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Mucosal immune probiotic vector vaccines: Development and prospects for application in COVID−19

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused a global pandemic of infectious disease characterized by significant morbidity, symptomatic manifestations, and severe outcomes. Vaccines have proven to be an effective means of impeding the transmission of SARS-CoV-2, and have been widely employed globally. Despite breakthroughs such as mRNA vaccine, intramuscular COVID-19 vaccine still faces numerous chanllenges, such as failure to completely avoid infections, high cost, and difficult transport conditions. The mucosa is the primary site through which SARS-CoV-2 invades the body and may induce effective immune responses. In the face of the increasing immune escape capacity of the Omicron variants and the potential outbreak of a new round of epidemics, it is imperative to develop a new generation of mucosal immune vaccines to prevent infection, which is mainly transmitted through the respiratory tract, along with its severe and fatal consequences. Probiotics have not only been used as safe raw materials for fermented foods but have also been developed as oral or nasal vaccine vectors for various pathogens in recent years, showing good mucosal and systemic immune effects in animals or humans. Although the antigen delivery technology based on protein anchoring on the surface of probiotics is rarely applied to COVID-19, probiotic-based vaccines aim at a new development direction for COVID-19 vaccines with low cost, high safety, and potential mucosal immune programs. In this review, we will discuss in detail the feasibility, advantages, and possible limitations of probiotics as a mucosal immune vaccine vector, which will be conducive to coping with the current situation of COVID-19.

KEYWORDS: Probiotics; Non-intramuscular vaccines; COVID-19; Mucosal immunity

1. Introduction

It has been three years since World Health Organization declared COVID-19 a pandemic[1]. As of August 30, 2023, the severe acute respiratory syndrome type 2 coronavirus (SARS-CoV-2) has resulted in a total of 770 million confirmed cases and 6.95 million deaths worldwide[2]. Given that the coronavirus can rapidly spread from person to person, measures to prevent or reduce transmission should be taken in high-risk populations[3]. In the three links of infectious disease prevention and control which include eliminating the source of infection, cutting off transmission routes, and protecting susceptible populations, vaccination is widely used as the most cost-effective way to block the spread of pathogens and safeguard public health[4].

The process of SARS-CoV-2 invading human cells is completed through the binding of SARS-CoV-2 S1/receptor-binding domain (RBD) and human angiotensin-converting enzyme (hACE2) receptor[5]. Therefore, developing RBD recombinant protein vaccines to immunize humans can achieve the production of

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neutralizing antibodies and antiviral effects in vivo[6]. The mucosal immune system is the largest component of the human immune system, and it is also the first immune barrier when SARS-CoV-2 invades the body. The mucosal immune response to nasopharynx-associated lymphoid tissue (NALT) or bronchial-associated lymphoid tissue (BALT) during SARS-CoV-2 infections, primarily in the upper respiratory tract, induces the production of secretory IgA antibodies against SARS-CoV-2, which are as important as serum circulating IgA and IgG[7]. The infectivity of SARS-CoV-2 mutants is greatly enhanced due to the RBD mutation, which increases the binding ability of the virus to ACE2[8], and because ACE2 is highly expressed in the nasal mucosa, the binding ability of omicron to nasal mucosal epithelial cells becomes stronger[9,10]. The latest researches indicate that motile cilia, microvilli, and mucociliary-dependent mucus flow are critical for efficient virus replication in nasal epithelia[11]. It is difficult to achieve the most timely and effective mucosal immune protection with the current intramuscularly based immunization method for COVID-19. The evaluation, based on the model of human airway and nasal organoids, indicates that the higher entry efficiency and fusogenic activity of BA.5 spike potentiated viral spread through syncytium formation in the human airway epithelium, leading to enhanced replicative fitness and immune evasion[12]. Vaccines immunized intranasally have the potential to respond effectively at the primary line of defense when the virus enters the body, thereby reducing the probability of infection, serious illness, and death[13]. At the same time, it was found that the gastrointestinal tract also plays a very important role in the process of COVID-19 infection. Due to the high expression of the ACE2 receptor in intestinal tissues, once infected and entering gastrointestinal cells, SARS-CoV-2 replicates in large quantities and causes digestive system symptoms such as diarrhea and vomiting[14]. Despite the development of various routes for COVID-19 vaccine administration, including oral, intra-muscular, intra-nasal, aerosol, and inhaled, few indirect injection vaccines have been applied on a large scale[15]. Given the lack of relevant summaries on the development and efficacy of COVID-19 mucosal immune vaccines and the latest summary of promising probiotic vector vaccines for intranasal and oral administration, we present here the advantages of the mucosal immunization vaccine application, together with an analysis of the difficult situation and future perspectives of using probiotics as antigen delivery vectors for the development of the next generation of COVID-19 vaccines.

