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REVIEW ARTICLE

Current Progress and Challenges in the Study of Adjuvants for Oral Vaccines

Bingming Ou[1] · Ying Yang[2] · Haihui Lv[1] · Xin Lin[1] · Minyu Zhang[1,3]

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Abstract

Over the past 20 years, a variety of potential adjuvants have been studied to enhance the effect of oral vaccines in the intestinal mucosal immune system; however, no licensed adjuvant for clinical application in oral vaccines is available. In this review, we systematically updated the research progress of oral vaccine adjuvants over the past 2 decades, including biogenic adjuvants, non-biogenic adjuvants, and their multi-type composite adjuvant materials, and introduced their immune mechanisms of adjuvanticity, aiming at providing theoretical basis for developing feasible and effective adjuvants for oral vaccines. Based on these insights, we briefly discussed the challenges in the development of oral vaccine adjuvants and prospects for their future development.

Key Points

Biogenic adjuvant materials used in the study of oral vaccines include bacteria-derived adjuvants, biologic proteins or peptides, intestinal immune cells targeting peptides, and some small-molecule immunomodulatory proteins.

Non-biogenic adjuvant materials used in oral vaccine studies mainly involve biodegradable polymers {such as poly(D,L-lactide-*co*-glycolide) [PLG], poly(D,L-lactic-*co*-glycolic acid) [PLGA], chitosan and their derivatives, alpha-galactosylceramide [α-GalCer], ulex europaeus agglutinin-1 [UEA-1]}, and some synthetic toll-like receptor agonists and their derivatives.

The combination of multitype materials has been used to design oral adjuvants; some protein vaccines (or biogenic adjuvants) are usually coated (or capsuled) with polymer-based microparticles/nanoparticles to prevent degradation in mucosa; some biogenic adjuvants are usually combined with the engineered living intestinal beneficial bacteria as a carrier to construct oral vaccine candidates.

1 Introduction

Pathogens initiate infection mainly by accessing the mucosal surface of the host, especially from oral-to-gastrointestinal tract (GIT). It is generally considered that direct vaccination via the mucosal surface at the initial site of infection is the most effective way to trigger protective mucosal immune response against pathogens [1–3], but the vast majority of vaccines are administered by injection [2]. Compared with parenteral vaccination (or traditional injection), peroral vaccination or administration requires less stringent regulatory requirements, allowing for the self-administration of oral formulations. For humans, oral vaccination will minimize the need for trained healthcare personnel [4, 5] and eliminate occupational needle-stick injuries, which could reduce blood-borne infectious diseases such as acquired immune deficiency syndrome (AIDS)/human immunodeficiency virus (HIV) and hepatitis [6, 7]. For animal husbandry and aquaculture, the use of oral vaccination for disease prevention and control can reduce the labor cost of animal management and reduce the stress response of animals. Therefore, oral vaccination is potentially easier, safer, more convenient, more time-saving, and more economical [8].

Bingming Ou and Ying Yang contributed equally to this work.

✉ Minyu Zhang
zhangminyu_zmy@126.com

[1] School of Life Sciences, Zhaoqing University, Zhaoqing, China

[2] College of Animal Science, Guizhou University, Guiyang, China

[3] School of Physical Education and Sports Science, South China Normal University, Guangzhou, China

However, oral vaccination is still challenging because most oral vaccinations universally could not trigger sufficient immune response, mainly because of an inadequate specific secretory immunoglobulin A (sIgA) response. There are two main reasons for this dilemma. First, the harsh conditions of the gastrointestinal environment, including hydrochloric acid, digestive enzymes, bile salts, mucus, antimicrobial peptides, and gastrointestinal peristalsis, would cause low bioavailability of antigens, which leads to acquired immune tolerance instead of stimulation [9–12]. Second, antigen-presenting cells (APCs) residing in the GIT are tolerogenic and hyporesponsive [13]; therefore, substantial impediments exist for oral vaccines to reach the inductive site of the mucosa-associated lymphoid tissues (MALT) and to trigger immune response, which critically hinders the effectiveness of oral mucosal immunization.

Given the poor immunogenicity (or low bioavailability) of oral vaccines, using appropriate and effective oral mucosal adjuvants may be critical to the success of peroral mucosal vaccination. Typical adjuvants, such as alum, complete Freund's adjuvant (CFA), and incomplete Freund's adjuvant (IFA), etc., have long been used in injectable vaccination but they do not work well in peroral mucosal immunity. To successfully stimulate intestinal mucosal immunity, oral vaccine adjuvants need to have two main properties—GIT delivery stability and intestinal mucosal adjuvanticity—because they must find an effective way to deliver vaccines (or antigens) to the dendritic cells (DCs), macrophages, and lymphocytes located in MALT through these natural barriers in GIT, and exert their adjuvant properties, while protecting the loaded vaccines (or antigens) from the harsh peroral mucosal environment. Therefore, it is necessary to explore effective peroral mucosal adjuvants to improve the effectiveness of oral vaccines; however, to date, no adjuvants have been included in licensed oral vaccines [14].

In recent years, many researchers have focused on finding safe and effective adjuvants (or delivery systems) to formulate oral vaccines, and have made great progress in the development of oral vaccine adjuvants. At present, some potential oral mucosal adjuvants have shown promising prospects, for instance modified bacterial enterotoxins (e.g., double-mutant heat-labile toxin [dmLT] of *Escherichia coli* and multiple mutant cholera toxin [mmCT]), some small molecule immunomodulatory proteins, and some non-biogenic biodegradable polymer materials {e.g., poly(D,L-lactide-*co*-glycolide) [PLG], poly(D,L-lactic-*co*-glycolic acid) [PLGA], alpha-galactosylceramide [α-GalCer], Ulex europaeus agglutinin-1 [UEA-1]}, and some synthetic toll-like receptor (TLR) agonists and their derivatives.

In this review, we systematically summarize various peroral adjuvant candidates, including the completed, ongoing, and planned study candidates. According to the physicochemical properties of these peroral adjuvant candidates, we classified the existing or emerging oral vaccine adjuvants as biogenic adjuvants (including bacteria-derived or biologic protein, peptide, or immunostimulants, small-molecule proteins, etc.), non-biogenic adjuvants (e.g., various biocompatible polymer-based microparticles/nanoparticles), and biogenic and non-biogenic composite adjuvants. We also introduced their general properties, mechanisms of adjuvanticity, origins and brief histories, preparation processes, and results of preclinical studies or even clinical studies, and discussed prospects for their application as oral vaccine adjuvants. This article reviews the research progress of oral adjuvants in recent years, aiming to promote the application prospects of oral vaccines.

2 Biogenic Type Oral Vaccine Adjuvants

At present, many effective oral vaccine adjuvants are still derived from biological material, such as bacteria-derived adjuvants (e.g., bacterial enterotoxins, bacterial flagellin, bacteria-derived enterocyte-targeting proteins, and some bacteria-derived proteins), protozoan-derived adjuvants, intestinal immune cells targeting peptide adjuvants, small molecular immunomodulatory proteins (SMIPs; e.g., cytokines and thymosin α-1 [Tα1]), Fc region of immunoglobulin (Ig) G, and adjuvants composed of multiple biogenic materials (Table 1).

2.1 Bacteria-Derived Adjuvants for Oral Vaccines

Targeting specific bacterial organelles or components, the host's immune system has evolved to recognize infections and activate the most potent immune cells to fight the pathogenic bacteria. When developing vaccines, adding appropriate bacterial organelles or components into vaccines would produce a stronger immune response to provide better and more enduring immune protection against infections. Bacteria-derived adjuvants have attracted particular interest for the development of oral vaccines because specific bacterial organelles or components have a role as immune stimulators.

2.1.1 Bacterial Enterotoxin Adjuvants

The most well-studied mucosal adjuvants to date are still the adenosine diphosphate (ADP)-ribosylating bacterial enterotoxins, such as cholera toxin (CT) produced by *Vibrio cholerae*, heat-labile toxins (LT) produced by enterotoxigenic *Escherichia coli* (ETEC), as well as their mutants or subunits. Initially, CT and LT were not only highly effective mucosal adjuvants but they were also very toxic, which precluded their clinical application. However, much effort has

Table 1 Current developments in biogenic adjuvants for oral administration vaccines

