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Review

Enhancement of live vaccines by co-delivery of immune modulating proteins

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ABSTRACT

Vaccines are very effective in providing protection against many infectious diseases. However, it has proven difficult to develop highly efficacious vaccines against some pathogens and so there is a continuing need to improve vaccine technologies. The first successful and widely used vaccines were based on attenuated pathogens (e.g., laboratory passaged *Pasteurella multocida* to vaccinate against fowl cholera) or closely related non-pathogenic organisms (e.g., cowpox to vaccinate against smallpox). Subsequently, live vaccines, either attenuated pathogens or non-pathogenic microorganisms modified to deliver heterologous antigens, have been successfully used to induce protective immune responses against many pathogens. Unlike conventional killed and subunit vaccines, live vaccines can deliver antigens to mucosal surfaces in a similar manner and context as the natural infection and hence can often produce a more appropriate and protective immune response. Despite these advantages, there is still a need to improve the immunogenicity of some live vaccines. The efficacy of injectable killed and subunit vaccines is usually enhanced using adjuvants such mineral salts, oils, and saponin, but such adjuvants cannot be used with live vaccines. Instead, live vaccines can be engineered to produce immunomodulatory molecules that can stimulate the immune system to induce more robust and long-lasting adaptive immune responses. This review focuses on research that has been undertaken to engineer live vaccines to produce immunomodulatory molecules that act as adjuvants to increase immunogenicity. Adjuvant strategies with varying mechanisms of action (inflammatory, antibody-mediated, cell-mediated) and delivery modes (oral, intramuscular, intranasal) have been investigated, with varying degrees of success. The goal of such research is to define adjuvant strategies that can be adapted to enhance live vaccine efficacy by triggering strong innate and adaptive immune responses and produce vaccines against a wider range of pathogens.

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1. Introduction

Vaccination is one of the most cost-effective ways to prevent disease in humans and livestock and improve productivity. Antibacterial vaccines reduce reliance on antibiotics and have the added advantage that they can prevent the development of antibiotic resistance. The challenge for modern vaccine development is to safely induce robust responses. Both live and killed or subunit vaccine are widely used in livestock. In this review, various immunomodulatory molecules and their role in inducing the immune system of animal models have been discussed in detail. It focuses on new developments in livestock vaccines and will focus on approaches to enhance the efficacy of live vaccines by using co-delivered immunomodulatory proteins.

1.1. Advantages and limitations of live over subunit vaccines

Live vaccines may be either attenuated pathogens, or live vector vaccines in which a vector (attenuated pathogen or commensal organism) is used to deliver a foreign antigen – effectively a type of subunit vaccine [1]. Live vaccines have key advantages because they mimic the route of entry of pathogens and stimulate an immune response specific to the location of the natural infection. Live vaccines can be administered via mucosal routes [2,3], which can be less invasive than parenteral routes of administration. By replicating, even to a limited extent, often intracellularly in epithelial cells or professional antigen presenting cells (APC), local innate response pathways are triggered resulting in chemokines and cytokines which attract further APC, neutrophils and eventually *T*-cells for establishment of adaptive immunity (Fig. 1). Induction of a mucosal immune response at one mucosal surface can result in migration of immune cells to other distant mucosal sites [2]. This mucosal homing feature is advantageous for vaccines targeting intestinal pathogens. Live bacterial vaccines are also relatively easy and cheap to manufacture as they do not require the downstream processing that some other vaccine formats need [1,4], and the cold chain may only be refrigeration, rather than freezing.

While live-attenuated vaccines are by definition unable to disseminate or cause disease in competent hosts, they may be shed for a period after vaccination in immunocompromised hosts [5], and the (unlikely) possibility of reversion to virulence also exists for some attenuated vectors [1]. Live attenuated vaccines might benefit from additional adjuvant strategies to improve efficacy. Natural immunity developed by infection with *Salmonella typhi*, *Campylobacter jejuni*, *Helicobacter pylori*, and mycobacterial infection does not confer protection against reinfection [6–8]; the generation of mucosal responses to additional (novel protective) antigens, or alternate immune responses is necessary.

Live-attenuated, and live-vectored vaccines that employ non-invasive organisms (e.g., *Lactobacillus*, *Lactococcus*), have the advantage of being robust and survive well in the gut. Surface displayed antigens may be more effective than secreted antigens to enhance recognition because they will be taken up and processed with the whole bacterium [2]. Live vectored vaccines are hypothesised to be recognised after uptake by microfold cells (M−cells) in the mucosal associated lymphoid tissue (MALT), facilitating presentation to patrolling professional antigen presenting cells (APC) such as dendritic cells (DC) and macrophages (Fig. 1). In both cases, local presentation of antigen permits the development of immune responses that mimic the natural infection.

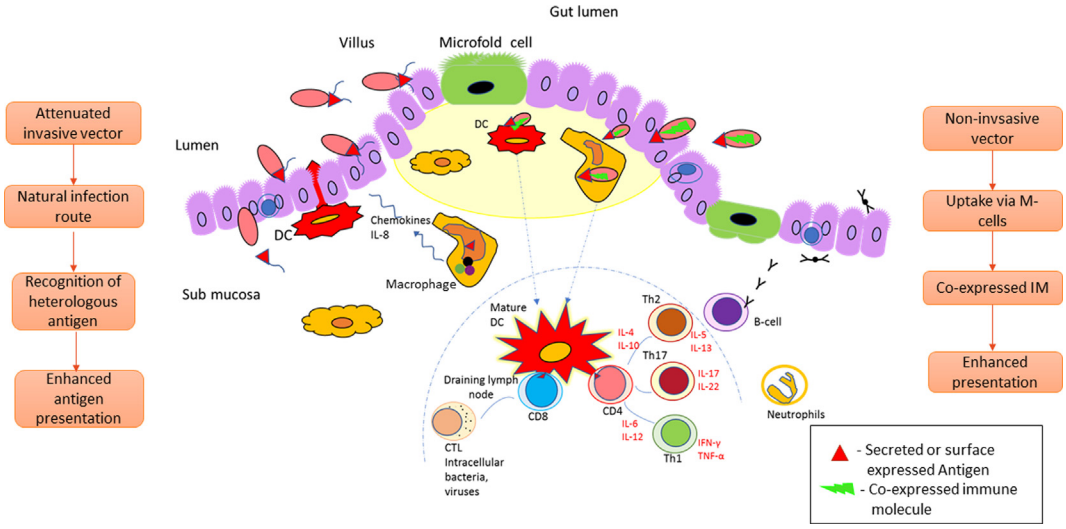

Fig. 1. Immunostimulatory molecules co-expressed by live vaccines enhance induction of mucosal immunity. The efficacy of both live attenuated and non-invasive commensal vectors can be enhanced by co-expression of immunomodulatory molecules. Attenuated pathogens invade via the natural route and replicate in a limited fashion in the submucosa and, in the case of *Salmonella*, also in antigen presenting cells (APC), macrophages. Co-expression of immunomodulatory molecules (IM, represented in green) can enhance antigen presentation and responses to vaccine antigen (represented in red). Non-invasive generally regarded as safe (GRAS) vectors are taken up via the microfold cells (M−cells) of mucosal associated lymphoid tissue (MALT) such as the Peyer's patches. Surface expressed antigens (red) are recognised by dendritic cells (DC). Co– expression of IM can enhance DC maturation, migration, and presentation in the draining lymph nodes. Depending on the antigen and co-expressed IM, Th1/17 responses are activated which are most effective at killing bacterial and fungal pathogens, Th2 responses promote B-cells and antibody production. Transport of secretory immunoglobulin A (sIgA) to the mucosal surface can help neutralize luminal pathogens.