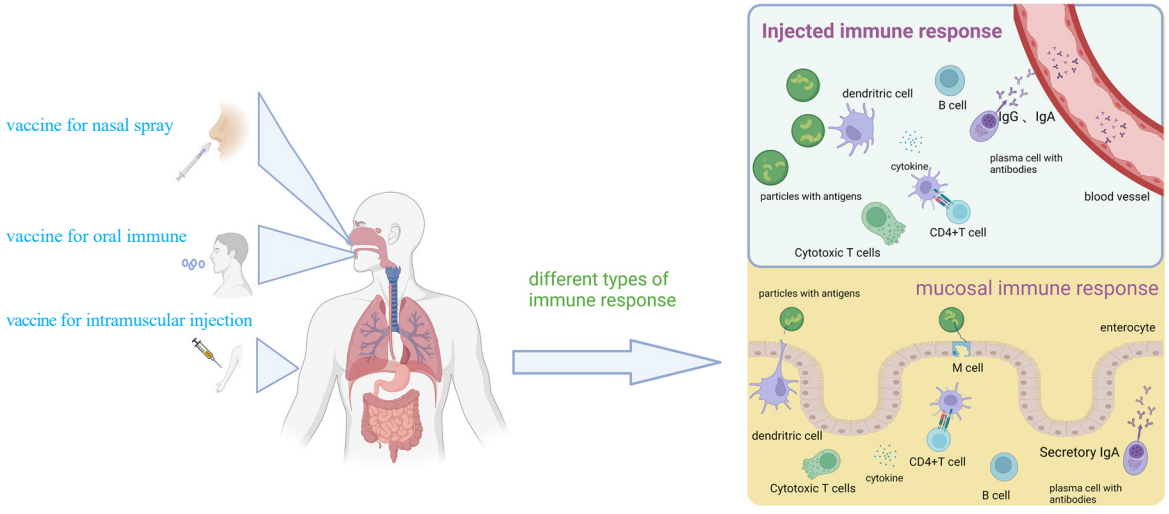

Figure 1. Immune responses induced by intranasal/oral and intramuscular vaccination.
At present, the listed COVID-19 vaccines mainly rely on intramuscular injection to induce systemic and local immune responses, which mainly generate circulating immunoglobulin G (IgG) antibodies to neutralize the virus through blood transmission. Vaccines immunized by nasal spray, inhalation, or oral administration have the advantages of safety and simplicity in application. What is more important is that compared to injectable vaccines, mucosal immune vaccines can effectively neutralize the virus for the first time by inducing immune responses in the body. Nasopharynx-associated lymphoid tissue (NALT) and intestinal lymphoid tissue can be stimulated by viral antigens to induce secreted IgA antibodies in the mucosa, directly countering the virus from respiratory or gastrointestinal infections.