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
Bacterial-derived oral vaccine adjuvants						
Bacterial enterotoxins						
dmLT	Double mutant (R192G L211A) heat-labile toxin from ETEC	Bacterial diarrhea in children and travelers (ETEC)	Oral vaccination with EtpA and dmLT	CD-1 mice	Significant protection against small intestinal colonization of ETEC strain The degree of protection correlated with fecal IgG, IgA, or total fecal antibody responses to EtpA	[199]
dmLT	As above	Bacterial diarrhea in children and travelers (ETEC)	Orally administered with an attenuated ETEC vaccine candidate (ACE527) and dmLT	Human trial	Challenge strain shedding was tenfold lower in those receiving the adjuvant than those receiving vaccine alone The unadjuvanted vaccine was not protective 83% showed significant mucosal IgA responses Significantly increased intestine-derived anti-CS6 responses compared with vaccine alone	[28–30]
dmLT	As above	Bacterial diarrhea in children and travelers (ETEC)	Orally administered with an oral ETEC vaccine (ETVAX) and dmLT	Phase I/II trial in Bangladesh	Enhanced the magnitude, breadth, and kinetics of immune responses in infants	[187, 188]
dmLT	As above	*H. pylori* infection	Orally administered with *H. pylori* lysate antigens and dmLT	C57BL/6 mice	Significant decrease in bacterial load compared with the unimmunized controls The same extent as CT as an adjuvant Enhanced *in vitro* proliferative and cytokine responses to *H. pylori* antigens	[200]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
dmLT	As above	*H. pylori* infection	Oral immunization with recombinant *H. pylori* protein antigens (NAP/UreA/UreB) and dmLT	BALB/c mice	Enhanced antigen-specific lymphocyte proliferation, and serum IgG and mucosal IgA responses Increased the proportion of $CD4^+$ $IL\text{-}17^+$ lymphocytes Enhanced the production of IL-17, IL-16, IL-6 and TNFα Confers more effective prophylactic protection against *H. pylori* infection	[201]
LTB	*Escherichia coli* heat-labile enterotoxin B subunit protein	H5N1 HPAI	H5N1 chimeric VLPs composed of the viral HA, NA, and M1 proteins and LTB	BALB/c mice	Conferred substantial protection against lethal challenge Showed tenfold higher virus-specific IgG titers than mice immunized with H5N1-VLPs lacking LTB	[39]
CT	Cholera toxin	Necrotic enteritis (*Clostridium perfringens type A*)	Orally administered live vaccine (non-virulent NetB-producing strain of *C. perfringens*) and CT	Broiler chickens	55% of vaccinated birds did not develop any lesions of NE after challenge, compared with 100% incidence in the unvaccinated group	[202]
mmCT	Multiple mutant cholera toxin	*H. pylori* infection	Intragastric immunizations with formalin-inactivated *H. pylori* whole cell vaccine admixed with mmCT	C57BL/6 mice	50- to 125-fold reduction in colonization of *H. pylori* Rise in both serum IgG and intestinal mucosal IgA anti-*H. pylori* antibody responses Strong T cell and IFNγ and IL-17A cytokine responses	[32]
Bacterial flagellin						
FliC	*Salmonella enterica* serovar Typhimurium flagellin	H5N1 HPAI	H5N1 chimeric VLPs composed of the viral HA, NA, and M1 proteins and FliC	BALB/c mice	Conferred substantial protection against lethal challenge Showed tenfold higher virus-specific IgG titers than mice immunized with H5N1-VLPs lacking FliC	[39]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
FliC and FljB flagellins	Diphasic *S. typhimurium* possesses two flagellins (phase I flagellin FliC and phase II flagellin FljB)	*Salmonellosis*	The recombinant attenuated *S. typhimurium* BRD509 strain (the *iacP* mutant-strain) expressing FliC and FljB flagellins	BALB/c mice	Strongly enhanced NF-κB activation and proinflammatory cytokine expression *in vitro* and *ex vivo*	[42]
Flagellin	*Salmonella enterica* subsp. flagellin	Rabies viruses	Recombinant rabies viruses expressing flagellin (LBNSE-Flagellin)	ICR mice	LBNSE-Flagellin is more immunogenic than the parent virus Induce greater activation and maturation of DCs and B cells	[34]
Flagellin	*S. typhimurium* flagellin	Fowl typhoid (*Salmonella gallinarum*)	*S. gallinarum* ghosts expressing *S. typhimurium* flagellin	Female layer brown nick chicks	Improved antigen-specific humoral and cell-mediated immune responses Enhanced protective efficacy against the virulent challenges	[41]
Flagellin	*Salmonella* flagellin	HBV	A live recombinant *Salmonella dublin* vaccine strain expressing HBsAg epitopes inserted in the hypervariable region of a cloned *Salmonella* flagellin gene	Guinea pigs and mice	Developed detectable antibodies specific to HBV epitopes	[45]
Flagellin	*Salmonella typhimurium* flagellin	*V. cholerae*	A flagellin-negative *S. dublin* strain expressing the chimeric *Salmonella* flagellin gene inserted with cholera toxin B epitopes	C57BL/6 mice	Produced high levels of antibody against cholera toxin	[46]
FliCd	*Salmonella enterica* FliCd flagellin	*Plasmodium yoelii*	A live *S. dublin* vaccine strain expressing the target $CS_{280–288}$ peptide fused at the central hypervariable domain	BALB/c mice	Primed $CS_{280–288}$-specific cytotoxic $CD8^+$ T cells	[47]
Bacterial-derived enterocyte cell-targeting proteins						
mInIA	The mutated form of internalin A of *L. monocytogenes*	Cow's milk allergy (bovine β-lactoglobulin)	*L. lactis* expressing mInIA and transformed with pValacBLG	BALB/c mice	The plasmid transfer in vitro was increased 10 times The number of mice producing BLG was slightly higher	[50]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
FnBPA	Fibronectin-binding protein A of *S. aureus*	NM	Recombinant invasive *L. plantarum* expressing FnBPA	C57BL/6 and BALB/c mice	Invasion ratios of *L. plantarum* strain on the IPEC-J2 cell line was about twofold that of the empty vector Induced specific humoral immune response	[51]
FnBPA	As above	Cow's milk allergy (bovine β-lactoglobulin)	*L. lactis* expressing FnBPA and carrying the plasmid pValac:BLG (LL-FnBPA$^+$ BLG)	BALB/c mice	Co-incubated with LL-FnBPA$^+$ BLG produced up to 30 times more BLG LL-FnBPA$^+$ increased the number of mice producing BLG	[52]
FnBPA	As above	Tuberculosis (*M. tuberculosis*)	*L. lactis* FnBPA$^+$ strain carrying the eukaryotic expression vector coding the ESAT-6 gene of *M. tuberculosis*	BALB/c mice	Significantly increased IFNγ production Significant increase in specific sIgA production	[53]
Additional bacteria-derived adjuvants						
MT	Muramyl dipeptide and tuftsin fusion protein	Transmissible gastroenteritis virus	*L. casei* expressing the MDP and tuftsin fusion protein repeated 20 and 40 times, and the D antigenic site of the spike protein of TGEV	BALB/c mice	Enhanced the anti-TGEV antibody immune responses of both humoral and T cell-mediated immune systems	[54]
PorA	An OMP of *N. meningitidis*	*H. pylori*	*L. lactis* strain expressing a PorA-HpaA hybrid	BALB/c mice	Enhanced the antibody response against the HpaA antigen approximately threefold	[57]
c-di-AMP	A bacterial second messenger	*T. cruzi* parasite	*L. lactis* strain expressing antigenic TScf combined with another *L. lactis* strain producing c-di-AMP	BALB/c mice	Elicit a TS-specific immune response	[58]
RCK	An OMP of *Salmonella enterica*	IBDV	A recombinant *L. lactis* co-expressing the major IBDV antigens VP2 and RCK protein	Chickens	Induced a specific neutralizing antibody-mediated immune response Conferred full protection against very-virulent IBDV challenge	[62]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
Protozoan-derived adjuvants						
VSPs	Variant-specific surface proteins covered on the *Giardia lamblia* surface	Influenza A (H5N1 or H1N1)	Chimeric VLPs decorated with VSPs and expressing influenza virus HA and NA	BALB/c, C57BL/6, C57BL/10ScNJ (TLR-4 KO) mice	Protected antigens from degradation and enhanced their immunogenicity Generated robust immune responses that protect mice from influenza infection and HA-expressing tumors	[63]
Intestinal immune cell-targeting peptide adjuvants						
M cell-targeting adjuvants						
Co1	M cell-specific peptide ligand	EGFP as model Ag	Co1-fused EGFP proteins	BALB/c mice	Co1-fused EGFP binds to M cells Transported effectively into the mucosal immune induction site	[78]
Co1	M cell-targeting peptide	PED (PEDV)	Genetically engineered *L. casei 393* strain fused expressing PEDV COE antigen and M cell-targeting peptide Co1 (pPG-COE-Co1/L393)	BALB/c mice	Effectively induce mucosal, humoral, and Th2-type cellular immune responses against PEDV infection	[79]
CKS9	M cell-targeting peptide ligand (CKSTHPLSC)	Swine dysentery (*Brachyspira hyodysenteriae*)	Cytoplasmic expression of a model antigen (BmpB) with M cell-targeting moiety in a recombinant *L. plantarum* strain	BALB/c mice	CKS9 could efficiently deliver its conjugated BmpB from the intestinal lumen into GALT via M cells Induce strong mucosal and systemic immune responses against BmpB	[80]
Gb-1	GP-2 (an integral membrane protein expressed specifically on M cells) binding peptides	EGFP as model Ag	Gb1-EGFP fusion proteins	BALB/c mice	Gb-1 showed high binding affinity to GP-2 Significantly increased the uptake of EGFP by M cells Induced efficient mucosal and systemic immune responses Induced a Th2-type immune response	[82]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
Cytokine receptor activator of NF-kB ligand (RANKL)						
RANKL	Cytokine receptor activator of NF-kB ligand	Porcine epidemic diarrhea (PEDV)	Oral vaccination with aP2 subunit vaccine-loaded HPMCP and RANKL-secreting *L. lactis* (HPMCP [aP2] plus LL RANKL)	Pregnant sows	Significantly increased titers of virus-specific IgA antibodies and neutralizing antibodies The survival rate of piglets delivered by sows vaccinated with HPMCP (aP2) plus LL RANKL was similar to those vaccinated with a commercial killed PEDV vaccine	[86]
DC-targeting adjuvants						
DCpep	DC-targeting peptide (FYPSYHSTPQRP)	*Bacillus anthracis* infections	*L. acidophilus* expressing a PA-DCpep fusion	A/J mice	Induced robust protective immunity against lethal challenge of *B. anthracis* The serum anti-PA titers, neutralizing PA antibodies, and the levels of IgA-expressing cells were comparable with the recombinant PA plus aluminum hydroxide vaccine administered SC	[70]
DCpep	As above	*M. tuberculosis*	Recombinant *L. plantarum* secreting and anchoring of *M. tuberculosis* antigens (Ag85B-ESAT6) fused with DCpep	Mice	The pro-inflammatory cytokines (IFNy and IL-17A) increased	[89]
DCpep	DC-targeting peptide	PED (PEDV)	A recombinant *L. casei* expressing a DC-targeting peptide fused with the PEDV core neutralizing epitope antigen	BALB/c mice, large white piglets	*Lactobacillus* vaccine elicits a specific systemic and mucosal immune response Promotes lymphocyte proliferation Effectively protects piglets against PEDV infection	[90, 91]
DCpep	DC-targeting peptide	H9N2 AIVs	Recombinant *L. plantarum* NC8 expressing HA and DCpep	BALB/c mice and white leghorn chickens	Elicited high serum titers of hemagglutination-inhibition antibodies in mice Induced robust T-cell immune responses in both mouse and chicken models	[92]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
SP	Chicken DC-binding peptides Candidate (SPHLHTSSP-WER)	IBDV	Recombinant *L. saerimneri* M11 delivering IBDV structural protein and protective antigen VP2 fused with SP	SPF chickens	Efficiently induced anti-IBDV mucosal and humoral immune responses Resulting in higher protective efficacy in the VP2-SP group than the VP2 group	[197]
SMIPs						
Cytokine adjuvants						
IL-1β	Murine IL-1β	Enteritidis (*Salmonella enterica*)	Intragastric immunization with heat-killed *Salmonella enterica* in combination with recombinant *L. casei* producing IL-1β	BALB/c mice and C3H/HeJ mice	Resulted in relatively high *Salmonella enterica*-specific antibody production	[95]
IL-1β fragment	A fragment of human IL-1β (VQGEESNDK peptide)	*Clostridium difficile*	Recombinant *Bacillus subtilis* spores presenting a chimeric Protein (*C. difficile* FliD protein and an IL-1β fragment)	BALB/c mice	Significantly changed the characteristics of elicited immune response	[96]
IL-2	Human IL-2	*H. pylori* infection	Recombinant *L. lactis* NZ9000 containing a common immunogen of *H. pylori* (UreB) as a chimeric protein fused with human IL-2	BALB/c mice	Elicited more anti-UreB antibody and more cytokines Had a lower *H. pylori* burden and urease activity than control mice	[98]
IL-2	Human IL-2	*H. pylori*	Coadministered with recombinant *B. subtilis* spores expressing the *Helicobacter acinonychis* UreB protein and another *B. subtilis* spore presenting IL-2	BALB/c mice	Elicited a strong cellular immune response	[99]
IL-2	Rabbit IL-2	Rabbit hemorrhagic disease	A DNA vaccine co-expressing IL-2 and VP60 and delivered by attenuated *Salmonella typhimurium*	Chinese white rabbits	Induced a higher level of antibodies to a significant extent Concentrations of IL-4 were markedly higher The fusion gene vaccine provided higher protection	[97]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
IL-12	Mouse IL-12	*Leishmania major* infection	*L. lactis*(*alr*-) co-expressing the protective *Leishmania* antigen (secLACK) and mouse secIL-12	BALB/c mice	Generated protective immunity against *Leishmania major* infections Induced Th1 immune response mediated by $CD4^+$ T cells	[100]
cGM-CSF	Canine GM-CSF	Canine corona virus	Coadministration CCV vaccine and *L. lactis* expressing cGM-CSF	Beagle puppies	Monocyte counts in hematology and serum IgA were higher Increased more CCV-specific IgG in serum	[101]
Tα1	Non-toxic immune-modifier peptide hormone from the thymus	CSFV	Recombinant *L. plantarum* bacteria expressing CSFV E2 protein in conjunction with Tα1 (*L. plantarum*/pYG-E2-Tα1)	Pigs	Effectively induced protective immune responses in pigs against CSFV infection Significant differences in the levels of immune responses between *L. plantarum*/pYG-E2-Tα1 and *L. plantarum*/pYG-E2	[102]
Fc region of IgG and Anti-DEC-205 antibody						
IgG Fc fragment	Fc fragment of mouse IgG2a	Influenza virus (H1N1 and H9N2)	Recombinant *L. plantarum* expressing the internal influenza viral protein M2e fused to an IgG Fc fragment	BALB/c mice	Markedly reduced the viral load in the lungs Protected against H1N1 influenza virus and mouse-adapted H9N2 AIV challenge	[112]
Fc fragments conjugated to nanoparticles	Fc fragment of IgG antibodies	Diabetes	Fc-targeted nanoparticles encapsulating insulin (insNP-Fc)	Wild-type mice and FcRn knockout mice	FcRn-targeted nanoparticles crossed the intestinal epithelium and reached systemic circulation with a mean absorption efficiency of 13.7%*h Elicited a prolonged hypoglycemic response in wild-type mice	[203]

Table 1 (continued)

Adjuvant name	Explanation	Disease model (pathogen)	Selected antigen or vaccine (or candidate) formulation	Animal model or clinical trials	Results of immune response in oral administration trials	Reference
aDec	Anti-DEC-205 antibody	NM	An engineered *L. plantarum* strain expressing aDec and containing a plasmid for expression of GFP under control of a eukaryotic promoter	C57BL/6 mice	DCs showed increased uptake of the engineered *L. plantarum* Increased internalization of *L. plantarum* and plasmid transfer in DCs	[204]
Multiple biogenic oral adjuvants						
DCpep and Co1	DC and M cell-targeting peptides	PED (PEDV)	Recombinant *L. casei* 393 strain expressing DCpep and Co1-targeting ligands fused with the PEDV COE antigen	BALB/c mice	Promoted stronger, more rapid antigen-specific immune responses	[114]
IL-6-CKS9	A recombinant cytokine conjugating an M cell-targeting peptide (CKS9) with C-terminus of the murine IL-6	Swine dysentery (*B. hyodysenteriae*)	M-BmpB protein combined with the recombinant *L. lactis* IL-1403 producing IL-6-CKS9	BALB/c mice	Induced both Th1- and Th2-type immune responses Enhanced induction of antigen-specific antibody in both mucosal and systemic immune response	[75]

The immune response results of the above examples are all the results of the oral immunization test

aDec anti-Dec-205 recombinant antibody, *Ag* antigen, *AIV* avian influenza virus, *BLG* β-lactoglobulin, *BmpB* Brachyspira membrane protein B, *CCV* canine corona virus, *Co1* M cell-specific peptide ligands, *CSFV* classical swine fever virus, *DC* dendritic cell, *EGFP* enhanced green fluorescence protein, *ETEC* enterotoxigenic *Escherichia coli*, *ETVAX* an oral, inactivated, enterotoxigenic *E. coli* vaccine, *GALT* gut-associated lymphoid tissue, *GM-CSF* granulocyte-macrophage colony-stimulating factor, *H5N1-VLPs* VLPs containing only HA, NA and M1, *HA* hemagglutinin, *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus, *HPAI* highly pathogenic avian influenza, *HPMCP* hydroxypropyl methylcellulose phthalate microspheres, *IBDV* infectious bursal disease virus, *Ig* immunoglobulin, *IFN* interferon, *IL* interleukin, *LACK* Leishmania homolog of activated C kinase, *LBNSE* a recombinant rabies virus, *M-BmpB* Brachyspira membrane protein B conjugated with CKS9, *NA* neuraminidase, *NE* necrotic enteritis, *NetB* necrotic enteritis toxin B-like, *NF-κB* nuclear factor Kappa B, *NM* not mentioned, *OMP* outer membrane protein, *PA* a *B. anthracis* protective antigen, *PED* porcine epidemic diarrhea, *PEDV* porcine epidemic diarrhea virus, *PEDV COE* core neutralizing epitope (COE) of the PEDV spike protein, *RANKL* receptor activator of NF-kB ligand, *RCK* Salmonella resistance to complement killing, *SC* subcutaneous, *sIGA* secretory immunoglobulin A, *SMIPs* small molecular immunomodulatory proteins, *Tα1* thymosin α-1, *TGEV* transmissible gastroenteritis virus, *Th* T helper, *TLR* toll-like receptor ligand, *TNF* tumor necrosis factor, *VLPs* virus-like particles

been devoted to developing variants of these enterotoxins that are low or non-toxic but still retain their adjuvant activity [15], such as LT (R192G or single-mutant LT [mLT]) [16], dmLT [17], and mmCT [18]. These enterotoxins (CT and LT) and their mutants (or subunits) [dmLT and mLT] can increase the generation of antigen-specific IgA antibodies, T-cell responses, and long-lasting memory when coadministered with antigens through the mucosal or transcutaneous routes [16, 19]. CT, LT, and some LT mutants could increase antigen capture in the small intestine by promoting DC migration from the subepithelial dome (SED) to the follicle-associated epithelium (FAE) between 1.5 and 12 h after oral administration [20]. Additionally, preclinical research showed that LT, dmLT, CT, and mmCT can all significantly raise T helper (Th) 17 responses and thus increase antibody responses (Fig. 1a) [16, 18, 19, 21].