1.2. Commercially available live veterinary vaccines

Veterinary vaccines, including the extensive use of attenuated live vaccines, have been commercially used to improve the health and welfare of companion animals, to increase production of livestock, to prevent the transmission of zoonotic diseases and to reduce reliance on antibiotics to control outbreaks [9,10]. Recent advances in technology have contributed to new generation vaccines which can combat viral, bacterial, protozoal, and multicellular pathogens in animals. Systematic reviews have listed a number of commercially available veterinary vaccines and non-specific effects of veterinary vaccines historically to combat infectious diseases [9–12]. Vaxsafe® (Bioproperties) are a group of successful live vaccines available for the control of a number of diseases like chronic respiratory disease, fowl cholera, Newcastle disease virus, infectious bursal disease, infectious bronchitis, and colonisation by *Salmonella* [13]. The mode of delivery for these vaccines varies from eye drop suspensions, intramuscular injection to mixing with drinking water. Besides the use of live vaccines to curb production losses in livestock industry, they can be used in large farm animals to improve the quality of life. Equilis StrepE (Intervet) is a live vaccine approved for use in the European union against a highly contagious and common respiratory disease in horses. This vaccine is a live attenuated strain of *Streptococcus equi* which is delivered as an injection and helps protect the horses from this debilitating infection [14]. Successful live vaccines are used worldwide for control of diseases in pigs [15], sheep [16], chickens [17], and turkeys [18]. There are strategies to increase the effectiveness of live vaccines using the adjuvanting approaches described below.

1.3. Co-expressed immunomodulators as adjuvants for live vaccines.

Adjuvants improve the effectiveness of vaccines by enhancing the strength and modifying the qualitative nature of the immune responses. Traditionally adjuvants, such as mineral salts, emulsions, and surfactant-based compounds, are administered by parenteral means and are not relevant to live vector vaccines [2,19]. For enhancement of the immune response of live-attenuated or live-vector vaccines, recombinant immune modulating proteins are an ideal choice as adjuvants. Immune potentiators or immune modulating proteins more specifically target the immune system through innate activation using pathogen pattern receptors (PRR), or enhance the immune response directly using immunomodulatory proteins (e.g., cytokines) (Fig. 1) [19–21]. The use of immunomodulator molecules (IM) such as cytokines has applications for live vaccines where the vaccine vector can be engineered to express both the antigen and the immunostimulatory molecule to enhance immune activation and recognition at the mucosal surface (Fig. 1).

Conceptually, inclusion of an IM as adjuvant has the advantage that when co-expressed with the antigen, it could promote activation of, or attract antigen presenting cells in the vicinity, resulting in initiation of a stronger response to the vaccine. Because the action occurs within the microenvironment of an applied live vaccine, only low levels of both antigen and IM might be required (discussed below, and examples are shown in Table 1).

For attenuated pathogen vectors that can invade the epithelium themselves (e.g., *Salmonella* spp.), co-expression of an IM can be expected to enhance activation of macrophages and promote the presentation of antigen. Indeed, this approach has shown promise in several studies (Table 2, 54–57).

Food grade bacteria are attractive potential vaccine vectors because they are generally regarded as safe (GRAS) and can be delivered orally. These commensal vectors might, in principle, be expected to be poorly recognised by the innate immune system, however a variety of species expressing heterologous antigens, with or without IM have been shown to induce antigen specific, cellular and humoral responses (Table 1-3). Detailed studies investigating the specific mechanisms of uptake have not been reported, however lactic acid bacteria (LAB) can propagate in the intestine, and theoretically bind to mucins and compete against pathogens [22]. This binding, and the expression of foreign antigens may be sufficient to ensure uptake by M−cells, or even recognition by DC extending projections through the epithelium [23,24] (Fig. 1).

Initial recognition of antigens is performed by professional APCs, dendritic cells (DC) and macrophages. Co-expressed IM can enhance activation of APCs, presentation of antigen and the development of T- and B-cell responses. Further, selection of specific IM can be used to direct the response to a Th1/17 or Th2 phenotype. Mature DC that secrete pro-inflammatory cytokines, such as IL-12 and IL-6, promote development of Th1 responses [25], and those that secrete IL-10, IL-4, or suppress IL-12, promote Th2 type responses [26]. In addition, once activated, the secretion of pro-inflammatory cytokines a such as TNFα and IL-12 can create an amplifying effect by attracting further recruitment of APCs to the site, ensuring strong immune priming. An alternative IM strategy is to activate pattern recognition receptors on DCs to promote DC maturation and presentation. This approach has been used successfully in a range of animal models by co-expressing Toll-like Receptor (TLR) ligands, or DC targeting peptides with antigens [10,11] (summarized in Table 2). To best take advantage of the potential for targeting adaptive responses, an understanding of the desirable protective mechanisms against the target pathogen is required.

Here we review the literature on the use of immune modulating proteins in live vaccines and discuss their applications, limitations, and future directions.

2. Immune potentiators as adjuvant strategies for live vaccines

2.1. Interferons

Interferon-γ, a type II interferon, is produced by natural killer cells (NK cells) and T lymphocytes, stimulates the production of pro-inflammatory cytokines, and upregulates the expression of class I and II major histocompatibility complex (MHC) molecules. IFN-γ regulates macrophage effector functions, and amplifies T helper (TH1) cell expansion [27] (Fig. 1). Studies that have used IFN-γ as a live vaccine adjuvant are summarized in Table 1 and some examples are discussed below.

Tuberculosis (TB) has, for many years, been controlled using the *Mycobacterium bovis* Bacille Calmette–Guérin (BCG) vaccine and this has dramatically reduced the impact of the disease. The protective efficacy provided by the BCG vaccine is however variable depending on age group, and geographical location [28]. Globally, TB continues to be the second leading cause of death by infectious disease [29]. One approach to improve the BCG vaccine has been by engineering the vaccine strain to produce cytokines and thus elicit a stronger immune response [21,30]. Overall the IFN-γ secreting BCG resulted in reduced tissue pathology, absence of fibrosis, and decreased bacterial load, which indicated that the delivery of IFN-γ promoted protective immunity [28].

Food-grade lactic acid bacterium *Lactococcus lactis* has been used to express and secrete biologically active, mature murine IFN-γ to circumvent the problem of sequestration into inclusion bodies in bacteria like *E. coli* [27]. *Lactobacillus lactis* does not produce lipopolysaccharide (LPS) or toxins and hence it is an ideal candidate to produce biologically active IFN-γ without any purification. The biological activity of *L. lactis* secreted IFN-γ was confirmed by the vesicular stomatitis virus (VSV) cytopathic effect reduction assay in a murine cell line (MoVS cells) infected with

Table 1
Adjuvant strategies using interferons and interleukins in live vaccines.