2. Development and application of mucosal immune vaccines

Currently approved vaccines for COVID-19 are mainly administered intramuscularly and can effectively induce the body to produce antibodies against severe disease, yet breakthrough infections have occurred[16,17]. According to information provided by the World Health Organization, as of March 30, 2023, there are 382 candidate vaccines under development, out of which 13 have been certified and listed as Emergency Use Listing(EUL)[15,18]. The vaccine routes developed for COVID-19 since its discovery in 2019, including inactivation, recombinant protein, viral vector, mRNA, etc., and they have been evaluated for their effectiveness[19]. This also includes the newly introduced mucosal immunization vaccines administered by inhalation or nasal spray (Table 1). The current COVID-19 vaccines mainly induce a Th1-biased response characterized by interferon-γ and tumor necrosis factor-α cytokine secretion by CD4 T cells and antibody production predominantly of IgG1 and IgG3 subclasses[20], and they also induce cytotoxic CD8 T cells but do not or only produce low-titer neutralizing IgA. Studies have shown that circulating IgG has challenging to penetrate the mucosal lumen and serum IgG concentrations induced by injectable vaccines do not truly reflect protection against respiratory tract infection[21]. An oral polio vaccine, developed by Albert Sabin in 1960 using three attenuated poliovirus strains, nearly eradicated polio worldwide and is considered as one of the safest vaccines in human history[22,23]. The safety and efficacy of oral rotavirus vaccines in children have also been demonstrated[24]. Huimin Yan's group developed an intranasal drip or spray mucosal vaccine called "P-KFD1" based on the human respiratory syncytial virus (RSV) phosphoprotein, which demonstrated effective antiviral effects in mice[25]. The vaccine is produced based on the expression of Escherichia coli, which offers the advantages of low cost and easy rapid mass production[26]. Only 28.5% of people in low-income countries have received at least one dose of the COVID-19 vaccine[27]. The low-cost production method of mucosal immune vaccines and the simplicity of nasal spray therapy are conducive to reducing the medical burden and will provide low-cost and safe alternatives for COVID-19 control in developing countries[28,29].

Table 1. Evaluation of different routes of COVID-19 vaccine effectiveness or immunogenicity.

Name	Manufacturer	Platform	Immunization way	Observation time	Effectiveness or immunogenicity	References
BNT162b2	BioNTech/Pfizer	mRNA	Intramuscular	19 days after 3-dose	95.3%	[30]
AZD1222 Vaxzevria	AstraZeneca	Recombinant ChAdOx1 adenoviral vector	Intramuscular	14 days after 2-dose	70.4%	[31]
Ad26.COV2.S	Janssen–Cilag International NV	Recombinant, replication-incompetent adenovirus type 26 (Ad26) vector	Intramuscular	14 days after 1-dose	66.9% (moderate to severe-critical cases);	[32]
mRNA-1273/Spikevax	Moderna Biotech	mRNA	Intramuscular	14 days after 2-dose	94.1%	[33]
BBIBP-CorV	Beijing Institute of Biological Products Co., Ltd	Inactivated, produced in Vero cells	Intramuscular	14 days after 2-dose	78.1%	[34]
NVX-CoV2373	Novavax	Recombinant nanoparticle prefusion spike protein	Intramuscular	7 days after 2-dose	86.3% (B.1.1.7); 96.4% (non- B.1.1.7)	[35]
Ad5-nCoV/Convidecia	CanSinoBIO	Adenovirus type 5 vector	Intramuscular	14 days after 1-dose	63.7%	[36]
Ad5-nCoV/Convidecia	CanSinoBIO	Adenovirus type 5 vector	Aerosol oral administration	14 days after 1-dose	Strong NAbs response and specific IgG and IgA antibodies	[37]
dNS1-RBD	Beijing Wantai et.al	Influenza virus vector	Nasal spray	30 days after 2-dose	Strong cellular immune response	[38]

Note: Among the routes of COVID-19 vaccines currently, on the market, intramuscular vaccines are dominant. By comparison, the viral vector vaccine using inhalation or nasal spray is still in its infancy. In terms of vaccine effectiveness (VE), mRNA vaccines have an unparalleled advantage. The effectiveness evaluation of mucosal immune vaccines is relatively lacking and has been reported only in terms of cellular or humoral immune effects. NAbs: Neutralising antibodies.