Double mutant heat-labile enterotoxin (dmLT) The most widely used and promising bacterial enterotoxin adjuvant to date is LT (R192G/L211A) or dmLT. In fact, dmLT is a genetically attenuated derivative of a wild-type ETEC heat-labile enterotoxin, which changes arginine to glycine at amino acid position 192 to disrupt the enzymatic and toxic activity of LT, and changes leucine to alanine at a potential pepsin-sensitive proteolytic cleavage site at amino acid position 211 [17]. This detoxified or attenuated form of LT retains its antigenicity and adjuvant properties. dmLT has been shown to be safe, well tolerated, and reasonably immunogenic in oral doses up to 100 μg in humans [22]. To date, dmLT has been an effective adjuvant that strongly potentiated the immune responses of various vaccines administered parenterally and mucosally against infectious pathogens (Table 1), e.g., *Streptococcus pneumoniae* [23], *Helicobacter pylori* [24, 25], tetanus toxoid [17], CT [18], and ETEC [26]. Noteworthy, when prophylactic immunization was performed with *H. pylori* lysate antigens, dmLT promoted strong B- and T-cell immune responses to *H. pylori* antigens and reduced the bacterial load in stomachs of *H. pylori*-infected mice [25]. Adding dmLT to an attenuated Salmonella-vectored ETEC vaccine improved its immunogenicity in mice [27]. Through preclinical studies, Holmgren et al. showed that adding dmLT to the multivalent ETEC vaccine (ETVAX) significantly improved both the anti-colonization factor (CF) and anti-LT responses following oral immunization [28]. Moreover, the phase I study of human volunteer trials proved that dmLT further enhanced the mucosal immune responses to CF antigens present in low amounts in this ETVAX vaccine [28, 29]. In addition, through clinical trials of human volunteers, Harro et al. demonstrated that the shedding of challenge strain (ETEC H10407) in those human volunteers orally administered ACE527 (the ETEC vaccine) and dmLT was tenfold lower than in those who received the vaccine alone, illustrating that dmLT can significantly contribute to vaccine efficacy to protect human volunteers against ETEC challenge [30]. In conclusion, dmLT is a well-tolerated and powerful mucosal adjuvant for coadministered antigens.

Multiple Mutant Cholera Toxin (mmCT) CT used to be an effective adjuvant, widely used to induce mucosal immune responses in animal models; however, the strong enterotoxicity of CT precludes its use in human or veterinary vaccines. The recently developed mmCT, which derived from CT with mutations in multi-sites in its A subunit and is fully resistant to proteolytic cleavage, is a strong, yet practically non-toxic novel mucosal adjuvant. Compared with native CT, the cAMP-inducing activity of mmCT decreased by >1000-fold [31]. Compared with dmLT, mmCT protein is more easily produced and purified in large quantities because mmCT is secreted from the extracellular medium of CT-deleted *V. cholerae*, while dmLT is located in inclusion bodies [19, 31]. mmCT possesses similar adjuvant activity and safety as dmLT, which promotes human Th17 responses via cAMP-dependent protein kinase A and caspase-1/inflammasome-dependent interleukin (IL)-1 signaling [18]. The study by Holmgren et al. [32] reported that intragastric immunization of *H. pylori* whole-cell vaccine (WCV) together with mmCT reduced the colonization of *H. pylori* in the stomach of mice by 50- to 125-fold, which was associated with rises in both the anti-*H. pylori* antibody responses of serum IgG and intestinal mucosal IgA and the responses of strong T cell and interferon (IFN)-γ and IL-17A cytokines. Moreover, its immune effect is similar to that of WCV together with CT, indicating mmCT, a non-toxic adjuvant, can replace CT as an adjuvant without loss in protective efficacy [32].

In conclusion, mmCT has no enterotoxicity but retains strong adjuvant activity, is economical and easy to be produced, and has great potential in designing oral vaccines.

2.1.2 Bacterial Flagellin

Flagellin, the main structural protein of bacterial flagella, is considered a pathogen-associated molecular pattern (PAMP). TLR5 can recognize flagellin, thus activating the production of inflammatory molecules, including chemokines and cytokines (Fig. 1b), and then triggering cellular immune responses, including DCs, through myeloid differentiation factor 88 (MyD88) signaling [33, 34]. In addition to TLR5 activation, flagellin can bind to cytosolic nucleotide binding oligomerization domain-like receptors, NLRC4, which activate the caspase-1 inflammasome [35]. TLR5 is extensively expressed in the lung, intestinal epithelial cells, monocytes/macrophages, and DCs [36], Because flagellin is easy to express, is stable and potently activates the adaptive immune response by binding to TLR5 [37, 38], it has attracted a lot of attention as a vaccine adjuvant. Oral administration of flagellin-based vaccines could induce effective immune protection in mice. In the study

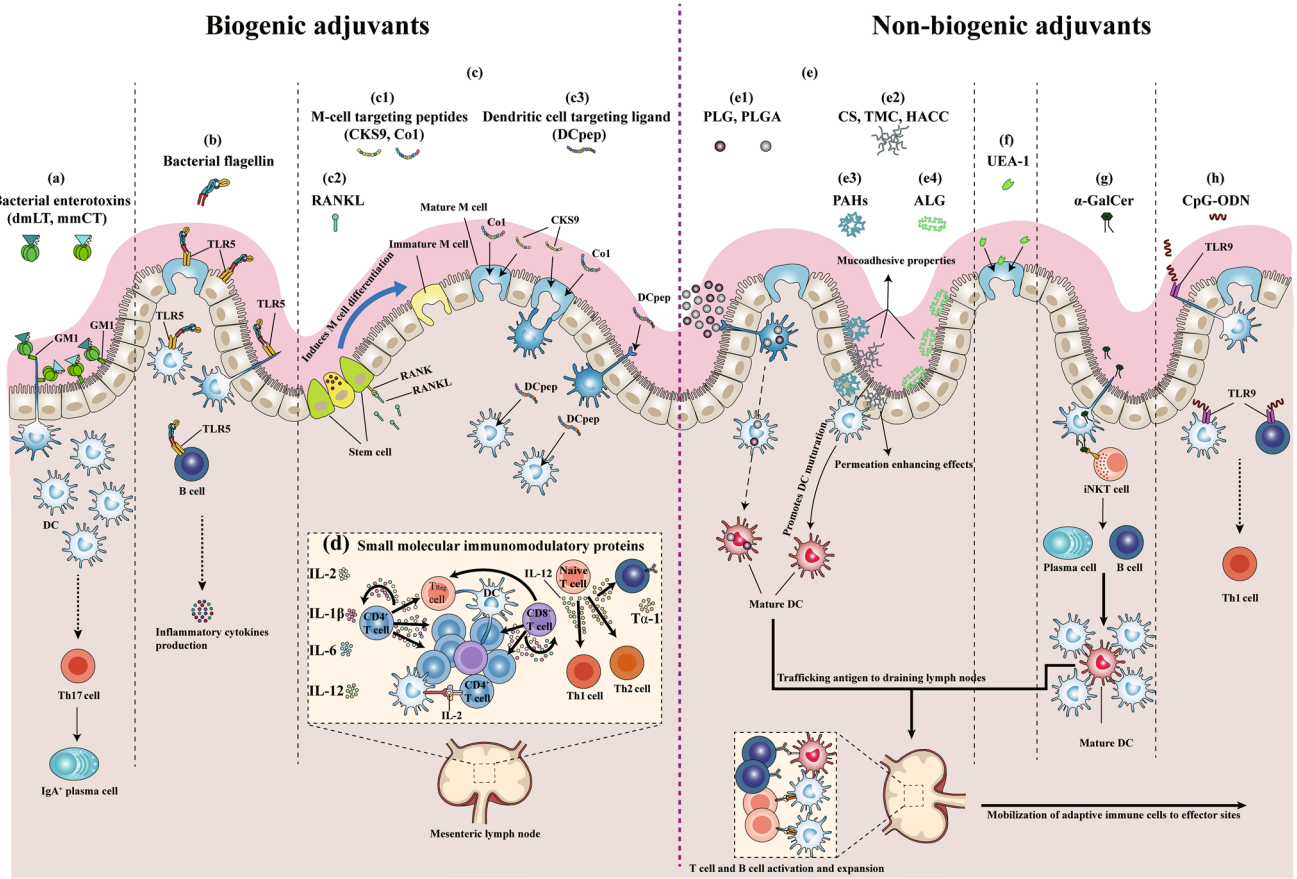

Fig. 1 Concise mechanisms of oral adjuvants at intestinal mucosal sites. **a** Bacterial enterotoxin (dmLT, mmCT) targets GM1 receptors, promotes Th17 response, and subsequently induces antigen-specific IgA antibodies. **b** Bacterial flagellin increases TLR5 stimulation that activates the production of inflammatory cytokines and subsequently augments innate and adaptive immune responses. **c1** M cell-targeting peptides (CKS9, Co1) specifically target and bind to M cells. **c2** RANKL, increasing the number of M cells. **c3** DC-targeting ligand (DCpep), specifically targets and binds to dendritic cells. **d** Small molecular immunomodulatory proteins (cytokines and Tα1) directly stimulate, attract immune cells, and induce immune response. **e1** PLG and PLGA protect antigens from degradation in GIT, allow the sustained and extended release of encapsulated antigens, and enhance antigen uptake by APCs, and subsequently the delivery of these microparticle-containing APCs to specific lymphoid compartments. **e2** CS and its derivatives (TMC, HACC) and **e3** PAHs possess mucoadhesive properties and permeation-enhancing effects. **e4** ALG possesses mucoadhesive properties. **f** UEA-1 specifically targets and binds to M cells. **g** α-GalCer activates the iNKT-cell. **h** CpG-ODN activates TLR9 on B-lymphocytes and DCs, stimulates antigen presentation and induction of antigen-specific immune response towards the Th1 phenotype. *CS* chitosan, *PAHs* polyanhydrides, *ALG* alginate, *iNKT-cell* invariant natural killer T cell

by Ren et al. [39], the H5N1 chimeric virus-like particles (VLPs) containing membrane-anchored FliC (FliC-VLP) were administered orally to mice, and the virus-specific IgG titers of immunized mice were tenfold higher than those of mice immunized with H5N1-VLPs lacking FliC, which significantly improved the protective immune response to lethal challenge from both homologous and heterologous H5N1 viruses. According to Zhou et al. [34], mice orally inoculated with LBNSE-Flagellin (the recombinant rabies viruses [rRABV] expressing flagellin of *Salmonella enterica* subsp.) could recruit/activate more DCs and B cells in the periphery, and trigger a stronger adaptive immune response (i.e., virus-neutralizing antibody level). LBNSE-Flagellin could shield more mice from LD_{50} challenge infection with rabies viruses strain CVS-24 compared with the parent virus LBNSE group. An innovative study by Girard et al. [40] revealed that plant-produced flagellin (flagellin of *Salmonella typhimurium* [FljB]) was a more potent and effective adjuvant for oral immunization. Beyond that, using plant-produced flagellin as an adjuvant for oral vaccine did not elicit an immune response against FljB.

By incorporating membrane-anchored flagellin into bacterial ghosts (BGs), it may be possible to create a more effective oral BG-based vaccine [41]. Moreover, synthesizing varied flagellins in an oral live bacterial vaccine strain is an attractive method for generating protective immunity. In the study by Eom et al., mice were shown to be protected against the virulent Salmonella SL1344 strain [42] after receiving an

oral immunization with attenuated *S. typhimurium* BRD509 vaccine strain that expressed FliC and FljB flagellins (diphasic *S. typhimurium* has two flagellin genes—the flagellin in phase I is FliC and the flagellin in phase II is FljB [43]). In addition, it is another promising way to develop oral probiotic live vaccine strains or oral attenuated (or non-virulent) *salmonella* live vaccine strains by integrating heterogeneous antigens into the hypervariable region of flagellin of probiotic strain [44] or attenuated (or non-virulent) *salmonella* strain [45–47].