Immune molecule (Interferon)	Vector/organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
IFN-γ (murine)	*Mycobacterium bovis* BCG	Mouse	+	IV injection	Tuberculosis	IHC, FM, HA	+	+	–	NM	+	Wangoo *et al.* 2000 [28]
IFN-γ	*Streptococcus gordonii*	Mouse	–	SC injection	Co-expression/adjuvant	CA, IB, SB, ELISA	NM	–	–	NM	–	Byrd *et al.* 2002 [93]
IFN-γ	*Lactococcus lactis*	Murine cell line - MoVS	–	*In vitro* expression	Vesicular stomatitis virus	ELISA, WB, AVA	NM	+	–	NM	NM	Bermudez-Humaran *et al.* 2007 [27]
IFN- γ (chicken)	*Mycoplasma gallisepticum*	Chicken	+	Eye drops	Avian respiratory mycoplasmosis	IB,	NM	+	–	NM	+	Muneta *et al.* 2008 [32]
IFN-γ	*Escherichia coli*	Mouse and pig	+	IM injection	*Taenia solium*	ELISA, CE, NOA	+	+	+	IgG1, IgG2a, IgG2b, IgE	+	Jing *et al.* 2010 [94]
IFN-γ	*Salmonella enterica. var* Typhimurium	Mice	+	IP injection	TLR-4 defective C_3H/H_eJ mice	ELISA, NOA	+	+	–	IgM, IgG2a	+	Al-Ojali *et al.* 2012 [33]

Immune molecule (Interleukin)	Vector/organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
IL-2, IL-6 (murine)	*L. lactis*	Mouse	+	IN injection	Therapeutic efficacy	ELISA, IA, IB	NM	NM	NM	IgG1, IgG2a, IgA	NM	Steidler *et al.* 1998 [37]
IL-2	*S. gordonii*	Mouse	–	SC injection	Co-expression/ adjuvant	CA, IB, SB, ELISA	NM	–	–	NM	–	Byrd *et al.* 2002 [93]
IL-10 (human)	*L. lactis*	Pig	–	Ileal loop injection	Inflammatory bowel disease	LM, ILI, PCR, WB, CA, HPLC	NM	–	+	NM	+	Steidler *et al.* 2003 [46]
IL-12	*L. lactis*	Mouse	+	IN inoculation	HPV-16	CA, ELISPOT TR, CE	+	+	–	IgG, IgA	–	Bermú dez-Humaŕan *et al.* 2005 [4]
IL-12	*M. bovis* BCG	Mouse	+	IV injection	Tuberculosis	LA, CXA CA, ELISA	NM	+	–	NM	+	Fan *et al.* 2006 [34]
IL-10	*L. lactis*	Human	–	Oral	Crohn's disease	qRT-PCR, CS, ES	NM	–	+	NM	NM	Braat *et al.* 2006 [44]
IL-12	*L. lactis, Lactobacillus plantarum*	Mouse	+	IN & IG inoculation	HPV-16	IB, IFM, ELISA ELISPOT, TR, CE	+	+	–	IgG, IgA	+	Cortes-Perez *et al.* 2007 [40]
IL-15 (murine)	*M. bovis* BCG	Mouse	+	IP injection	Tuberculosis	ELISA, ICS, CE	+	+	–	NM	+	Tang *et al.* 2008 [36]

Immune molecule (Interleukin)	Vector/organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
IL-1β (murine)	*L. casei*	Mouse	–	Oral	Adjuvant effect	, IB, CC, RT-PCR, LIL, ELISA	NM	NM	NM	IgG, IgA	+	Kajikawa *et al.* 2009 [38]
IL-4 (porcine)	*E. coli*	Mouse and pig	+	IM injection	*Taenia solium*	ELISA CE, NOA	+	+	+	IgG1, IgG2a, IgG2b, IgE	+	Jing *et al.* 2010 [94]
IL-12p70	*M. bovis* BCG	Mouse	+	SC injection	Tuberculosis	ELISA, FC, WB, CE, HP	–	+	–	IgG2a/IgG1	+	Deng *et al.* 2011 [35]
IL-6 (chicken)	*M. gallisepticum*	Chicken	+	Eye drops	Avian respiratory mycoplasmosis	SAT, BWG, HP, ASL, CE, CC,	+	NM	NM	NM	+	Shil *et al.* 2011 [95]
IL-2	*Lactobacillus. rhamnosus*	Mouse	+	Oral	Evaluate immune response	ELISA, ELISPOT, FC, CXA, EM	NM	+	–	IgG, IgA	+	Kandasamy *et al.* 2011 [39]

Immune molecule (Interleukin)	Vector/organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
IL-12	*L. lactis*	Mouse	+	Oral & SC injection	Leishmaniasis	WB, CE, CA, FC, ELISA	+	+	+	IgG, IgG1, IgG2a	+	Hugentobler *et al.* 2012 [41,42]
IL-2 (chicken)	*L. lactis*	Mouse	+	Oral	Avian influenza	RT-PCR, WB, DB, ELISA	NM	+	–	IgG, IgA	NM	Szatraj *et al.* 2014 [43]
IL-1β(murine)	*Lactobacillus. acidophilus*	Mouse	+	Oral	HIV-1	Western blot, FC, ELISA ELISPOT, MMK	NM	+	–	IgA, IgG, Ig2b	NM	Kajikawa *et al.* 2015 [96]
IL-12	*L. plantarum L. lactis*	Mouse	+	Oral	Tuberculosis	ELISA, IFM, BWG	NM	+	–	IgG	+	Mustafa *et al.* 2018 [30]
IL-35	*L. lactis*	Mouse	–	Oral	Collagen induced arthritis	Cytokine ELISA, FC, CE	+	–	+	NM	+	Maddaloni *et al.* 2018 [45]

'+' – Antigen present; '-' – Antigen absent; AA – Adhesion assay; AL – Air sac lesion; AT – Agglutination test; AVA – Antiviral assay; BWG – Body weight Gain; CA – Cytokine assay; CC – Cell culture; CE – Challenge experiment; CS – Colonoscopy; CT – Cloning and Transformation; CXA – Cytotoxicity assay; DB – Dot blot; ELISA – Enzyme-linked immunosorbent assay; ELISPOT – Enzyme-linked immunospot assay; EM – Electron microscopy; ES – Endoscopic score; FM – Fibriosis model; FC – Flow cytometry; HA – Hydroxyproline Assay; HP – Histopathology; IA – Immunoassay; IB – Immunoblot; ICS – Intracellular cytokine staining; IFM – Immunofluorescent microscopy; IHC – Immunohistochemistry; ILI – Ileal loop incubation; Ig – Immunoglobulin; IM – Intramuscular; IP- Intraperitoneal; IV – Intravenous; LA – Lymphoproliferation assay; LM – Laparotomy; LIL – Ligated Intestinal loop assay; LS – Lesion score; LVT – Lung viral titre; MB – Microbial studies; MMK – Milliplex Map kit; NA – Neutralization assay; NM – Not measured; PEA – Protein expression assay; qRT-PCR – Quantitative reverse transcription polymerase chain reaction; SAT – Serum Agglutination test; SB – Streak blot; SC – Subcutaneous; Th – T helper cells; TCP – *T*-cell Proliferation assay; TLR – Toll-like receptor; TNA – Toxin Neutralization assay; TP – Tissue pathology; TR – Tumour regression; WB – Western blot.