The orally administered aerosolized Ad5-nCoV vaccine that encodes the SARS-CoV-2 spike protein, which was developed by CanSino Biologics, has been approved for emergency use as a booster in China in September 2022. According to the results of the published phase I clinical data, the greatest neutralizing antibody response against the Omicron variant was generated at day 28 post-vaccination, as well as the greatest IFN-γ T-cell response at day 14 after booster vaccination[37,39]. On December 2, 2022, the dNS1-RBD nasal spray influenza virus vector COVID-19 vaccine, jointly developed by Xiamen University, the University of Hong Kong, and Wantai Bio, was approved for emergency use. The vaccine is a temperature-sensitive replicating live virus vector vaccine constructed by inserting a gene encoding the RBD of the spike protein of the SARS-CoV-2 prototype strain based on a doubly attenuated influenza virus vector (CA4-dNS1), which is administered by nasal spray to form a local first-line protective immune response against the new coronavirus in the respiratory tract, with rapid onset of action, durable protection, broad-spectrum anti-mutation, easy vaccination, and good safety[40].

3. Probiotic vector vaccines

Non-living antigen delivery systems, such as cationic liposomes, have made great strides in vaccine development[41]. Living delivery systems include genetically engineered virus[42], bacteria[43], and protozoa[44]. Bacterial vector vaccines include attenuated bacterial and probiotic vector vaccines, and the applications of the former include *Listeria*, *Salmonella*, and *Vibrio cholerae*[45–47]. Although attenuated bacterial vector vaccines have been developed to some extent to stimulate durable humoral and cellular immunity in the body, their safety risks must be considered[48]. Food-grade probiotics are a kind of potential live antigen delivery vector that can be used as a live carrier to deliver heterologous proteins of SARS-CoV-2. Many strains of Lactobacillus and Lactococcus, which are the main raw materials and ingredients of fermented foods, have been generally regarded as safe (GRAS) by the US Food and Drug Administration (FDA) and have been consumed as symbiotes originating from the human gut for a long time. Studies have shown that *Lactobacillus lactis* does not colonize in mice for a long time after oral administration[49], and there are no critical changes in the gut microbiome after oral administration of probiotic cell extracts[50]. One of the advantages of the bacterial vector vaccine is that immune cells recognize the lipoteichoic acid (LTA), peptidoglycan (PG), and cytomyl dipeptide on the bacterial surface through TLR 2/6, leading to the production of mucosal antibodies, especially IgA[51]. Most probiotics possess the characteristics of bile salt and gastric acid tolerance, ensuring their retention activity in the gastrointestinal tract (GIT)[52,53]. It is well documented that lactic acid bacteria (LAB) provide immunomodulatory/immunostimulatory activity and contribute to health maintenance[54]. Probiotics have also been extended as nutritional modifiers for chronic metabolic diseases and can be effective in improving the physical status of patients with diseases such as diabetes and Parkinson's[55,56]. The substitute of probiotics as antigen delivery vehicles allows direct contact between the antigen and the human immune system to better induce an immune response while overcoming the risk of reversion and residual virulence due to the use of live attenuated enterovirus strains. Therefore, this technology has been considered and developed very early[57]. In addition, although controversial in human clinical trials, the selection of specific strains of probiotics as vaccine adjuvants to enhance immune efficacy has been demonstrated in vitro and in animal models[58,59]. An interesting randomized, double-blind controlled trial of unvaccinated COVID-19 outpatients receiving a specifically formulated throat spray with *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus rhamnosus* was conducted. A trend towards a more rapid decrease in positivity compared to controls was observed, suggesting an anti-SARS-CoV-2 effect of probiotics[60]. However, no significant effect seems to have been observed in the prevention of COVID-19 infection[61]. In infected patients, the viral load in the nasal cavity is higher than that in the throat, indicating that early invasion of the virus may be achieved through upper respiratory tract infections[62]. The failure of nasal epithelial anti-viral immunity at the beginning of infection is an important factor in the development of COVID-19[63]. The mucosal immune system is responsible for functioning as a first line of physical and immunological defense against invading pathogens[64]. In the face of Omicron mutant strains, which are predominantly respiratory infections, inhalation vaccines via the mouth or nose are even more important[65]. Considering the characteristics of safety, low cost, non-immunosuppression, and the ability to induce mucosal and systemic immune responses of vaccines orally administered by lactic acid bacteria, the application of LAB in the mucosal immune procedure for COVID-19 vaccines is very attractive.