In summary, it is commonly acknowledged that flagellin can boost an antigen-specific immune response when used as an adjuvant. This will facilitate the development of flagellin-based vaccines that are safer and more effective, as well as their entry into oral clinical trials.

2.1.3 Bacteria-Derived Enterocyte Targeting Proteins

Expression of enterocyte binding proteins derived from some pathogenic bacteria on the surface of probiotic strains as adjuvants to deliver eukaryotic expression plasmids into host intestinal epithelial cells could be an effective oral DNA vaccine strategy [36]. Internalin A (InIA) in *Listeria monocytogenes* (*L. monocytogenes*) and fibronectin binding protein A (FnBPA) in *Staphylococcus aureus* (*S. aureus*) are well-known enterocyte targeting proteins. InlA is a cell wall protein that allows *L. monocytogenes* to bind to and be internalized by epithelial cells [48]. And FnBPA is an epithelial cell binding protein that can bind to fibrinogen, elastin, and fibronectin allowing for internalization of *S. aureus* into non-phagocytic cells [49]. When InlA (or mInlA, the mutated form of InlA [Ser192Asn and Tyr369Ser]) [50] and/or FnBPA [51–53] were expressed on the surface of lactic acid bacteria (LAB) strains, these recombinant strains acquire the ability to invade mammalian cells through the interaction between InlA and/or FnBPA and cellular receptors, resulting in the increase of targeted antigens cDNA in the intestinal lumen and the enhancement of host immune response [50–53].

2.1.4 Other Bacteria-Derived Proteins

Rarely investigated as mucosal adjuvants, some bacterial proteins and messengers still lack a clear understanding of how exactly they trigger immunity. However, they could be candidates for oral vaccine adjuvants because of their capacity to facilitate the immune response to antigens.

Muramyl dipeptide (MDP) is part of the bacterial cell wall and is delivered as a dipeptide with tuftsin, a biologically active compound [54, 55]. Although their roles in oral immune adjustment have not been fully elucidated, it has been demonstrated that MDP and tuftsin can activate APCs [55]. In the study by Jiang et al. [54], the fusion protein of MDP and tuftsin was utilized as an adjuvant to modify the *Lactobacillus casei* vaccine strain. The results showed that antibody and T-cell responses were improved after oral administration in BALB/c mice.

PorA is an outer membrane protein (OMP) from the *Neisseria meningitidis* [56]. It is remarkable that PorA has an important feature of oral protein adjuvants, namely resistance to proteolytic enzymes in the GIT [57]. It has the potential to act as an oral adjuvant when conjugated to antigens. For example, when PorA was fused with the *H. pylori* HpaA antigen and expressed in *Lactococcus lactis*, PorA could significantly enhance the antibody response against the HpaA antigen after oral administration in mice [57].

3'5'-Cyclic di-adenosine monophosphate (c-di-AMP) is a bacterial second messenger that has strong mucosal adjuvant activity and numerous effects on the immune system, including type I IFN responses, promotion of Th1 and Th2 responses, increasing lymphocyte proliferation, and activation of APCs [58, 59]. Oral administration of recombinant *L. lactis* strains co-producing c-di-AMP and an anti-*Trypanosoma cruzi* antigen resulted in a *T. cruzi*-specific immune response

The Salmonella resistance to complement killing (RCK) protein plays an important role in interfering with complement killing and invading cells, including epithelial cells and APCs [60, 61]. The use of RCK as an oral adjuvant for the *L. lactis* vaccine strain successfully increased immune responses, conferring full protection against very-virulent infectious bursal disease virus (IBDV) challenge [62].

2.2 Protozoan-Derived Adjuvant Variant-Specific Surface Proteins

A novel oral adjuvant candidate could be achieved from parasitic protozoa, *Giardia lamblia*, which colonizes in the lumen of the upper small intestine of many vertebrate hosts. Serradell et al. [63] reported the variant-specific surface proteins (VSPs) from the *Giardia lamblia* surface can not only resist proteolytic digestion and extreme pH in GIT, as well as temperatures, but also stimulate host innate immune responses in a TLR-4-dependent manner. They constructed chimeric VSP-pseudotyped VLPs expressing hemagglutinin (HA) and neuraminidase (NA) of the influenza virus. These VSP-pseudotyped VLPs, but not plain VLPs, produced robust immune responses, protecting mice from influenza infection and HA-expressing tumors after oral immunization. This versatile oral vaccine adjuvant based on VSPs can be applied to antigens from different infectious agents or tumors and facilitate their use in remote areas where cold-chain for vaccine is not guaranteed.

2.3 Intestinal Immune Cells Targeting Peptide Adjuvants

Microfold cells (M cells), a unique subset of epithelial cells found in the epithelia covering MALT, such as Peyer's patches, are used by the mucosal immune system to sample antigens in the GIT [64]. A variety of substances, including bacteria, viruses, and antigens, can be transported by M cells from the lumen to the underlying lymphoid tissues thanks to their great transcytotic ability [65–68]. Additionally, various antigens delivered by M cells can be sampled and captured by DCs positioned inside or beneath the epithelium [69, 70]. In addition, DCs can extend their probing dendrites into the lumen to sample commensal or microbial immunogens after passing through tight junctions to reach the gut epithelia [71]. These DCs subsequently migrate into the lymphoid follicles, where processed antigens are presented to B and T cells to sequentially trigger humoral (IgA) and T-cell immune responses [68, 72]. The aforementioned immunologic mechanisms of M cells and DCs can be exploited for the development of oral vaccine adjuvants. Therefore, targeting intestinal immune cells (such as M cells and/or DCs) is a promising strategy for developing oral vaccine adjuvants.

2.3.1 M Cell-Mediated Oral Adjuvants

In peroral mucosal vaccination, targeting M cells is considered a frontline prerequisite for effectively inducing antigen-specific immunostimulatory effects [73]. In the GIT, M cells are the antigen-collecting portals located on the FAE of Peyer's patches and the gut-associated lymphoid tissue (GALT) of different species, which facilitate to transport antigens from gut lumen to the submucosal immune system [68, 74, 75]. M cells are believed to play a role in controlling gastrointestinal infection and immunity [73]. Therefore, M cell targeting might be a promising strategy for developing effective oral vaccine adjuvants [76].

M cell-targeting peptides Through phage display technology, Cho and colleagues [77] identified an M cell-homing peptide, CKS9 (CKSTHPLSC), which can facilitate the transcytosis of target antigen in M cells. In addition, according to Kim et al. [78], fusion of enhanced green fluorescence protein (EGFP) with another M cell-homing peptide, Col, could direct EGFP to bind to M cells and effectively transport it to mucosal immune induction sites to improve immune induction. Soon afterwards, with M cell-targeting peptides (Co1 or CKS9) as an oral vaccine adjuvant and LAB strain as an oral delivery vector, researchers tried to develop probiotic-derived oral vaccines against porcine diarrheal diseases, including porcine epidemic diarrhea (PED) [79] and swine dysentery [80], and obtained encouraging experimental outcomes after oral administration in mice.

In addition, the efficient uptake of antigens by M cells requires specific surface receptor molecules. Targeting the inherent receptors specifically expressed on the surface of M cells is another way to target M cells to deliver antigens to improve vaccine efficacy. Glycoprotein-2 (GP-2) is a glycosylphosphatidyl inositol anchoring protein that is specifically expressed on M cells and serves as a transcytotic receptor for luminal antigens [81]. Therefore, targeting GP-2 with specific ligands should increase antigen delivery to the immune initiation sites. Khan et al. [82] selected a GP2-binding peptide ligand, Gb-1, through phage library screening, which showed high binding affinity to GP-2. When fused with EGFP, Gb-1 significantly enhanced the uptake of EGFP by M cells compared with EGFP alone. Likewise, the Gb1-EGFP fusion induced effective mucosal and systemic immune responses after oral administration in mice. Therefore, exploiting the GP2-binding peptide Gb-1 for oral vaccine delivery would be a realistic approach.

Cytokine receptor activator of nuclear factor kappa B (NF-kB) ligand (RANKL). The proportion of M cells in intestinal epithelial cells is very low, accounting for approximately 1% of the total intestinal surface [68]. Therefore, if the number of M cells could be increased, it would be a promising technique to improve the effect of oral vaccines. It has been well-documented that the cytokine receptor activator of the nuclear factor Kappa B (NF-kB) ligand (RANKL) is a prevalent control factor for inducing M cells to differentiate from intestinal epithelial precursor cells by interacting with the cytokine receptor activator of NF-kB (RANK) expressed on the sub-epithelium of Peyer's patches in the intestinal tract [83–85]. It has been proven that systemic administration of exogenous soluble RANKL (sRANKL) can correct the M-cell deficiency and uptake impairment in the Peyer's Patch [73]. In this regard, oral immunization by administering RANKL to induce the supraphysiological amount of M cells and then administering M cell-targeting antigens may be a viable approach to enhance the effect of oral vaccination.

A recombinant *L. lactis* IL-1403 producing and secreting soluble RANKL (sRANKL-LAB) constructed by Kim et al. could increase the expression of M cells in mice to be 1.51-fold higher than that in the untreated group through oral administration [83]. Maharjan et al. firstly administered intraperitoneally (or systemically) transmembrane RANKL (mRANKL) to mice and then delivered microparticulate antigen orally, which significantly increased the expression of M cells in FAE, showing similar effect as sRANKL-LAB [85]. They also demonstrated that RANKL-mediated transcytosis of antigens through M cells can enhance mucosal and humoral immunity. Choe et al. constructed RANKL-secreting *L. lactis* (LL RANKL) as an oral adjuvant for the aP2 subunit (soluble recombinant partial spike S1 protein from PEDV) vaccine loaded in hydroxypropyl methylcellulose

phthalate (HPMCP) microspheres (HPMCP [aP2] plus LL RANKL) [86]. Their results showed that titers of virus-specific IgA antibodies in colostrum, and neutralizing antibodies in serum of sows vaccinated with HPMCP (aP2) plus LL RANKL increased significantly, and the survival rate of newborn suckling piglets delivered by sows vaccinated with HPMCP (aP2) plus LL RANKL was similar to that of piglets delivered by sows vaccinated with a commercial PED killed vaccine. These preclinical studies show that oral administration of RANKL is a promising adjuvant strategy, which could be used for effective oral vaccination and even oral therapeutic administration.

2.3.2 Dendritic Cell-Targeting Ligands

DCs represent the interface of the innate and adaptive immunity, and DCs play a pivotal role in priming T-cell immune responses against the inoculated antigen. Therefore, DCs are the major determinants of vaccination, so targeting oral vaccines to DCs is another strategy to enhance vaccination efficacy [87–89]. With DC-targeting peptides (DCpep, FYPSYHSTPQRP) as adjuvant, many researchers tried to utilize various LAB strains (including *Lactobacillus plantarum, L. casei, Lactobacillus acidophilus*, *Lactobacillus saerimneri*, *L. lactis*, etc.) as oral delivery vectors to develop oral vaccines for zoonotic or veterinary infectious diseases, such as *Bacillus anthracis*, and obtained good preclinical research results in animal model experiments of various diseases (Table 1). These research cases showed that modifying and specifically targeting a certain antigen to DCs can enhance antigen uptake.

2.4 Small Molecular Immunomodulatory Proteins

SMIPs are synthesized and secreted by a variety of tissue cells (mainly immune cells). They have many biological functions, such as regulating innate immunity and adaptive immunity, hematogenesis, cell growth, pluripotent stem cells and damaged tissue repair. To date, SMIPs used in the research of peroral vaccine adjuvants are mainly cytokines and Tα1.

2.4.1 Cytokine-Derived Oral Adjuvants

Cytokines are small proteins released by various cell types. Their functions are to stimulate, attract, and regulate the activity of immune cells (especially T cells), enhance the signal transduction of APCs, and sequentially improve the immune response to pathogens. They play a critical role in the regulation of innate and adaptive immunity [93, 94]. Cytokines have already been used orally to steer the immune system towards an increase in local cytotoxic T lymphocyte (CTL) activity and/or increased IgG and IgA titers. Some cytokines have been investigated as adjuvants for oral vaccines, and success has been reported in various preclinical studies, in which IL-2 is the most widely used oral adjuvant (Table 1). In particular, by genetically modifying probiotic strains (*L. casei* strain or *Bacillus subtilis* spores) to express corresponding host cytokines (such as IL-1β [95, 96], IL-2 [97–99], IL-6 [75], IL-12 [100], and granulocyte-macrophage colony-stimulating factor [GM-CSF] [101]), and oral coadministration with antigens or vaccine strains could significantly stimulate the production of specific antibody response in animals compared with the control groups. In some animal challenge tests, obvious protective immunity could be produced to fight against various infectious diseases, such as *H. pylori* infection [98, 99], *Leishmania major* infection [100], rabbit hemorrhagic disease (RHD) [97], and canine corona virus (CCV) [101]. Despite the above promising results, the potential safety concerns of cytokines need to be considered before using them as adjuvants [93]. According to the immunological properties of target antigens (or diseases), selecting specific and suitable cytokines as oral adjuvants needs to be based on the expected immune response of vaccination and its known influence on immune cells, but this is still one of the challenges of current immunological research. Overall, the optimal regimen of cytokines should be determined before starting clinical studies.