Table 2
Adjuvant strategies using pattern recognition receptor and dendritic cell targeting peptides in live vaccines.

Immune molecule (Pattern recognition receptor)	Vector/ organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
TLR5 ligand - flagellin	*Lactobacillus. casei & L. plantarum*	Mouse	–	Oral	Comparison studies of two bacteria as antigen delivery vehicles	WB,FC, ELISA, ICS, CE	+	+	–	IgG, IgA	NM	Kajikawa *et al.* 2007 [48]
TLR5 ligand - flagellin	*Lactobacillus casei*	Mouse	+	IP injection	Evaluate immune response	IB, ELISA FACS, CC	NM	+	+	IgG, IgG1, IgG2a	NM	Kajikawa and Igimi. 2010 [47]
TLR5 ligand - flagellin	*Lactobacillus gasseri*	Mouse	–	Oral	Evaluate immune response	WB, FC, ELISA, CA	NM	–	+	IgA, IgG	+	Stoeker *et al.* 2011 [49]

Immune molecule (DC targeting peptide)	Vector/ organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
DCpep	*L. acidophilus*	Mouse	+	Oral	Anthrax	WB, ELISA, CC, CA, TNA	+	+	–	IgA	NM	Mohamadzadeh *et al.* 2009 [50]
DCpep	*L. gasseri*	Mouse	+	Oral	Anthrax	WB, TNA, CE	+	+	–	NM	NM	Mohamadzadeh *et al.* 2009 [51]
DCpep	*L. plantarum*	Chicken	+	Oral	Newcastle disease virus (NDV)	ELISA, FACS, CE, HS, HP	+	NM	NM	IgA	+	Jiang *et al.*2015 [63]
DCpep	*L. plantarum*	Mouse, chicken	+	Oral	H9N2	ELISA, ICS, HP, FC, TCP	+	+	+	IgG, IgA	+	Shi *et al.* 2016 [56]
DCpep	*L. plantarum*	Mouse	+	Oral & IM injection	Avian influenza	CC, ELISPOT, CXA, CE, FC, ICS, HP, TCP	+	+	–	IgA	+	Yang *et al.* 2016 [57]
DCpep	*L. plantarum*	Chicken	+	Oral	Avian coccidiosis	LS, HP, CE, ELISA	+	NM	NM	IgG, IgA	+	Yang *et al.* 2017 [59]
DCpep	*L. plantarum*	Chicken	+	Oral	H9N2 avian influenza	FC, HP, CE, ELISA	+	NM	NM	IgA, IgG	+	Yang *et al.* 2017 [55]
DCpep	*L. acidophilus*	Mouse	+	Oral	Botulinum neurotoxin	FC, CE, ELISA	+	NM	NM	IgG, IgA	+	Sahay *et al.* 2017 [61]
DCpep	*L. plantarum*	Mouse	+	Oral	Enterotoxigenic *E. coli*	AA, ELISA, CA, HS	+	NM	NM	IgG, IgA	+	Yang *et al.* 2017 [62]

Immune molecule (DC targeting peptide)	Vector/ organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
DCpep	*L. casei*	Mouse	+	Oral	PEDV	FACS, ELISA, CA, LA	–	+	+	IgG, IgA	+	Wang *et al.* 2017 [52]
DCpep	*L. casei*	Mouse	+	Oral	PEDV	WB, TNA,ELISA	–	+	+	IgG, IgA	+	Ma *et al.* 2018 [23]
DCpep	*L. casei*	Pig	+	Oral	PEDV	FC, IA,ELISA, qRT-PCR, HP, CE	+	+	–	IgA, IgG	+	Hou *et al.* 2018 [53]
DCpep	*L. plantarum*	Mouse	+	Oral	PEDV	WB, FC, ELISA, NA	–	+	+	IgA, IgG	–	Huang *et al.* 2018 [54]
DCpep	*L. casei*	Mouse	+	Oral	BVDV	ELISA, LA, NA, CE, FACS	+	+	+	IgA, IgG	+	Wang *et al.* 2019 [60]
DCpep	*L. lactis*	Chicken	+	Oral	Avian coccidiosis	WB, ELISA, LS, CE, PEA, HP	+	+	–	IgG, IgA	+	Li *et al.* 2020 [58]

'+' – Antigen present; '-' – Antigen absent; AA – Adhesion assay; APC- Antigen presenting cells; BVDV- Bovine viral diarrhoea virus; BWG – Body weight Gain; CA – Cytokine assay; CC – Cell culture; CE – Challenge experiment; CXA – Cytotoxicity assay; DC- Dendritic cell; ELISA- Enzyme-linked immunosorbent assay; ELISPOT- Enzyme-linked immunospot assay; FACS- Fluorescence-activated cell sorting; FC – Flow cytometry; HP – Histopathology; IA – Immunoassay; IB – Immunoblot; ICS – Intracellular cytokine staining; Ig- Immunoglobulin; IM- Intramuscular; IP- Intraperitoneal; LA – Lymphoproliferation assay; LS – Lesion score; NA – Neutralization assay; NM – Not measured; PP- Peyer's patch; PEA – Protein expression assay; PEDV- Porcine epidemic diarrhoea virus; qRT-PCR- Quantitative reverse transcription polymerase chain reaction; Th- *T*-helper cell; TCP – *T*-cell Proliferation assay; TNA – Toxin Neutralization assay; WB – Western blot.

Table 3
Adjuvant strategies using other immune active molecules in live vaccines.

Immune molecule (Tumour necrosis factor)	Vector/ organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
CD40L	*Salmonella* Enteritidis	Chicken	+	Oral	Avian influenza	NA, CE, ELISA	+	+	NM	IgG	+	Layton *et al.* 2009 [67]
CD40L	*S.* Enteritidis	Chicken, turkey	+	Oral	Foodborne *Salmonella* infection	ELISA, AT, TP, PCR,	+	NM	NM	IgG	+	O'Meara *et al.* 2010 [66]
CD40L	*S.* Enteritidis	Chicken	+	Oral	Avian coccidiosis	LS. BWG, CE	+	NM	NM	NM	+	Shivaramaiah *et al.* 2010 [69]
CD40L	*E. coli*	Chicken	+	IM injection	Avian coccidiosis	ELISA, ELISpot	+	+	+	IgG, IgA	+	Yin *et al.* 2015 [97]
CD40L	*Salmonella* Gallinarum	Chicken	+	Oral	Fowl typhoid and H9N2	qRT-PCR, ELISA, HP, LA, CE	+	+	+	IgG, IgA	+	Hajam *et al.* 2018 [68]
RANKL	*L. lactis*	Mouse	–	Oral	Evaluate immune response	qRT-PCR, ELISA, CC, SDS-PAGE, IHC	NM	–	–	IgG1, IgG2a, IgA	NM	Kim *et al.* 2015 [65]
RANKL	*L. lactis*	Pig	+	Oral/ Intramuscular	PEDV	EM, qRT-PCR, HP, BWG	+	NM	NM	IgA	+	Choe *et al.* 2020 [70]

