with those of the negative controls ($P = 0.003$, $P = 0.004$). However, there was no significant difference in anti-*T. gondii* IgG antibodies between mice immunized with or

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without CTA2/B as a genetic adjuvant. The group vaccinated without the adjuvant obtained a strong Th2 response; however, with the CTA2/B adjuvant, the *T. gondii*-specific response was predominantly Th1. When immunized mice were intraperitoneally challenged with 10^3 tachyzoite of the virulent strain of *T. gondii*, the survival time of mice immunized with the vaccine with CTA2/B adjuvant was longer than that of other vaccine groups ($P = 0.003$), and a survival rate of 40% was achieved.

In another study, Cong and colleagues [22] used live-attenuated *S.* Typhimurium to deliver a vaccine encoding several epitopes derived from tachyzoite proteins SAG1, GRA1, ROP2, GRA4, and bradyzoite proteins SAG2C, SAG2X of *T. gondii*, along with (CTA2/B). This live-attenuated recombinant *Salmonella* vaccine was evaluated for BALB/c mice administered via the oral and nasal routes or by intramuscular injection, using plasmids named (pVAX1-MEG-CTX A 2 /B). The higher levels of anti-*T. gondii* were detected in the serum of mice immunized orally and nasally than in the serum of mice injected intramuscularly with RASV ($P < 0.05$). CD4+ and CD8+ T lymphocyte subsets in immunized mice were analyzed by flow cytometry, resulting in 28.54 ± 0.92% and 30.01 ± 1.78%, respectively, in mice immunized intramuscularly and intranasally. Cytokines released in the culture of mice vaccinated with the RASV intramuscularly demonstrated a preferential production of IFN-γ and IL-2, significantly higher than in the control groups ($p < 0.05$). However, they were significantly lower compared to animals immunized orally and intranasally with RASV ($P = 0.02$). The levels of IL-4 and IL-5 produced by the immunized mice were low, and there were no statistically significant differences between the vaccinated groups and control groups ($P > 0.05$). Furthermore, the proliferation activity of antigen-specific lymphocytes was significantly increased in the oral and intranasal immunization groups compared to the intramuscular immunization group ($P < 0.05$). Four weeks after the last immunization, mice were challenged with 100 μL of 1×10^3 tachyzoite of the *T. gondii* strain intraperitoneally. All control mice died. Although there was a survival rate of 20% (2/10) for mice immunized with the pVAX1-MEG-CTXA2/B plasmid intramuscularly. The RASV intranasal immunization had a survival rate of 40% (4/10) after 10 days. The highest survival rate of 60% (6/10) was achieved in mice orally immunized with the RASV.

Benitez, McNair, and Mead [142] utilized strains of live-attenuated *S.* Typhimurium expressing Cp23 and Cp40 from *Cryptosporidium parvum*, recognized as surface immunodominant antigens, as they are acknowledged by serum antibodies from humans and many other animals. In the study, mice were orally immunized with 5×10^9 CFU/mouse 0.2 mL of PBS intragastrically for each vaccine, which included a vector with an empty plasmid, 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. 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 in mice immunized with 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.

Another vaccine using live-attenuated *S*. Typhimurium was studied by Chen and colleagues [143]. They employed active promoters within the intracellular environment of antigen-presenting cells, such nitrite reductase B (*nir*B) and *pho*P activated gene C (*pag*C) or pMohly, to express the bivalent antigen Sj23LHD-GST from *Schistosoma japonicum* through the *Salmonella* type III secretion system or α-hemolysin. Mice were orally immunized with 0.2 mL of PBS containing 10^9 CFUs for the vaccine groups: recombinant *S.* Typhimurium and the empty vector pQE30, with a negative control receiving only PBS. Three doses were administered at two-week intervals. The Sj23LHD-GST antigen induced a moderate level of IgG in mice immunized with *S.* Typhimurium containing the *nir*B promoter. Unlike the total IgG level, the IgG2a:IgG1 ratio in mice immunized with *S.* Typhimurium harboring the *nir*B promoter was the highest. The predominant presence of the IgG2a isotype strongly suggests that the strain with *nir*B triggered a Th1-type specific response. The expression of CD44 in splenocytes of mice immunized with the RASV of *S.* Typhimurium was increased (25 ± 2%) when delivered by the *Salmonella* type III secretion system driven by the *nir*B promoter, which was

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significantly higher than that found in mice immunized with strains of *S.* Typhimurium containing the *pag*C promoter (12±1%) or pMohly1 (11±1%) or vector (11±1%) ($P < 0.01$). The mice were challenged with *S. japonicum* cercariae by penetration into the abdominal skin and examined for worm and egg burdens in the mesenteric vein and liver six weeks after challenge. Recombinant strains of *S.* Typhimurium containing the *pag*C, *nir*B, or pMohly1 promoters caused a reduction of 30.07%, 57.71%, and 40.46% reduction in the number of eggs. Moreover, animals immunized by the prime-boost method with protein driven by the *nir*B promoter, delivered by the *Salmonella* type III secretion system, caused a reduction in parasite burden by 51.35% and 62.59% in *S. japonicum* egg burden, significantly higher than that of recombinant protein alone or before boosting. These results demonstrate the in vivo protective efficacy of antigens delivered by the *Salmonella* type III secretion system driven by the *nir*B promoter.

6. Conclusion and Future Directions

The development and commercialization of RASVs represent 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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