2.4.2 Thymosin α-1

Tα1 is a non-toxic immunomodified peptide hormone secreted by the thymus. It plays a very important role in cellular immune response by triggering T-cell maturation, augmenting T-cell function, developing antibody production, promoting reconstitution of immune defects, and increasing cytotoxic cells, Th1 and Th2 cytokine production, and IgG and intestinal sIgA production [102–104]. On account of its adjuvant attributes, by conjoining with the CSFV-E2 antigen and displaying it on the surface of *L. plantarum*, Tα1 could be used as an adjuvant of oral vaccine against classical swine fever virus (CSFV), which showed that Tα1 molecule adjuvant could enhance immune response and augment specific lymphocyte functions [102]. Therefore, Tα1 will be a promising adjuvant strategy in the development of an oral LAB vaccine [36].

2.5 Fc Region of Immunoglobulin G

As a potential adjuvant, the Fc region of IgG has attracted considerable attention. More and more evidence has demonstrated that fusion of the Ig Fc domain with the desired protein can facilitate dimerization of the protein, thus potently

elevating the pharmacological and immunological characteristics of the protein [105–107], because the Fc region of IgG specifically binds to the FcRn (neonatal Fc receptor for IgG), which mediates IgG transport across the polarized epithelial cell lining on the mucosal surfaces [108]. As we know, IgG plays a predominant role in providing immune defense against foreign pathogens. Therefore, researchers have tried to target pathogenic antigens to FcRn as a new strategy to overcome intestinal epithelial barriers for mucosal vaccine delivery and drug therapy. Fc fusion proteins, or the recombinant proteins constructed by fusing the desired pathogenic antigens with the Ig Fc domain, have recently been utilized to produce vaccine candidates against infectious agents, including herpes simplex virus (HSV; gD-Fc) [109], pseudorabies virus (PRV) (gB-IgG2aFc) [110], HIV (Gag-Fc) [111], influenza A (H1N1) virus (3M2e-Fc) [112] and classical swine fever virus (CSFV) (E2-Fc) [113]. The aforementioned Fc fusion proteins could improve humoral and cellular immune responses by oral or intranasal immunization.

2.6 Biogenic Composite Oral Adjuvants

By combining two or more biogenic adjuvant materials to form a new composite adjuvant regimen, it is possible to improve mucosal immunity of target antigens in the intestine lumen. In this way, the advantages of each adjuvant could be fully utilized to enhance the overall immune effect. Until now, only the combination of intestinal immune cells targeting peptides and cytokines or the combination of two intestinal immune cells targeting peptides have been used as composite biogenic adjuvants in the development of oral vaccines, achieving good preclinical results. In particular, Li et al. [75] reported a novel biogenic composite mucosal adjuvant, IL-6-CKS9, which was a recombinant cytokine produced by conjugating an M cell-targeting peptide (CKS9) with the c-terminus of murine IL-6. Oral administration of recombinant *L. lactis* IL-1403 vaccine strain containing the above composite adjuvant promoted mucosal immune response. In addition, through combining the M cell-targeting peptide (Col) and DC-targeting peptide (DCpep) as a composite adjuvant, Ma et al. [114] genetically engineered a *Lactobacillus* vaccine strain that could target intestinal M cells and DCs and express COE antigen of PEDV. The recombinant strain efficiently induced anti-PEDV mucosal, humoral, and cellular immune responses in mice after oral administration. This suggests that the combination of Col and DCpep is a promising adjuvant strategy for oral probiotic vaccines. It is believed that more biogenic composite oral adjuvants will appear in the future.

3 Non-biogenic Oral Vaccine Adjuvants

Non-biogenic oral vaccine adjuvant materials are mostly polymeric microparticles/nanoparticles. They have many advantages, such as good biocompatibility, biodegradability, easy processing and modification, controllable surface properties, etc., and they could deliver and protect DNA and antigen protein of oral vaccines (or drugs) and control their release. Beyond that, they also possess mucosal absorptivity and immunostimulatory activity to activate or enhance immunity. Therefore, the application of non-biogenic adjuvant materials in oral vaccine research, and even in biomedical research, has become increasingly popular, showing great application prospects (Table 2).

3.1 Alum

Alum, also referred to as ‘aluminium salts’, encompass aluminium potassium sulphate, aluminium hydroxide, aluminium phosphate, and amorphous aluminium hydroxyphosphate sulfate [115]. Alum is one of the most widely accepted vaccine adjuvants and is a component of several licensed parenteral vaccines [116]. Kapusta et al. [117] reported oral administration with nanogram doses of alum-adjuvanted hepatitis B surface antigen (HBsAg) in mice-induced humoral immune response at the protective level. However, alum is unable to enhance cell-mediated Th1 or CTL responses, which are vital to control most intracellular pathogens [118]. Furthermore, alum is considered a poor inducer of mucosal immunity [37].

3.2 Polymer-Based Microparticle/Nanoparticle Oral Adjuvants

To overcome the harsh environment of the GIT, different types of polymer-based nanoparticles (including synthetic and natural polymers) have been widely studied for the preparation of various microparticle/nanoparticle vaccines (or nanoparticle adjuvants) for the GIT due to their biocompatibility, biodegradability, non-toxic nature, and ease of modification into desired shapes and sizes, as well as protecting the vaccine bioactivity from adverse situations [2, 119]. Polymer-based nanoparticle adjuvants are made of polymers such polyanhydride, poly (ethylene-glycol), PLG, PLGA, poly(lactic acid) [PLA], chitosan, alginate, and their derivatives, among others, and they have demonstrated enhancement of intestinal immune responses in vaccines for preventing various infections and treating various inflammatory diseases [7]. In this part, the application and research progress of polymer-based microparticles/nanoparticles as adjuvants for the peroral vaccines were reviewed.

Table 2 Current developments in non-biogenic adjuvants for oral administration vaccines

Adjuvant names	Explanations	Disease models (pathogens)	Vaccines (or candidate) formulations or antigens	Animal models	Results or immune responses in oral administration trials	References
Alum						
Alum	Aluminium salts	HBV	Alum-adsorbed recombinant HBsAg from the commercial vaccine Engerix B®	BALB/c mice	Induced immune response at the protective level (≥10 mIU/mL)	[117]
PLG and PLGA						
PLG	Poly(D,L-lactide-*co*-glycolide)	*H. pylori* infection	*H. pylori*-loaded PLG NPs	BALB/c mice	Induced the *H. pylori*-specific mucosal and systemic responses Enhanced Th2-type responses	[136]
PLG	As above	*Rabies* (CRV)	Encapsulated PLG^+ CRV	Swiss albino mice	Showed significantly higher anti-rabies virus IgG titer, virus-neutralizing antibody titers, and IgG2a and IgG1 titers The stimulation index of the lymphoproliferation assay was significantly higher The humoral, cellular immune response, and survival rates were significantly higher	[138]
PLGA	Poly(D,L-lactic-*co*-glycolic acid) NPs	*Aeromonas hydrophila*	Recombinant OmpW of *A. hydrophila* encapsulated in PLGA NPs	*Labeo rohita*	Protected against lethal challenge with *A. hydrophila* in rohu Inhibited *A. hydrophila* growth by sera from the high antigen group	[205]
PLGA	As above	CMA	18-Aaβ-lactoglobulin-derived peptides loaded PLGA NPs	C3H/HeOuJ mice	Inhibited *ex vivo* whey-stimulated proinflammatory cytokine TNFα release Induced a dose-related partial prevention of CMA symptoms upon challenge to whole whey protein Silenced whey-specific systemic immune response	[206]

Table 2 (continued)

Adjuvant names	Explanations	Disease models (pathogens)	Vaccines (or candidate) formulations or antigens	Animal models	Results or immune responses in oral administration trials	References
Chitosan and its derivatives						
Chitosan NPs	Prepared by ionotropic gelation	Photobacteriosis (*Photobacterium damselae subsp. Piscicida*)	DNA vaccine (pPDPimpdh) conjugated with CS-TPP NPs	Senegalese sole juveniles	Significantly increased the concentration of lysozyme The non-specific immune responses and the specific humoral and cell-mediated immunity were observed	[147]
Chitosan NPs	As above	Cystic echinococcosis	The multi-epitope vaccine (e.g. antigens combined with B cell, CTL and Th epitopes) encapsulated by chitosan NP	Mice	The concentration of multi-epitope antigen merged with microfold cells was high	[149]
Chitosan NPs	As above	Cow mastitis (*E. coli*)	Purified OmpA encapsulated with chitosan	Kunming mice	Obtained the anti-serum titer (1:3200) Immune protection rate was 71.43% Downregulated the inflammation-related gene expression and the antioxidant factors Reduce injury in the liver and kidney	[150]
Chitosan	As above	*Salmonella* serovar Enteritidis infection (*S. enteritidis*)	*Salmonella* subunit vaccine containing OMPs and flagellin protein loaded and flagellin protein surface-coated chitosan NPs	White leghorn layer chicks	Increased TLRs, and Th1 and Th2 cytokine mRNA expression Enhanced specific systemic IgY and mucosal IgA antibody responses Reduced the challenge *Salmonella* load in the intestines	[151]
Chitosan	As above	OVA-sensitized asthma	Chitosan-formulated OVA particles	OVA-sensitized BALB/c mice	Increases specific T-cell proliferation and IFNγ/IL-10 secretion Enhanced tolerance induction in mice with asthma Dramatically reduced AHR, lung inflammation, eosinophil numbers Induced antigen-specific Th2 responses	[207]

Table 2 (continued)

Adjuvant names	Explanations	Disease models (pathogens)	Vaccines (or candidate) formulations or antigens	Animal models	Results or immune responses in oral administration trials	References
Chitosan	As above	Enterovirus 71	Recombinant enterovirus 71 VP1 formulated with chitosan	ICR mice	Induced VP1-specific IgA antibodies and serum-specific IgG and neutralization antibodies Induced high levels of Th1-, Th2- and Th3-type immune responses Conferred survival rate up to 30%	[208]
Chitosan NPs	As above	*Edwardsiella tarda* infection	Recombinant outer membrane protein A of *Edwardsiella tarda* encapsulated in chitosan NPs	*Labeo fimbriatus*	Produced higher antibody levels Had superior protection over the inactivated whole cell *E. tarda* vaccine Conferred improved protection against *E. tarda* mortality	[209]
N-trimethyl chitosan (TMC)	Prepared by ionic complexation with pentasodium TPP	Brucellosis (*Brucella melitensis*)	Recombinant *B. melitensis* Omp31 loaded onto TMC NPs	BALB/c mice	Increased vaccine residence time in the intestine Enhanced vaccine permeation and immunogenicity Stimulated maturation of DCs Induced specific IgG2a production, high levels of IFNγ, IL-12, IL-17 and Th1–Th17 production Increased IgA levels Significantly protected against *B. melitensis* 16M	[153]
Mannosylated chitosan nanoparticles (MCS NPs)	Prepared by the ionic gelation method with TPP	NM	BSA-loaded Eudragit® L100-coated MCS NPs	Sprague Dawley rats and BALB/c mice	MCS NPs were accumulated more specifically into PPs Elicited strong systemic IgG antibody and mucosal IgA responses	[155]
UEA-1						
UEA-1	Ulex europaeus agglutinin 1	Hepatitis B	HBsAG encapsulated liposomes conjugated with UEA-1	BALB/c mice	Enhanced binding to M cells Induced higher sIgA and cytokine levels	[161]

Table 2 (continued)

Adjuvant names	Explanations	Disease models (pathogens)	Vaccines (or candidate) formulations or antigens	Animal models	Results or immune responses in oral administration trials	References
Alpha-Galactosylceramide (α-GalCer)						
α-GalCer	Synthetic iNKT-cell agonists	AIDS (HIV)	Combination of the CTL-inducing HIV envelope peptide (R15K peptide) and the synthetic glycolipid α-GalCer	BALB/c mice	Induced efficient and broader systemic and mucosal antigen-specific immune responses Led to immune recognition of the cognate HIV envelope protein Repeated dosing of α-GalCer does not adversely affect the peptide-specific CTL responses	[162]
α-GalCer	As above	Diarrheal infections (ETEC and cholera)	SmPill® vaccine formulation that combines α-GalCer coating whole cell killed *E. coli* overexpressing JT-49	BALB/c mice	Promoted CFA/I-specific IgA responses in the intestinal mucosa in addition to serum IgG	[164]
α-GalCer	As above	Severe diarrheal disease (*V. cholerae*)	SmPill® minispheres contained formalin-killed *V. cholerae* Hikojima MS1242 bacteria and α-GalCer	C57BL/6 and BALB/c mice	Significantly enhanced intestinal and serum antigen-specific antibody responses	[14]
α-GalCer	As above	Chronic gastric infection (*H. pylori*)	Intragastric immunization with a whole-cell killed *H. pylori* antigen candidate vaccine with α-GalCer	C57BL/6, IL-17RA$^{-/-}$ and IL-1RI$^{-/-}$ mice	Induced effective immune protection against *H. pylori* infection with similar magnitude as cholera toxin as adjuvant Enhanced intestinal antigen-specific IgA responses to a whole-cell killed *H. pylori* antigen	[165]
Synthetic TLR-agonist adjuvants						
GS-986	TLR7 agonist	SIV	Ad26/MVA vaccination and GS-986 administration	Rhesus monkeys	Improved virologic control and delayed viral rebound following ART discontinuation Led to innate immune stimulation and cellular immune activation	[168]