Immune molecule (Bacterial toxins)	Vector/ organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
Cholera toxin subunit A1 (CTA1)	*L. casei*	Mouse	+	Oral, IN	Influenza A	WB, IFM, HP, ELISA, ELISpot, LVT	+	+	+	IgG, IgG1, IgG2a, IgA	+	Chowdhury *et al.* 2013 [76], Li *et al.* 2015 [75]
Cholera toxin subunit B (CTB)	*L. casei*	Mouse	+	IN	*Bordetella pertussis* (Whooping cough)	CT, AT, ELISA	NM	NM	NM	IgG, IgA	NM	Colombi *et al* 2006 [73]
Cholera toxin subunit B (CTB)	*L. lactis*	Mouse	+	Oral	Avian influenza (H5N1)	WB, FC, IFM, CE, ELISA, ELISpot	+	+	+	IgG, IgA	NM	Lei *et al.* 2011 [74]
Cholera toxin subunit B (CTB)	*L. casei* *Lactobacillus reuteri*	Mouse	–	IN	Cholera	SDS-PAGE, IB, ELISA	NM	NM	NM	IgG	NM	Okuno *et al.* 2013 [72]
Heat-labile enterotoxin B (LTB)	*L. casei*	Mouse	+	Oral	Porcine rotavirus	, WB, NA, ELISA,	NM	NM	NM	IgA, IgG	NM	Qiao *et al.* 2009 [79]
Heat-labile enterotoxin B (LTB)	*L. casei*	Mouse	+	Oral	Enterotoxigenic *E. coli*	WB, ELISA, CE	+	NM	NM	IgG, IgA	NM	Yu *et al.* 2016 [77]
Heat-labile enterotoxin B (LTB)	*L. plantarum*	Mouse	+	Oral	Avian influenza (H9N2)	WB, ELISA, FACS, ELISA, HP	+	+	+	IgG, IgA	+	Jiang *et al.* 2017 [78]

Other Immune molecules	Vector/ organism	Animal model	Ag	Delivery	Application	Techniques	Protective efficacy from challenge	Th1 response	Th2 response	Antibody measured	BW & organ analysis	Reference
C3d3	*L. casei*	Mouse	+	Vaginal inoculation	Enhance contraceptive efficiency	WB, CLM, ELISA, ELISPOT, FC	NM	NM	+	IgG, IgA	NM	Yao *et al.* 2007 [84]
CKS_9	*L. lactis*	Mouse	+	Oral	Evaluate immune response	CC, IHC, ELISA, ELISpot	NM	+	+	IgG, IgA	NM	Li *et al.* 2015 [83]
DEC-205	*L. plantarum*	Mouse	+	Oral	Bacterial internalisation	FACS, WB, ILA, CMA	NM	NM	NM	NM	+	Christophe *et al.* 2015 [81]
GM-CSF	*M. bovis BCG*	Mouse	+	SC injection	Tuberculosis	ELISA, CAFC, LA	+	+	–	IgG, IgG2a, IgG1	+	Yang *et al.* 2011 [80]
IgG-Fc	*L. plantarum*	Mouse	+	Oral	H1N1 influenza virus	WB, CE, FC, HP, ELISA, ELISPOT	+	+	–	IgA	+	Yang *et al.* 2016 [82]
N-glycan	*E. coli*	Chicken	+	Oral	Gastroenteritis	ELISA, WB, FACS, NMR, CE, MBS	+	NM	NM	IgY	+	Nothaft *et al.* 2016 [86]

'+' – Antigen present; '-' – Antigen absent; AT – Agglutination test; BWG – Body weight Gain; CC – Cell culture; CE – Challenge experiment; CLM – Chemiluminescence; CMA – Competition assay; CT – Cloning and Transformation; CTL- Cytotoxic *T*-lymphocytes; DC- Dendritic cell; ELISA- Enzyme-linked immunosorbent assay; ELISPOT- Enzyme-linked immunospot assay; EM – Electron microscopy; FACS- Fluorescence-activated cell sorting; FC – Flow cytometry; hCG – Human chorionic gonadotrophin; HIV- Human immunodeficiency virus; HPLC- High performance liquid chromatography; HP – Histopathology; IB – Immunoblot; IFM – Immunofluorescent microscopy; IHC – Immunohistochemistry; IG- Intragastric; ILA – Internalization assay; IN- Intranasal; IP- Intraperitoneal; IV- Intravenous; Ig- Immunoglobulin; LA – Lymphoproliferation assay; LS – Lesion score; LVT – Lung viral titre; MBS – Microbiome studies; MLN- Mesenteric lymph nodes; NMR- Nuclear magnetic resonance; NM – Not measured; PBMC- Peripheral blood mononuclear cells; PEA – Protein expression assay; PP- Peyer's patch; qRT-PCR- Quantitative reverse transcription polymerase chain reaction; SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis; SC- Subcutaneous; Th- *T*-helper cell; TP – Tissue pathology; WB – Western blot.

VSV [27]. *Mycoplasma gallisepticum* (MG) is a poultry pathogen known to cause respiratory disease and loss of eggs. A live-attenuated vaccine, Vaxsafe® MG (strain ts-11), has been used to control this disease worldwide and is administered to birds as eye drops [31]. The vaccine strain results in upper respiratory tract colonisation only. In a study aimed at enhancing response to this vaccine, chicken IFN-γ was cloned into a MG ts-11 strain using a transposon-based delivery vector. Chickens immunized with MG ts-11 expressing IFN-γ produced greater heterophil infiltration of the tracheal epithelium. Given that heterophils are the avian equivalent of mammalian neutrophils, this indicated an enhanced mucosal cellular immune response. This study lacked challenge experiments and so did not demonstrate that the overall effectiveness of the modified vaccine had been enhanced [32].

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) contains more than 100 virulence genes which can potentially be mutated to produce an attenuated strain that confers immunity. In a study by Al Ojali *et al.* 2013 [33], a recombinant *S.* Typhimurium strain was used to express murine IFN-γ to induce immunity in immunodeficient mice. A Toll-like receptor 4 (TLR4), member of the PRR family, deficient C3H/HeJ mouse strain which is hypersusceptible to *Salmonella* was used to test for an immune response. After immunization of the C3H/HeJ mice with *S.* Typhimurium expressing murine IFN-γ at the optimal dose, they were challenged with virulent strains and deaths were scored. The results from this study showed that at optimal dosage, immunization conferred protection in 100 % of vaccinated mice when compared to the unvaccinated control group [33].

The immunomodulatory functions of IFN-γ give it strong potential as an adjuvant when delivered using recombinant vectors and has been shown to be effective in animal models and some livestock vaccines.