4. Delivery principle based on probiotic vectors

Fixation and display of antigenic proteins on the bacterial surface, as well as cytoplasmic transport, would both be ideal delivery forms for mucosal immunity in the gastrointestinal tract and nasal cavity. Gram-negative bacteria are typically invested as delivery vehicles but are limited by their inevitable retention of invasiveness[66]. Non-pathogenic gram-positive bacteria overcome this drawback. Probiotics applied for surface display of heterologous antigenic

proteins include *Lactobacillus casei*, *Lactococcus lactis*, *Lactobacillus acidophilus*, and *Lactobacillus plantarum et al*[67–70] (Table 2). There are five types of proteins that can be used as anchoring components present on the surface of gram-positive bacteria, including transmembrane proteins, lipoproteins, covalently bound cell wall-associated proteins, non-covalently bound cell wall associated proteins, and S-layer proteins. The attachment of ankyrin to bacterial surface structures can be classified as covalent or noncovalent[71,72]. According to the different surface structures of anchoring bacteria, ankyrin or anchoring methods can be divided into the following five categories: poly-γ-glutamate synthase A (pgsA) proteins from *Bacillus subtilis* anchor to the cytoplasmic membrane[73–75]; lipid cassette motifs that mediate antigen anchoring to lipoproteins[76]; LysM motifs, and SH3b motifs noncovalently anchor to the cell wall(CW)[77,78]; or LPXTG-type anchor proteins and M6 proteins covalently to the CW[79,80]; and SlpA proteins anchor to the S-layer of the bacterial surface[81,82]. The basis of the transmembrane delivery mode is to design the target protein to fuse with the C-terminal of ankyrin, and then the N-terminal of ankyrin with transmembrane helix anchors will be embedded in the bacterial surface structure, which makes the fusion protein located between the cell surface and the membrane[83]. Successful examples of antigen coupling to the C-terminal of pgsA from *Bacillus subtilis* and displayed on the surface of *Lactobacillus casei* and *Lactobacillus lactis* have been reported[84,85]. The N-terminal signal peptide of lipoproteins is cleaved off by the specific signal peptidase to form covalent binding with cell membrane phospholipids, which is another membrane anchoring method. Compared with the above anchoring methods, the target protein fused with the LPXTG type protein containing the C-terminal anchoring motif is covalently attached to the cell wall peptidoglycan. The method of delivering antigens using probiotics as carriers includes not only surface display but also cytoplasmic storage and transportation. Due to limitations in protein expression, the surface presentation shows unexpected immune responses despite better immunological advantages[86]. Two potential reasons may lead to the situation: the discrepancy introduced by different immunization routes (intramuscular, intranasal, and oral), and the low antigen protein content on the surface fail to reach the immune threshold[87]. In addition, probiotic spores have also been developed to serve as new delivery carriers to enhance the vaccines' ability to withstand high temperatures and preserve time[88]. Similar to the surface presentation of antigens on probiotics, the expression of heterologous antigens or proteins on the surface of *Bacillus subtilis* spores can also be utilized for oral vaccination[89]. Due to the unique advantages of high resistance to harsh environmental conditions, stability, and low cost of bacterial spores, oral vaccines against *Helicobacter pylori* infections and clonorchiasis have been developed using *Bacillus subtilis* spores, and good immune responses have been observed in mice[90].

Table 2. Applications for probiotic surface display.