Table 2 (continued)

Adjuvant names	Explanations	Disease models (pathogens)	Vaccines (or candidate) formulations or antigens	Animal models	Results or immune responses in oral administration trials	References
CpG-ISS (or CpG ODN 1018)	TLR9 agonist	NV	Oral co-delivery of NV VLPs with CpG-ISS	BALB/c mice	Augmented local VLP-specific fecal IgA titers	[175]
CpG ODN 1826	TLR9 agonist	OVA-sensitized asthma	OVA in combination with CpG-ODN	BALB/c mice	Induced OVA-specific T-cell proliferative response, IgG, and IgA	[171]
Multiple non-biogenic-composite (or multiple microparticles/nanoparticles) adjuvants						
Mannosylated PNPs	Mannosamine-coated poly(anhydride) NPs	OVA-sensitized asthma	OVA-loaded mannosylated polyanhydride NPs	BALB/c mice	Elicited higher and balanced systemic-specific antibody responses Elicited higher level of intestinal sIgA compared with SC administration Strong, long-lasting systemic and mucosal immune responses	[179]
UEA-MPL/lipid NPs	UEA-1 conjugated PLGA-lipid nanoparticles containing a TLR-agonist monophosphoryl lipid A	OVA-sensitized asthma	OVA-UEA-MPL/lipid NPs	BALB/c mice	Almost exclusively adhered to M cells Led to specific absorption and continuous retention in the PP Effectively transported by M cells and captured by mucosal DCs Stimulated effective mucosal IgA and serum IgG antibodies	[160]
UEA-1 LACNP	UEA-1 lectin-anchored alginate-coated chitosan NPs	NM	UEA-1 LACNP-BSA	BALB/c mice	Induces efficient systemic and mucosal immune responses against BSA	[210]
MPLA/PLGA	Immunostimulant MPL incorporated in PLGA NPs	OVA-sensitized asthma	OVA and the MPLA incorporated in PLGA	BALB/c mice	Induced a stronger IgG immune response than the control formulations Generated significantly higher IgA titers	[178]
LTA-PLGA NPs	Lectin-anchored PLGA NPs	Hepatitis B	LTA-grafted PLGA nanoparticles encapsulating hepatitis B surface antigen	BALB/c mice	Shown fourfold increase in the degree of interaction with the BSM Elicited strong mucosal and systemic response	[180]

Table 2 (continued)

Adjuvant names	Explanations	Disease models (pathogens)	Vaccines (or candidate) formulations or antigens	Animal models	Results or immune responses in oral administration trials	References
HP55/PLGA NPs	Acid- resistant HP55/PLGA NPs	*H. pylori* infection	*H. pylori* recombinant antigen CCF encapsulated acid-resistant HP55/PLGA NPs	BALB/c mice	Induced high levels of urease-specific antibodies and memory T-cell responses 43% of mice were completely protected after *H. pylori* challenge	[142]
Chitosan-alginate capsules	NM	KHV disease	Probiotic vaccine (pYG-KHV-ORF81/LR CIQ249 expressing KHV ORF81 protein) encapsulated by chitosan-alginate capsules	Koi carp	Effectively induced antigen-specific IgM Displayed effective KHV-neutralizing activity Provided 85% protection rate for koi carp against KHV challenge	[148]
Chitosan/alginate microparticles	NM	Fowl typhoid (*S. gallinarum*)	Live 9R vaccine coated with chitosan/alginate microparticles	Chicks	Upregulated IFNγ expression 100% protection No significant difference between oral and subcutaneous administrations Prevented vaccine destruction in the GIT	[211]
Alginate-coated chitosan NPs	NM	Hepatitis B	Recombinant hepatitis B vaccine with alginate-coated chitosan NPs	Mice	Have potential use as a delivery system for oral vaccination with recombinant HBsAg	[182]
CpG ODN-loaded alginate coated chitosan NPs	NM	Schistosomiasis (*Schistosoma mansoni*)	SmRho-CpG ODN-loaded alginate-coated chitosan NPs	C57BL6 mice	Showed significant modulation of granuloma reaction Presented significant levels of protection against infection challenge with *S. mansoni* worms	[181]
CpG ODN-loaded PLGA NPs	NM	Campylobacteriosis (*C. jejuni*)	Combination of PLGA-encapsulated CpG and *C. jejuni* lysate	Commercial broiler chicks	Reduced bacterial counts in cecal contents by 2.42 $\log_{10}$ Anti-*C. jejuni* IgG antibody titers were significantly higher	[183]

Table 2 (continued)

Adjuvant names	Explanations	Disease models (pathogens)	Vaccines (or candidate) formulations or antigens	Animal models	Results or immune responses in oral administration trials	References
ALG-CHT-LDH NPs	Alginate-chitosan coated layered double hydroxide nanocomposites	NM	ALG-CHT-LDH@BSA	NM	Significantly enhance the attachment and internalization of proteins in the Caco-2 cells and macrophages	[157]
PLG/PLA microsphere	Poly-(D,L-lactide-*co*-glycolide) and poly-(L-lactic acid)	Seven common respiratory pathogens	LW 50020 encapsulated into PLG and PLA microsphere	BALB/c mice	Enhanced immune response Immunomodulation was statistically significant compared with free LW 50020	[137]
CS/PLGA-NPs	Chitosan-coated poly (lactic-*co*-glycolic) acid NPs	NDV	pFDNA-CS/PLGA-NPs	Chickens	Induced stronger cellular, humoral, and mucosal immune responses	[212]

The immune response results of the above examples are all the results of the oral immunization test

AHR airway hyperresponsiveness, *AIDs* acquired immune deficiency syndrome, *ART* antiretroviral therapy, *BSA* bovine serum albumin, *BSM* bovine submaxillary mucin, *CMA* cow's milk allergy, *CpG-ODN* CpG oligodeoxynucleotides, *CRV* concentrated rabies virus, *CTL* cytotoxic T lymphocyte, *DCs DC* dendritic cells, *ETEC* enterotoxigenic *Escherichia coli*, *GIT* gastrointestinal tract, *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus, *HIV* human immunodeficiency virus, *IFN* interferon, *Ig* immunoglobulin, *IL* interleukin, *iNKT* invariant natural killer T cell, *KHV* Koi herpes virus, *MPLA* monophosphoryl lipid A, *MVA* modified vaccinia virus Ankara, *NDV* Newcastle disease virus, *NM* not mentioned, *NPs* nanoparticles, *NV* Norwalk virus, *OMPs* outer membrane proteins, *OmpA* outer membrane protein A, *OmpW* outer membrane protein W, *OVA* ovalbumin, *PNPs* polyanhydride nanoparticles, *SC* subcutaneous administration, *sIGA* secretory immunoglobulin A, *SIV* simian immunodeficiency virus, *Th* T helper, *TLRs* toll-like receptors, *TNF* tumor necrosis factor, *TPP* tripolyphosphate, *VLPs* virus-like particles

3.2.1 Polyanhydride-Based Oral Adjuvant Materials

Polyanhydrides (PAHs), a class of synthetic biodegradable, non-cytotoxic, biocompatible polymers, are polymerized by methyl vinyl ether and maleic anhydride [120, 121]. PAHs are inherently highly reactive to water, thus leading to relatively rapid hydrolytic degradation, breaking down into carboxylic acids without cytotoxicity [121]. PAHs have been used in vaccine delivery systems for a long time, and polyanhydride nanoparticles (PNPs) are licensed for oral drug delivery in the UK [121–123]. In fact, PAHs are also a promising oral vaccine encapsulating material with the function of adjuvant and carrier. First, polyanhydride particles are cleaved in the gut to expose carboxylic acid groups that form hydrogen bonds with the hydroxyl groups of glycoproteins in the gut mucus, giving polyanhydride particles their mucoadhesive properties [124, 125]. Second, it has been reported that polyanhydride particles possess intrinsic adjuvant properties, which can activate APCs and regulate the immune responses [121, 126]. Furthermore, polyanhydride particles have been demonstrated to be able to provide sustained release of protein antigens via surface erosion [121, 125]. In addition, polyanhydride materials can be made into nano-encapsulated formulations by nanotechnology, which can exert better adjuvant effects. PNP-based vaccines have been shown to successfully encapsulate and release antigens, activate B and T cells, and induce both antibody- and cell-mediated immunity towards a variety of immunogens [127]. Moreover, PNPs act as agonists of various TLRs (TLR2, 4, and 5) [10, 126], innate immunity, complement system, and APCs to modulate the immune responses and induce long-lasting immunity [121, 126, 128]. Renu et al. reported that mucoadhesive PNPs could protect the vaccine cargo and deliver it to intestinal immune sites to elicit robust mucosal immunity and mitigate *Salmonella* colonization and shedding [125]. Overall, PNPs have potent immune adjuvant properties when administered orally and can target immune cells of chickens [125], mice [129, 130], rats [131, 132], and other animals.

3.2.2 Poly(D,L-Lactide-*co*-Glycolide) and Poly(D,L-Lactic-*co*-Glycolic Acid)

PLG is a biodegradable and biocompatible polymer [133]. Microparticles prepared from PLG have been proven to be effective adjuvants for a variety of antigens because microencapsulation of PLG can protect antigens from adverse degradation, allow sustained and prolonged release of antigens for a long time, and enhance uptake of antigen by APCs [134]. These APCs containing PLG-microparticles are then delivered to specific lymphoid compartments, such as the spleen and mesenteric lymph nodes, where they effectively present antigenic epitopes to T lymphocytes, especially Th1 and Tc, thus inducing strong specific cell-mediated immunity (Fig. 1e1) [134, 135], which is urgently needed for eliminating intracellular pathogens in host cells. Kim et al. reported that using *H. pylori* lysates encapsulated in PLG nanoparticles as an oral vaccine candidate could induce the *H. pylori*-specific mucosal and systemic responses in mice, and enhanced Th2-type responses [136]. Kofler et al. reported that the pulmonary and serum immune responses of BALB/c mice were enhanced by oral immunization with LW50020 encapsulated with PLG microspheres [137]. Ramya et al. used PLG microspheres as an oral delivery system for β-propiolactone inactivated concentrated rabies virus (CRV) and found that Th1-mediated cellular immunity was activated after oral administration of PLG+CRV in mice [138]. In addition, PLG microspheres also have many potential advantages in gene therapy [133].

PLGA nanoparticles are US FDA-approved biocompatible and biodegradable polymers, which are widely used in preclinical vaccine delivery. PLGA has the functions of delivery device, protection, sustained release of encapsulated antigen, and enhancement of antigen uptake during vaccination [139–141]. In addition, PLGA combined with pH-responsive materials can adapt to the extreme GIT more efficiently and has the potential to become an oral vaccine adjuvant. Tan et al. designed an acid-resistant PLGA nanoparticle (HP55/PLGA-CCF) using pH-responsive material, HP-55, which was an effective immunomodulator and an oral carrier to enhance the efficacy of subunit vaccines. Mice immunized with HP55/PLGA-CCF nanoparticles could induce high levels of urease-specific antibodies and memory T-cell responses [142]. As pointed out by Munang'andu and Evensen [143], adjuvants that serve as antigen delivery vehicles and immunostimulants are able to enhance antigen uptake by APC. Furthermore, PLGA has the above two inherent adjuvant properties [144]. PLGA NP-rOmpW (i.e., the outer membrane protein W [OmpW] of *Aeromonas hydrophila* encapsulated in PLGA nanoparticles) provided dose-dependent protection against *A. hydrophila* infection in Rohu (*Labeo rohita* Hamilton) after oral administration [140]. In general, the design of PLGA nanoparticles as an oral immune adjuvant is a promising strategy to improve antigen uptake and vaccine efficiency.

3.2.3 Chitin, Chitosan and Their Derivatives

Chitin particles possess TLR-2-dependent adjuvant activity and can augment the Th1, Th2, and Th17 antigen-specific immune responses when admixed with protein antigens [145]. Chitosan (CS), a deacetylated form of chitin, is a polysaccharide composed of *N*-acetyl-D-glucosamine and D-glucosamine [146]. Because of its low toxicity, excellent biocompatibility, biodegradability, antimicrobial activity, mucoadhesive properties, and permeation-enhancing effects,

chitosan has been widely used as a potential excipient for the oral delivery of DNA, peptides, and live attenuated virus [147–151]; however, its limited mucoadhesive strength and low water solubility at neutral and basic pHs are considered as two major drawbacks of its biomedical applications. The chemical modification of chitosan results in quaternized chitosan [152] or its derivatives, such as *N*-trimethyl chitosan (TMC) [153], O-2′-hydroxypropyltrimethyl ammonium chloride chitosan (O-2′-HACC) [154], and mannosylated chitosan (MCS) nanoparticles [155]. This enhanced the mucoadhesive properties of chitosan. In addition, many researchers are trying to optimize chitosan nanoparticles and combine them with other nano-materials for composite adjuvants, which can promote a more efficient immune function and serve as a promising carrier for oral protein vaccine delivery [146].