2.2. Interleukins

Interleukins are a class of cytokines secreted by lymphocytes that promote the development and maturation of T- and B-cell populations. Interleukin 2 is secreted by activated CD4 and CD8 cells and plays a key role in inflammatory responses. An approach to enhance the immunogenicity of the existing BCG vaccine was to incorporate IL-2 as an adjuvant, resulting in a strong Th1 response and IFN-γ production. A study by Fan *et al.* (2006), concluded that recombinant BCG (rBCG) that expressed IL-2 enhanced the Th1 immune response in immunized mice compared with native BCG [34]. Other studies utilizing BCG have used IL-12 and IL-15 (both pro- inflammatory interleukins) as adjuvants. Recombinant BCG expressing IL-12 elicited a stronger Th1 type cell-mediated immune response in mice compared to the conventional BCG and rBCG antigens alone [35]. In a study by Tang *et al.* 2007, rBCG expressing IL-15 immunized mice showed enhanced $CD8^+$ and $CD4^+$ *T*-cell responses when compared with the antigen only control group. There was robust protection in the lungs when the mice were intratracheally challenged with *M. tuberculosis* [36]. Steidler *et al.* (1998), used *L. lactis* to express IL-6 in mice to compare with a naïve, non-immunized group of animals. It was found that IgG and serum IgA levels were higher than in the control group. Recombinant *L. lactis* secreting biologically active IL-2 or IL-6 elicited significantly higher (10–15 fold) antibodies than antigen alone control groups [37].

In addition to the use of interleukins as adjuvants in challenge models, there have been qualitative studies to evaluate the immunological and beneficial effects of interleukins. *Lactobacillus casei* was genetically engineered to express interleukin-1β (IL-1β) [38]. *Lactobacillus rhamnosus* strain GG (LGG) is a probiotic organism which has been used to express a fusion protein of green fluorescent protein (GFP) and interleukin-2 (IL-2) to examine bacterial uptake and immune response. IL-2 fusion with GFP increased antibody production and also increased CTL activity which led to the conclusion that even low levels of adjuvant were enough to enhance immune responses [39].

Live recombinant LAB was used to deliver the E7 antigen from human papilloma virus type 16 (HPV-16), the leading cause of cervical cancer, and interleukin-12 (IL-12) as an adjuvant and was shown that mucosal vaccination evoked a strong antigen-specific cellular immune response, which led to anti-HPV-16 tumour effects [4]. In the study by Cortes-Perez *et al.* (2007), a comparison was made between intragastric and intranasal routes of administration and two different LAB strains (*L. lactis* and *Lactobacillus plantarum*). It was confirmed that the intranasal route of immunization by *L. plantarum* was more efficient than intragastric immunization to induce antigen-specific mucosal and systemic immune responses in mice [40]. In addition *L. lactis* vectors were used to deliver antigens and IL-12 for immunization of mice against leishmaniasis [41,42], and IL-2 in the prevention of avian influenza in poultry [43]. Other studies aimed at adjuvant therapy to reduce inflammation have used recombinant *L. lactis* to deliver antigens with the anti- inflammatory IL-10 in the control of Crohn's disease in humans [44], IL-35 to ameliorate collagen-induced arthritis in mice [45], and IL-10 in the treatment of inflammatory bowel syndrome [46].

The studies reviewed here show that co-expressed interleukins have been successfully used as adjuvants in live-vectored vaccines which provide other beneficial effects like increased survival of antigens. The details and results of additional interleukin studies are summarized in Table 1.

2.3. Pattern recognition receptor ligands

Toll-like receptors (TLR) are a type of pattern recognition receptor (PRR) which are an important activator of the innate immune system.

Despite not being an immune modulator *per se*, flagellin, a component of bacterial flagella, is an important TLR5 ligand and stimulates production of chemokines and cytokines through myeloid differentiation factor 88 (MyD88) signalling [2]. Flagellin binds to the cytosolic nucleotide binding oligomerization domain-like receptors (NLR) NLRC4, which leads to caspase-1 inflammasome activation. A fusion protein of *Salmonella* flagellin FliC, which is flagellar antigen, and a *Salmonella enterica* antigen, SipC, involved in translocation of effectors and actin modulation, was expressed by *L. casei* and tested in a mouse model to determine its immunological properties. Results from the study concluded that adaptive immunity, mainly of the Th-1 type, was elicited and also high levels of IFN-γ were induced [47]. A similar study by Kajikawa *et al.* 2007, using purified orally administered FliC and recombinant *L. casei* expressing *Salmonella* FliC in mice showed that the *L. casei* vaccine induced IFN-γ production more efficiently than immunization with purified FliC, and significantly higher protective immunity against *Salmonella* infection [48].

In a study by Stoeker *et al.* (2011), genetically modified *Lactobacillus gasseri* expressing intracellular FliC was used. Mice immunized orally with the recombinant *L. gasseri* had increased proinflammatory cytokine/chemokine release, high IL-10/IL-12 ratios in myeloid DC, activation of TLR2/6 and TLR5, and diversification of B-cell populations in the colon, compared to mice immunised with wild type *L. gasseri.* Immunization also increased the T_{reg}/Th17 ratio of lymphocytes in the colonic lamina propria [49].

TLR5 is highly expressed in the lung and intestinal epithelial cells and professional APC such as DC and macrophages. Flagellin therefore has potential as a mucosal adjuvant to facilitate immune activation and delivery of antigen to APC [2,47] (Table 2).

2.4. Dendritic cell (DC) targeting adjuvants

DC targeting peptides (DCpep) binds to ligands on DC and has been shown to stimulate *T*-cell production and cell-mediated immunity in studies to elicit protection against various diseases. It facilitates the efficient delivery of antigen to DC and therefore act as a vaccine adjuvant to trigger strong immune responses.

Bacillus anthracis protective antigen (PA) fused to DCpep have been tested for efficacy against anthrax. A *Lactobacillus acidophilus* vaccine candidate designed to deliver PA-DCpep activated DC, which in turn induced neutralizing antibodies, IgA secretion, and *T*-cell immunity against *B. anthracis* [50]. Oral administration of *L. gasseri*, which expressed PA-DCpep, elicited robust toxin neutralizing antibodies, and showed protective efficacy in challenge studies. The results showed that expression of the PA-DCpep fusion skewed the response towards a Th1 response and confirmed that oral administration of the vaccine led to mucosal and systemic immune responses [51].

Porcine epidemic diarrhea virus (PEDV), an enteric coronavirus, is the causative agent of porcine epidemic diarrhea (PED) characterised by damage to intestinal epithelial cells and leads to 100 % mortality in piglets [52]. In a series of studies, genetically engineered *L. casei* expressing PEDV core neutralizing epitope (COE) antigen fused to DCpep was tested as a vaccine candidate in mice [23,52,53]. Results confirmed that this live vaccine elicited sIgA-based mucosal and IgG-based humoral immune responses via oral vaccination. Recombinant *L. plantarum* expressing the spike protein (S) of PEDV capsid and DCpep fusion was used to induce DC activation and high production of sIgA and IgG in experimental animals but lacked any challenge studies to determine the protective efficacy of the vaccine candidate [54].

L. plantarum NC8 was used to present vaccine antigens such as hemagglutinin (HA), core nucleoprotein (NP) and matrix protein (M1) against avian influenza fused to DCpep in mouse and chicken models [55–57]. Recombinant *L. lactis* expressing *Eimeria tenella* 3-1E protein, a conserved surface protein of both merozoites and sporozoites, fused to DCpep was used to orally immunize chickens with subsequent challenge studies using sporulated oocysts to determine protective efficacy [58]. Another study focused on using genetically engineered *L. plantarum* expressing *E. tenella* SO7 protein, an abundant protein in sporozoites associated with the refractile bodies, fused to DCpep, in broiler chickens and challenged with sporulated oocysts [59].

Recombinant LAB have been used to induce immune responses of mice against bovine viral diarrhoea virus (BVDV) [60], lethal doses of botulinum neurotoxin A [40], and enterotoxigenic *E. coli* [62], and in protection of chickens against Newcastle disease virus (NDV) [63]. DCs located in the lamina propria mucosa are able to extend dendrites through the basement membrane and between epithelial cells into the gut lumen of the host [50]. In this way DC can efficiently capture foreign and self-antigens and present them to *T*-cells and thereby elicit a Th1, Th2, Th17, and/or T_{reg} response based on the circumstances [55]. Hence, DCs are considered a bridge between host innate and adaptive immunity and a DC targeting strategy has been gaining more interest in the field of vaccine design [50,55]. Details and results from the studies involving DCpep as an adjuvant are listed in Table 2.

2.5. Tumour necrosis factor ligand

The CD40 ligand (also called CD154), which belongs to the TNF superfamily ligands, is a glycoprotein expressed on the surface of activated *T*-cells, mast cells and basophils. CD40L/CD154 binds to CD40 on APC and can promote B-cell maturation and induction of primary and secondary CD8 + cytotoxic T lymphocyte responses [64]. The receptor activator of NF-kappa-beta ligand (RANKL), a member of the TNF superfamily ligands, has been shown to be important in M−cell differentiation in Peyer's patches and thereby indicates a role in inducing gut mucosal immunity. RANKL interacts with receptor activator of NF-kB (RANK) expressed on the sub-epithelium of Peyer's patches and this molecular signalling is an essential regulator of bone remodelling, and establishment of the microenvironment of the thymus and lymph nodes [65].

Attenuated *Salmonella* Enteritidis strains expressing avian influenza (AI) matrix 2 protein, extracellular domain (M2e antigen), along with immune-enhancing CD154 (CD40L) have been tested for protective efficacy in chickens and turkeys and found to elicit an IgG-mediated humoral immune response, and reduced organ invasion and colonization in commercial birds [66,67]. In another study, oral delivery of *Salmonella* Gallinarum (SG, causative agent of fowl typhoid) with the M2e antigen and CD40L, to elicit immunity against avian influenza (AI) and fowl typhoid (FT) resulted in M2e-specific humoral and cell-mediated immunity. The immunized birds challenged with AI virus exhibited lower lung inflammation and reduced viral loads in cloacal and lung samples [68]. This study demonstrated that vaccination with live vaccines can be used to elicit protection against more than one disease using suitable antigens. Vaccination with attenuated *Salmonella* strains expressing antigens augmented with cytokine adjuvants has also been used in poultry to protect against coccidiosis, an enteric disease caused by *Eimeria* spp. parasites [69].

L. lactis IL1403 was used as a live carrier for RANKL (RANKL-LAB) because of its inherent benefit of protecting the recombinant protein from low pH and enzymatic degradation in the gut of mice. The adjuvant effect of sRANKL-LAB was measured by administration of M−BmpB, model subunit antigen developed against *Brachyspira hydrosenteriae,* to check for protection against mucohaemorrhagic dysentery [65]. Immunoglobulin levels in mouse faecal extracts and intestinal lavage fluid were analysed and it was found that vaccination led to enhancement of systemic and mucosal immune responses by increasing M−cell numbers and thus improving transcytosis [65]. *Lactobacillus lactis* expressing spike protein from PEDV and immunomodulating RANKL was used as an oral vaccine candidate in pregnant pigs to compare the survival rate with a commercial, killed vaccine [70].

The CD40 ligand and RANKL have shown potential as a powerful immunological adjuvant in various studies. They have been shown to stimulate humoral and cell-mediated immune responses [66,68]. The details and results from various studies are presented in Table 3.

2.6. Bacterial toxins as adjuvants

Bacterial exotoxins are biological proteins that are secreted by bacteria like *Vibrio cholera, Escherichia coli, Bordetella pertussis, and Pseudomonas aeruginosa* that assist the bacteria to invade and damage tissues [71]. These toxins possess ADP-ribosylating activity and their non-toxic form has an ability to bind to receptors on the epithelial cell surface, thereby, facilitating their delivery to the underlying lymphoepithelial tissue. Cholera toxin (CT) secreted by *Vibrio cholera* and heat-labile enterotoxin (LT) secreted by *E. coli* are the most extensively studied mucosal adjuvant in animal models, belonging to the AB_5 class of bacterial exotoxins and consist of a receptor-binding pentameric B subunit and an enzymatically active A subunit [71,72].

In a study by Columbi *et al.* (2006), CT subunit B (CTB) has been expressed in *L. casei* along with *Bordetella pertussis* filamentous haemagglutinin adhesin (FHA) to protect against whooping cough [73]. CTB was used as a vaccine adjuvant in an avian influenza vaccine where recombinant *L. lactis* was used to express a gene fusion of hemagglutinin (HA) from H5N1 and Poly-γ-Glutamic Acid Synthetase A (PgsA) from *Bacillus subtilis* as a C-terminal anchor [74].

This live vaccine was found to elicit HA-specific serum IgG and faecal IgA immune responses and protection upon challenge by H5N1 virus [74].

CT subunit A1 (CTA1) has been used as an adjuvant in the study by Li *et al.* (2015), along with influenza antigens on the surface of *L. casei*. Oral and nasal administration of recombinant *L. casei* resulted in IgG, IgG1, IgG2a and IgA. Seven months after immunization, the mice were found to show antigen specific antibodies and low viral titres in their lungs after challenge, indicating long-lasting immunity [75]. In another approach, recombinant *L. casei* expressing CTA1 conjugated with matrix protein-2 (sM2) on the surface confirmed that *L. casei* expressing CTA1-conjugated sM2 protein on its surface eicited protective immune responses against diverse influenza subtypes [76].

The well-studied bacterial protein heat-labile toxin (LT) of *E. coli* consists of subunit A (LTA), and subunit B (LTB) act as an adjuvant to increase mucosal and adhere to epithelial surfaces by different mechanisms, activate DC, aid in APC presentation, and APC-T cell interactions [77,78].

Porcine rotavirus infection leads to diarrhea in piglets, morbidity and mortality in swine. A live vaccine *L. casei* utilizing outer capsid protein VP4 and LTB was used to orally immunize mice and secretions from gastrointestinal tract, vagina and eye were used to test for IgA and IgG response. There was no efficacy studies carried out as part of this study which would be the next step in determining the usage in a porcine model [79]. Enterotoxigenic *E. coli* (ETEC) infections leads to diarrhea and mortality in piglets leading to large economic losses in pig farming. Recombinant *L. casei* expressing fimbrial adhesin (FaeG), a non-toxic form of LTA obtained by amino acid mutation of 63rd amino acid serine to lysine (LTAK63), and LTB has been used to orally immunize mice. Upon challenge with ETEC, the antigen alone immunized mice showed severe diarrhoea with mortality in 80 % of mice and the mice immunized with antigen and LT adjuvant showed 100 % protection against ETEC with only mild diarrhea lasting 2–3 days [77].