3.2.4 Alginate and Its Derivatives

Alginate is a non-toxic, biodegradable, low cost, readily available polysaccharide copolymer containing (1-4)-linked β-D-mannuronate and α-L-guluronate residues, and is a mucoadhesive, biocompatible, non-immunogenic substance [2]. Alginate has been widely used in drug delivery because of its ability to contract in the stomach and release its cargo in the intestine. Alginate polymer as a single component is rarely used as an adjuvant. Alginate would usually be anchored/coated with chitosan or other electropositive materials by chemical modification to develop alginate-based composite adjuvant formulations for oral protein antigens (or vaccines) delivery (Tables 2, 3), such as alginate-coated chitosan microparticles (ACMs) [156] and alginate-chitosan coated layered double hydroxide nanoparticles (LDHs) nanocomposites (ALG-CHT-LDH) [157].

3.3 M Cell-Targeting Polymeric Particles (Ulex Europaeus Agglutinin-1)

UEA-1 is a lectin with specific binding activity to epitopes containing α-L-fucose [158]. UEA-1 can exclusively bind to M cells of mouse small intestine [159] and has been identified as an M cell-selective molecular marker [160]. Bioactive UEA-1 has been explored in the present investigation for targeted oral immunization. Gupta and Vyas reported UEA-1 conjugated liposomes as an oral M cell-targeted vaccine delivery vector [161]. In their study, the UEA-1 conjugated liposomes were predominantly targeted to the M cells. The serum anti-HBsAg IgG titer was obtained after oral immunization with HBsAg-encapsulated liposomes conjugated with UEA-1 for 3 consecutive days. The boosting immune effect was comparable with the titer recorded after single intramuscular immunization with alum-HBsAg [161]. Moreover, UEA-1-conjugated liposomes induced higher sIgA levels in mucosal secretions and cytokine levels in the spleen homogenates [161].

3.4 Alpha-Galactosylceramide

α-GalCer, a synthetic glycolipid, is a potent inducer of the invariant natural killer T (iNKT) cells, which are an important innate immune cell type [162]. α-GalCer can be presented by the CD1d molecules on the APC to NKT cells [163], which leads to activation and expansion of NKT cells, and subsequently induces full maturation of DCs in the spleen after immunization [162]. Therefore, α-GalCer is identified as a non-toxic oral adjuvant. It has recently been shown that α-GalCer acted as an oral active adjuvant to induce T-cell immunity against pathogenic bacteria and viruses through efficient activation/maturation of DCs. According to studies, α-GalCer potentiated mucosal immune responses to the HIV model envelope peptide (R15K peptide) [162], ETEC vaccine [164], *V. cholerae* vaccine [14], and whole-cell killed (WCK) *H. pylori* candidate vaccine [165] through oral immunization. The study by Davitt et al. demonstrated that α-GalCer was as effective as the 'gold standard' mucosal adjuvant CT in promoting intestinal IgA responses against a novel ETEC antigen [164]. In another study by Davitt et al., the addition of α-GalCer enhanced mucosal immunogenicity of Dukoral®, the most widely licensed oral cholera vaccine (OCV) internationally, and significantly increased intestinal anti-LPS and anti-cholera toxin B subunit (CTB) IgA responses against *V. cholerae* infections [14]. Longet et al. demonstrated that oral immunization of *H. pylori* WC antigen adjuvanted with α-GalCer significantly reduced bacterial loads in the stomach of *H. pylori*-infected mice; this reduction was IFNγ- and CD1d-dependent, similar to CT as adjuvant [165]. In conclusion, α-GalCer is an effective mucosal adjuvant for oral immunization and can enhance the mucosal responses of IgA and Th1 in mice, but its safety and efficacy in humans still warrant further evaluation. In addition to its impressive oral adjuvant effects in mice, α-GalCer has been tested in clinical trials for the treatment of cancer and hepatitis, in which its safety has been assessed [14].

3.5 Synthetic Toll-Like Receptor (TLR)-Agonist Molecules

As mentioned earlier, TLR molecules have been the target of many new mucosal vaccine candidates. Targeting one or more TLR(s) might activate sensors of innate TLR pathogens and promote intracellular signaling cascades that lead to upregulation of the production of chemokines and cytokines required for DC maturation, which results in increased magnitude and quality of immune responses [93, 166]. Some synthetic TLR ligands could also activate

Table 3 Current developments in biogenic and non-biogenic combined composite adjuvants for oral administration vaccines

Adjuvant names	Explanations	Disease models (pathogens)	Selected antigens or vaccines	Animal models or cell models	Results or immune responses	References
PLGA-IL-2, PLGA-IL-18, PLGA-GM-CSF	PLGA NPs combined with plasmid encoding IL-2, IL-18, or GM-CSF	FMD	PLGA-VP013/IL-2, PLGA-VP013/IL-18, PLGA-VP013/GM-CSF	Guinea pigs	Elicited significantly higher FMDV-specific antibody levels Significantly increased neutralizing antibodies Dramatically enhanced cellular immunity	[186]
CKS9-CNs	CKS9-immobilized chitosan NPs	None	None	*In vitro* transcytosis assay and closed ileal loop assay	Transported more effectively across the M cell model and accumulated more specifically into PP regions	[77]
Flagellin-PNPs	Polyanhydride nanoparticles coated with *Salmonella enteritidis*-derived flagellin	OVA-sensitized asthma	OVA-loaded flagellin nanoparticles	BALB/c mice	Elicited higher and balanced systemic specific antibody responses Elicited higher level of intestinal sIgA compared with SC administration Strong, long-lasting systemic and mucosal immune responses	[179]
F-PNPs	*S. enteritidis* flagellar protein-coated polyanhydride NPs	Salmonellosis (*S. enteritidis*)	*Salmonella* OMPs and flagellar protein-entrapped and surface flagellar protein-coated PNPs	Austra White laying chicks	Induced higher OMP-specific IgG response and secretion of Th1 cytokine IFN-γ Enhanced $CD8^+/CD4^+$ cell ratio Increased OMP-specific lymphocyte proliferation Upregulated the expression of TLR2 and 4, TGF-β, and IL-4 cytokine genes Cleared Salmonella cecal colonization in 33% of vaccinated birds	[125]
CS NPs-F	Flagellin protein-coated CS NPs	Salmonellosis (*S. enteritidis*)	OMPs-F-CS NPs	Chicks	The particles were localized in ileal Peyer's patches Induced significantly higher OMP-specific mucosal IgA and lymphocyte proliferation response Increased the expression of TLR2, TLR4, IFN-γ, TGFß and IL-4 mRNA expression	[213]

Table 3 (continued)

Adjuvant names	Explanations	Disease models (pathogens)	Selected antigens or vaccines	Animal models or cell models	Results or immune responses	References
CKS9-WSC-PLGA MPs	Porous PLGA MPs coated with M cell-homing peptide (CKS9)-coupled water-soluble chitosan	Swine dysentery (*B. hyodysenteriae*)	Membrane protein B of *B. hyodysenteriae* loaded into porous PLGA MPs coated with the WSC conjugated with CKS9	BALB/c mice	Enhanced M cell targeting and transcytosis ability Showed elevated secretory IgA responses and systemic IgG responses Induced both Th1- and Th2-type responses	[185]
AlgChiPs	Alginate-coated chitosan particles	HBV	Recombinant HBsAg encapsulated into AlgChiPs	C57BL/6 mice	Induced serum anti-HBsAg IgG and anti-HBsAg sIgA	[214]

The immune response results of the above examples are all the results of the oral immunization test

CS chitosan, *FMD* foot and mouth disease, *GM-CSF* granulocyte-macrophage colony-stimulating factor, *HBsAg* hepatitis B surface antigen, *HBV* hepatitis B virus, *IFN* interferon, *Ig* immunoglobulin, *IL* interleukin, *MPs* microparticles, *NPs* nanoparticles, *OMPs* outer membrane proteins, *OVA* ovalbumin, *PLGA* poly(D,L-lactic-*co*-glycolic acid), *SC* subcutaneous, *sIGA* secretory immunoglobulin A, *TGF* transforming growth factor, *Th* T helper, *TLR* toll-like receptor

TLR signals and subsequently promote immune responses, which have been exploited as potential adjuvants of mucosal vaccines. For example, incorporation of the TLR4 agonist monophosphoryl lipid A (MPL) into nanoparticle vaccines could contribute to triggering TLR signaling with mucosal DCs and subsequently improve the capture efficiency of vaccines [160]. The TLR 7/8 agonists R848 have showed great potential as oral vaccine adjuvants because they can directly activate APCs and enhance both humoral and cellular immune responses, especially Th1 responses [167]. According to Borducchi et al., oral administration of Ad26/MVA combined with the TLR7 agonist GS-986 could decrease the level of SIV viral DNA in lymph nodes and peripheral blood, as well as control and delay virologic rebound following antiretroviral therapy discontinuation in SIV-infected Rhesus Monkeys [168].

In addition, CpG oligodeoxynucleotides (CpG-ODN) are another promising synthetic TLR-agonist adjuvant. They are short single-stranded synthetic DNA molecules that can activate the immune system and have been found to be effective in the prevention and treatment of infectious diseases, allergies, and cancers [16, 169]. CpG-ODN, a ligand of TLR9, can activate TLR9 on B-lymphocytes and DCs, showing potent activity in stimulating antigen presentation and inducing antigen-specific immune response towards the Th1 phenotype [170]. Alignani et al. reported CpG-ODN-loaded ovalbumin (OVA) induced specific mucosal and systemic immune response in mice after oral administration [171]. CpG-ODN has different classes, such as CpG-ODN 2007 [172], CpG-ODN 1668 [173], and CpG-ODN 1826 [174], and has also shown potent mucosal adjuvant activity. Hjelm et al. [175] used a panel of TLR agonists (PIC [TLR3], FLAG [TLR5], GARD [TLR7], CpG [TLR9], CpG-ISS [CpG 1018, alternate CpG motif, TLR9], and CL097 [TLR7/8]) as adjuvants combined with Norwalk VLPs (NV VLPs) coadministered to mice through intranasal and oral routes to determine the mucosal adjuvant activity of these immunomodulators. Of these, intranasal co-delivery of VLPs with TLR7 or TLR9 agonists (i.e., GARD or CpG) produced the most robust and broad-spectrum immune response, but oral administration with other TLR agonists (i.e., PIC, FLAG, and CL097) could not consistently enhance VLP-specific immune responses in mice.

According to our knowledge, there are no human trials using TLR agonists as oral vaccine adjuvants. These above studies are preclinical studies, indicating that TLR plays an important role in inducing immune response in the oral route.

3.6 Composite Non-biogenic Material Adjuvants

Through chemical modification or nanotechnology, the physicochemical properties of non-biogenic adjuvant materials

(or polymeric nanoparticles) can be improved by combining them with another non-biogenic adjuvant material. Their advantages can be complementary, which is beneficial to enhance the interaction between nanoparticle adjuvant and intestinal endocytosis pathways [176, 177]. For instance, UEA-1 is the M-cell selective molecular signature, which could exclusively adhere to M cells, and MPL is a TLR agonist. With a combination of UEA-1 and MPL, Ma et al. [160] reported that the composite material, UEA-MPL-conjugated PLGA-lipid nanoparticles, can be effectively transported by M cells and captured by mucosal DCs, showing the potential of an attractive oral vaccine delivery system for boosting oral immunity. Ma et al. found that OVA-UEA-MPL/lipid nanoparticles stimulated the most effective mucosal IgA and serum IgG antibodies during oral vaccination [160]. Sarti et al. used MPL-conjugated PLGA nanoparticles as an oral adjuvant of OVA in mice [178], and compared with the control formulation group, it generated significantly higher IgA titers, which indicated that MPL-PLGA nanoparticles had the ability to induce mucosal immunity. Salman et al. reported that mannosamine-coated poly(anhydride) nanoparticles as an oral composite adjuvant of OVA induced strong, long-lasting systemic and mucosal immune responses than the non-conjugated vectors [179]. Moreover, Mishra et al. demonstrated that LTA (*Lotus tetragonolobus* from *Winged or Asparagus pea*)-anchored PLGA nanoparticles could elicit strong mucosal and systemic response and hence could be a promising M cell-targeting adjuvant for oral mucosal immunization against hepatitis B [180]. Alginate-coated chitosan nanoparticles are of interest because of their great stability and immunostimulatory properties. They can effectively transport antigens into the M cells and subsequently induce significant immune responses in serum IgG and mucosal sIgA levels [156, 181]. Borges et al. demonstrated that alginate-coated chitosan nanoparticles showed potential as a delivery system for oral recombinant HBsAg [182]. Most recently, Yu et al. demonstrated that alginate-chitosan coated layered double hydroxide nanocomposites (ALG-CHT-LDHs) showed great potential in oral protein vaccine delivery [157]. In addition, Taha-Abdelaziz et al. reported that oral administration of PLGA-encapsulated CpG ODN, and *Campylobacter jejuni* lysate reduced cecal colonization by *C. jejuni* in chickens [183].

The above studies show that the physicochemical properties of single non-biogenic adjuvant material can be improved by comprehensive combination of double or triple, or even quadruple, adjuvant materials through nanotechnology, so as to correctly match the size, electric charge, hydrophobicity, and other physicochemical properties of antigen, and so that antigen can cross the mucosal barriers and target APCs [184]. Through the appropriate combination of a variety of non-biogenic adjuvant materials, its advantages could be developed and its disadvantages could be avoided, so as to construct a universal and powerful oral vaccine carrier or adjuvant.

4 Biogenic and Non-biogenic Combined Composite Material for Oral Adjuvants

Nowadays, using conjugation techniques to combine biogenic adjuvants (such as M cell-targeting peptides, bacterial flagellin, or cytokines, etc.) with other non-biogenic adjuvants to create composite adjuvants can give full play to the advantages of the activity of each adjuvant, thus enhancing the overall activity of adjuvants (Table 3). The composite adjuvant, PLGA microparticles coated with chitosan-coupled M cell-homing peptide (CKS9), can strengthen the targeting ability to M cells, and the mucosal and systemic immune responses were induced when it was used to deliver swine dysentery vaccine [185]. As mentioned previously, PNPs are natural mucoadhesive polymers that could efficiently deliver antigens to the GALT [179], and flagellar protein possesses potent immune adjuvant activity. Renu et al. designed a *Salmonella* subunit vaccine (OMPs-F-PNPs) that consisted of PNPs containing immunogenic *Salmonella* OMPs and entrapped flagellar (F) protein and surface F-protein-coated PNPs. The vaccine could induce specific immune response to mitigate *Salmonella* colonization in the intestines of chickens vaccinated orally [125]. Yang et al. used PLGA nanoparticles combined with cytokine as adjuvant to deliver DNA vaccine of foot and mouth disease, which significantly enhanced its immunogenicity than naked DNA [186]. Generally, the conjugation of biogenic adjuvant and non-biogenic adjuvant as composite adjuvants is another frequently used and promising way to assist the delivery of oral vaccine and enhance its immunogenicity.

5 Concluding Remarks, Challenges, and Future Perspectives

The complex and harsh environment in GIT leads to the weak immunogenicity of peroral mucosal vaccines. Preparation of effective adjuvants to enhance the immune response is an integral part of the development of oral vaccines. Over the past few decades, several biogenic and/or non-biogenic adjuvants have been used in the trials of various peroral mucosal vaccines to enhance their immune responses. At present, among the aforementioned adjuvant candidates of peroral vaccines, only dmLT has undergone human clinical trials and further passed clinical phase I and II trials [187, 188], while other adjuvant candidates are still in animal (or veterinary or aquatic) experimental stage (Fig. 2). In this review, some adjuvants have been tested on farm animals, such as pigs, birds, fish, etc., to develop veterinary vaccines

(or adjuvants) (Fig. 2). For the development of veterinary vaccines (or adjuvants), the target animals are the most ideal animal models. Mice (especially BALB/c mice) are commonly used animal models for preclinical trials of adjuvants (or vaccines) in animals or humans (Fig. 2). Animal models play a critical role in the in vivo study of the immunology and pharmacology of oral adjuvant (or vaccines) candidates, as well as the evaluation of potential applications in humans. However, it is undeniable that many animal models used in oral adjuvant (or vaccines) tests have limited predictive value for the human response to oral adjuvants (or vaccines) in terms of both efficacy and toxicology [189]. Among the examples of oral adjuvants (or candidates) we summarized (Tables 1, 2, 3), there were few reports focusing on the purposeful selection of animal models to evaluate the efficacy of oral adjuvants. Many researchers chose animal models for their animal experiments based on the disease types they were studying, instead of the oral adjuvants, which made it difficult to translate the positive effects of oral adjuvants on animal models to humans and required considerable analysis and debate. It is necessary to improve the existing animal models to make them more predictive for humans.

An obvious advantage of biogenic adjuvants is that they can be constructed or optimized by genetic engineering. On the one hand, gene editing technology is used to remove their toxicity and optimize their adjuvant performance, such as from LT to dmLT (R192G/L211A) and from CT to mmCT. On the other hand, the biogenic adjuvants could be fused with the target antigens by DNA recombination technology to construct the fusion protein vaccines. In addition, most biogenic adjuvants could be encoded and expressed in beneficial bacterial strains, such as probiotics (e.g., LAB), attenuated live bacteria, or gut commensal bacteria, as a protective strategy across the GIT against degradation from gastric acid and proteases, etc. This is also another promising way to develop oral vaccines. Ideally, adjuvants should not induce adaptive immune responses against themselves, but should promote appropriate immune response to accompanying antigens [190, 191]. However, some well-known protein-based oral adjuvants listed above, including FliC [192, 193], CTB [194], FnBPA [51], PorA [195], and even the well-studied dmLT [196], have been reported to produce a certain degree of immune response against themselves in the host, thereby potentially affecting their effectiveness as adjuvants. However, FljB has been reported to produce no immune response against itself [40], and an appropriate oral dose of dmLT is still safe, well tolerated, and reasonably immunogenic [196].

Using non-biogenic adjuvants, protein antigens could be coated in a variety of ways, such as lipidation, nanoparticle encapsulation (using polymersomes, for example), adsorption and conjugation to polymer-based microparticles/nanoparticles (using PLGA, chitosan, alginate, etc.) and/or additive/synergistic admixture [167]. In addition, combining two or more types of adjuvant materials (including biogenic and non-biogenic materials) to construct composite adjuvants can make up for inherent flaws of some biogenic adjuvants, such as their short half-life and ease of degradation in GIT, and strengthen their immune effects in the intestinal tract. There are also some nanomaterials that can control the slow release of vaccines. For example, some TLR ligands are often chimeric with other nano-adjuvant materials, therefore the adjuvant effect is better.

Undoubtedly, in order to exert the adjuvant activity for peroral mucosal vaccines, more attention should be focused on the endogenous immune activation mechanisms of adjuvants and the immunological and pharmacological relationship among adjuvants, vaccines (or antigens) and the host gastrointestinal mucosal immune system. Although there are various adjuvant strategies, they should all be studied in detail before selecting the optimum formulation. The formulation of vaccines and adjuvants should not only maintain the immunogenicity of the vaccine but also protect their adjuvant activity. This is an issue that needs meticulous consideration when designing oral vaccines.

The development of oral adjuvants still presents many challenges. As mentioned earlier, the GIT is a complex and harsh environment, which leads to the instability of adjuvants, especially biogenic adjuvants, and hinders the interaction between oral adjuvants and intestinal epithelial cells. On the other hand, the low proportion of M cells in the intestinal epithelium would limit the effect of M cell-mediated adjuvants. However, we believe that more and more oral adjuvants, similar to RANKL, that can induce M-cell differentiation will emerge in the future to change this dilemma. The potential safety concerns of adjuvants, such as cytokine-derived adjuvants [93], are another challenge. The effective targeting, pharmacokinetics and nanotoxicology of some potential oral adjuvants need to be further evaluated.

As mentioned previously, the targeting peptides that target intestinal immune cells (or receptor proteins on their cell surface) can improve the binding ability of antigens to bind to intestinal DCs or M cells (or receptors). Therefore, in addition to the targeting peptides of human and/or mouse intestinal immune cells (and their receptor proteins), some researchers are trying to screen and identify specific M cells or DC-binding peptides of other animal species for studies in veterinary or comparative medicine by using the cell-based phage display technique combined with high-throughput sequencing; for instance, the chicken DC-binding peptide (SPHLHTSSPWER, named SP) [197] and the porcine TLR2-targeting peptide ligand (NAGHLSQ) [198] [porcine TLR2 is highly expressed in M cells and plays an important role in pig mucosal immune responses]. Aiming at M cells or DCs (or receptor proteins on their cell surface), it will

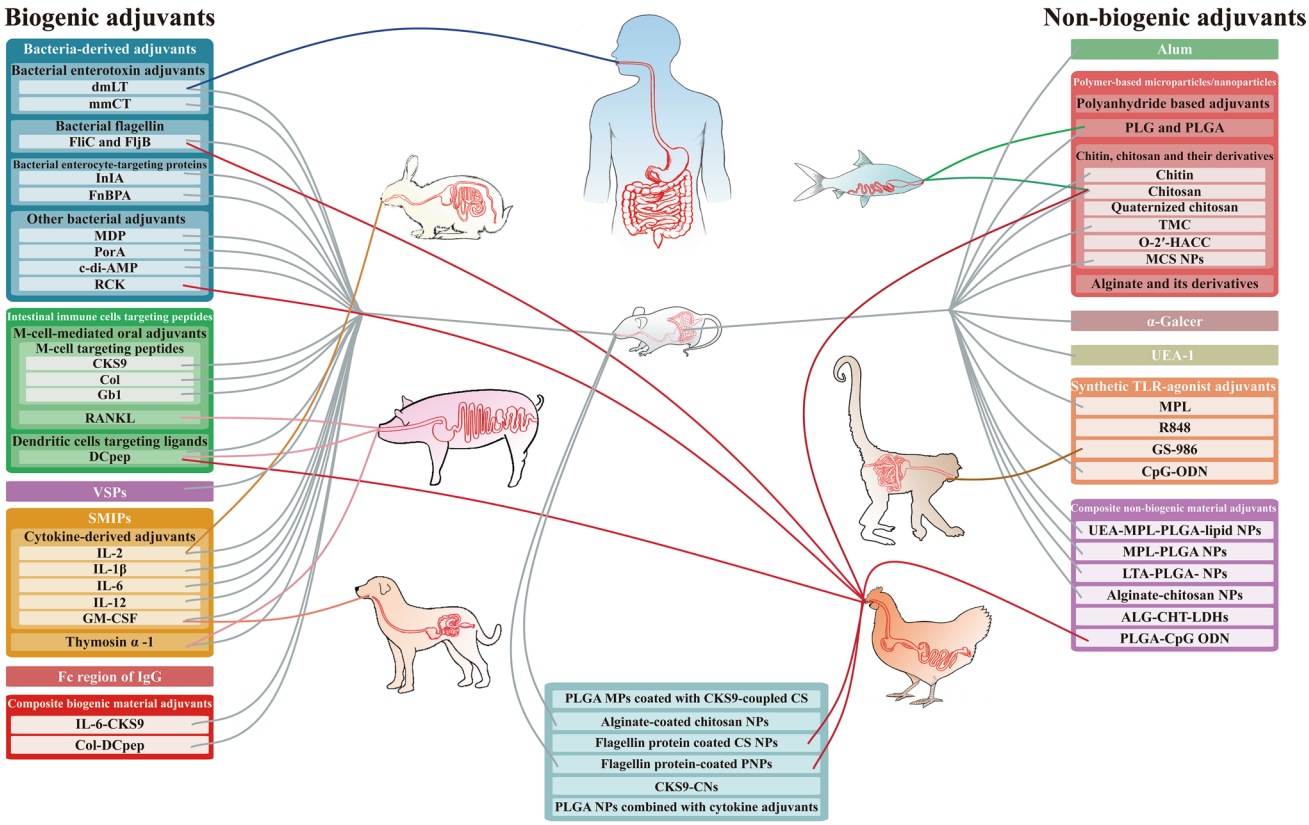

Fig. 2 List of oral adjuvant candidates developed and their corresponding in vivo tests. According to the physicochemical properties, oral adjuvants could be divided into biogenic, non-biogenic, and a biogenic and non-biogenic combined composite. In vivo tests for oral adjuvant development have involved humans, rabbits, fish, rodents, pigs, primates, canines, and chickens. No connection means the in vivo test has not yet been carried out. *α-GalCer* alpha-Galactosylceramide, *c-di-AMP* 3′5′-cyclic di-adenosine monophosphate, *CKS9* M cell-targeting peptide, *Co1* M cell-specific peptide ligands, *CpG-ODN* CpG oligodeoxynucleotides, *DCpep* dendritic cell-targeting peptide, *dmLT* double-mutant heat-labile toxin, *FnBPA* fibronectin binding protein A, *GM-CSF* granulocyte-macrophage colony-stimulating factor, *MCS NPs* mannosylated chitosan nanoparticles, *MDP* muramyl dipeptide, *mmCT* multiple mutant cholera toxin, *MPL* monophosphoryl lipid A, *O-2′-HACC* O-2′-hydroxypropyltrimethyl ammonium chloride chitosan, *PLG* poly(D,L-lactide-*co*-glycolide), *PLGA* poly(D,L-lactic-*co*-glycolic acid), *RANKL* receptor activator of NF-kB ligand, *RCK* Salmonella resistance to complement killing, *SMIPs* small molecular immunomodulatory proteins, *TMC* trimethyl chitosan, *UEA-1* ulex europaeus agglutinin-1

be a trend to develop more targeting peptides for different animal species, especially in the prevention and control of veterinary infectious diseases. Furthermore, with further understanding of the mechanisms of action of some less-studied candidate adjuvants, such as muramyl dipeptide and tuftsin fusion protein (MT) [54, 55], *N. meningitidis* PorA [57], c-di-AMP [58], RCK protein [62], etc., these may be the future development direction of oral adjuvants.

With the development of oral adjuvants in recent years, it is believed that more reasonable and effective oral adjuvants will appear in the future and hence solve the challenges mentioned.

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Declarations

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Conflict of interest Bingming Ou, Ying Yang, Haihui Lv, Xin Lin, and Minyu Zhang declare that they have no competing interests.

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