LTB has been used as an adjuvant in the live vaccine against H9N2 virus with surface displayed haemagglutinin subunit 2 (HA2) as a viral antigen. Challenge studies were carried out to test for protective effects of recombinant *L. plantarum* and results showed protection against AIV challenge, body weight gains, increase of cytokines and decreased lung lesions in histopathologic analysis [78]. The details and results from various studies using bacterial toxins as adjuvants are presented in Table 3.

2.7. Other immunomodulatory proteins as adjuvants

Several additional immune modulating molecules have been used as adjuvants in live vectors. Selected examples are discussed below, and the studies are summarized in Table 3.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been used as an adjuvant to enhance the immunogenicity of the conventional BCG vaccine in a mouse model. GM-CSF can recruit DC and stimulate *T*-cells and NK cells, and stimulates upregulation of MHC class II and co-stimulatory molecules such as CD80 and CD86 on APC [80].

DEC-205 is a C-type lectin receptor involved in recognition of ligands expressed during apoptosis and necrosis of cells upon recognition of CpG oligonucleotides and in antigen processing. Several studies have used this receptor as a target in vaccine improvement and in increasing tolerance to tumours and autoimmune diseases [81].

The neonatal Fc receptor (FcRn) of immunoglobulin G, a cell surface molecule of immunoglobulin Fc fragment, is extensively expressed on the surface of effector cells, accessory cells, and mucosal epithelial cells in adulthood. Before the neonatal immune system matures, FcRn transports maternal IgG, which plays an important role in providing immune defence against pathogens. Previous studies have reported that FcRn can improve humoral and cellular immune responses [82].

A M−cell targeting peptide ligand, CKS_9, consists of 9-amino acid in cyclic conformation which improves the transport of antigens from the gut lumen into the cell. In the study by Li *et al.* (2015), recombinant *L. lactis* was used to express IL-6 and CKS_9 in mice and various immunological tests were carried out to test for immune response [83].

In a contraceptive vaccine study by Yao *et al.* 2007, to enhance contraceptive efficiency, human chorionic gonadotrophin (hCG)-beta subunit acted as a target antigen for the treatment of trophoblastic disease [84]. C3d is a fragment of the complement complex which plays a key role in humoral immune regulation by interacting with its receptor CR2 on B-cells. Fusing antigens to C3d can promote immune recognition. *Lactobacillus casei* was used as a live vector to express human chorionic gonadotrophin (hCG)-beta subunit (hCGβ-C3d3) protein to immunize mice. This study provides a theoretical foundation to solve one of the shortcomings of the hCG immune-contraceptive vaccine, variable and inadequate responses in some individuals [84].

N-glycosylation can greatly influence immune responses and increase the immunogenicity of antigens. The attachment of glycans enables recognition by endocytic receptors like the mannose receptor (MR) and show increased uptake by DC thereby leading to DC activation [85]. Furthermore, glycosylated proteins can be presented by MHC-I and MHC-II, leading to recognition by *T*-cells and glycan-dependent Th cell and CTL responses [86].

2.8. Vaccination strategies to target B cell memory

T follicular helper cells (Tfh) are a specialised subset of $CD4^+$ T cells located in secondary lymphoid organs that play an essential role in the formation of germinal centres (GC) [87]. GC are distinct structures which form in secondary lymphoid organs and produce long-lived antibody secreting plasma cells and memory B cells [87,88]. Several studies have shown that there are ways to directly target these cells and compartments using immune modulating proteins. A recombinant rabies viral vaccine expressing IL-21 was found to induce virus neutralising antibodies (VNA) by activation of Tfh cells and GC B cells in a mouse model lethal challenge [88]. B-cell activating factor (BAFF) and (a proliferation-inducing ligand) APRIL belong to the TNF superfamily ligands and are *T*-cell independent pathways which leads to GC stimulation and higher chances of protection during a re-infection. These pathways were targeted in a DNA adjuvant approach, where BAFF or APRIL were expressed as multitrimers using surfactant protein D as a scaffold. When these were administered with IL-12 as an adjuvant system for Human Immunodeficiency virus (HIV) Env proteins, enhanced GC activity, and neutralizing antibodies were detected in a mouse model [89]. More recently, BAFF and APRIL were incorporated into Virus-like Particles (VLPs) by fusion with the influenza haemagglutinin in an experimental multimeric subtype vaccine [90].

Dendritic cell (DC) based vaccination is an emerging immunotherapeutic strategy against cancer and shows promising results using a cocktail of cytokines for antigen priming [91]. As an attempt to increase the longevity of DC response and survival of DCs during immune response, an anti-apoptotic B-cell lymphoma 2 (Bcl-2) family member has been used [92]. anti-tumour T cell priming cytokine IL-12 was used in conjunction with Bcl-2 to improve the efficacy of DC vaccine and was found to enhance $CD8^+$ T cells secreting IFN-γ and TNF-α. This combination approach of using a cytokine cocktail for antigen priming, anti-apoptotic Bcl-2, and anti-tumour IL-12 provides a stronger immunotherapeutic strategy against cancer [92].These human vaccine approaches use

a combination of immune modulating molecules with adjuvant strategies to enhance immunogenicity, and have the potential to be applied in veterinary applications.

3. Discussion and conclusion

Despite the widespread use of vaccines, there is still much to learn about how best to induce appropriate, strong, and reproducible effects in target hosts. The use of live organisms to deliver antigens to mucosal surfaces provides an efficient mode of delivery to APC and assists in priming of the innate immune system. This review has surveyed research that has investigated how co-delivery of proteinaceous immunomodulatory molecules have been used to enhance live bacterial antigen delivery. Delivery of a wide variety of immunomodulatory proteins have been shown to modify immune responses by mechanisms including the release of cytokines and chemokines, activation of Th cells and B-cells, and stimulation of IgG and IgA mediated effector functions. This approach has been show to induce both $CD4^+$ and $CD8^+$ immune responses leading to protective efficacy upon pathogen challenge [26] and regression in tumour models [4,36,41]. In some studies, antigens have not been used and only the immune active molecules have been delivered. Such studies have aimed to understand the degree of immune stimulation induced by the immune active molecules without necessarily connecting them to protection [27,38,44–46,48,93]. Unless clear immune response that correlates to protection has previously been determined, it can be difficult to draw conclusions about the effectiveness of delivery. Studies employing challenge experiments provide an opportunity to link immune responses to protection and this can help determine whether any increased immune activity enhanced vaccine efficacy.

The adjuvant strategies reviewed here resulted in increased immune responses to live vaccines. Live vector vaccines have various advantages over conventional vaccination, including low cost of production, possibility of using GRAS organisms in feed or water, and stimulation of mucosal immune responses. However, the immune response to live delivered antigens is often weaker than required to produce good levels of protection. Protective efficacy months after immunization is the decisive factor for a successful live vaccine.

Vaccines for livestock, particularly low unit value, high volume food animals such as chickens, present particular challenges in that they must not only be safe for downstream consumption, but also be feasible for simple low-cost bulk administration, preferably in feed, water, or spray application. Live vector vaccination strategies lend themselves to these applications. Co-delivery of immune active proteins with vectored antigens promises to enhance vaccine efficacy and extend the range of pathogens that can be successfully addressed with live vaccines.

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Data availability

This is a review article. Ne new experimental data is included.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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