Vector	Ankyrin	Anchor site	Antigen	References
Lactobacillus casei 525	pgsA (*Bacillus subtilis*)	Cytoplasmic membrane	Porcine epidemic diarrhea viral antigens	[73]
Lactococcus lactis	pgsA (*Bacillus subtilis*)	Cytoplasmic membrane	Avian influenza virus HA1 antigens	[74]
Lactobacillus casei	pgsA (*Bacillus subtilis*)	Cytoplasmic membrane	HPV16 L2	[75]
Lactobacillus casei	pgsA (*Bacillus subtilis*)	Cytoplasmic membrane	HPV 16 E7	[84,91]
Lactococcus lactis	Usps (*Lactococcus lactis subsp. cremoris* MG1363)	Lipoprotein	NUC	[71]
Lactobacillus plantarum (WCFS1)	Lipobox (Lp_1261, Lp_1452) (*Lactobacillus plantarum*); SP (Lp_1568); LysM (Lp_3014) (*Lactobacillus fermentum* temperate bacteriophage)	Lipoprotein; Cytoplasmic membrane; CW	Invasin from the enteropathogenic bacterium *Yersinia pseudotuberculosis*	[76]
Lactobacillus plantarum (WCFS1)	LP_XTG: LP_2578 (*Lactobacillus plantarum*)	Peptidoglycan	Oncofetal antigen	[92]
Lactococcus lactis/*Lactobacillus brevis*	SlpA (*Lactobacillus brevis*)	S layer	*Escherichia coli* β-lactamases	[72]
Lactobacillus casei (MCJ)	SlpA (*Lactobacillus brevis*)	S layer	Green fluorescent protein	[93]
Lactococcus lactis (MG1363)	AcmA-LysM	CW	Enterovirus type 71	[94]
Lactobacillus acidophilus (NCFM)	PrtP/Mub	CW	FliC	[95]
Lactobacillus plantarum (WCFS1)	LPXTG: LP_2578 (*Lactobacillus plantarum*)	Peptidoglycan	Fes	[96]
Lactococcus lactis	Ubiquitin specific peptidase 45 (*Streptococcus pyogenes*/ *Lactococcus lactis*)	CW	HPV-16 E7	[97]

Note: *Lactobacillus casei*, *Lactococcus lactis*, and *Lactobacillus plantarum* are commonly the choices of antigen delivery carriers. The cell walls (CW) and plasma membranes of bacteria are served as targets to be linked and fixed by ankyrin that couples antigen proteins to achieve the purpose of surface display. Ankyrin which is derived from probiotics interacts covalently or noncovalently with components of the cell membrane or the CW on the surface of bacteria. The antigens used for display are very extensive, including virus components such as swine influenza virus, avian influenza virus, and HPV. pgsA: The poly-gamma-glutamate synthetase A. Nuc: the Staphylococcus aureus secreted nuclease. HPV: Human papillomavirus. FliC: *Salmonella enterica* serovar Typhimurium flagellin.

5. Non–COVID–19 vaccines applications

The developments and researches of mucosally administered live bacterial vector vaccines in non-COVID-19 fields have been very extensive, mainly focusing on vaccines against avian influenza, swine influenza, rotavirus, and HIV, etc[73–75,98]. The immune efficacy of these vaccines has mostly been validated in mouse experiments which induce immune responses in both systemic (serum IgG) and local (saliva, nasal mucosa, and alveolar lavage fluid IgA) settings. Specific mucosal immune responses and the pathological downgrade have been observed in the treatment of patients with cervical intraepithelial neoplasia grade 3 (CIN3), for whom therapeutic probiotic vector vaccines using HPV type 16 E7 protein as an exogenous antigen anchored to the surface of *Lactobacillus casei* have been accepted[91]. It has also been shown that *Lactobacillus plantarum* may have an intrinsic adjuvant effect as a vaccine vector in tumor suppression studies in mice, which is of great significance for enhancing the humoral immune function[99]. In terms of improving the ability to target antigen-presenting cells (APCs), most investigators take advantage of the addition of DC-targeting peptides, as this strategy increases the chance of antigen recognition and presentation by dendritic cells[76,100]. Primarily located within follicle-associated epithelia (FAE) of the Peyer's patches in the gut-associated lymphoid tissue (GALT), as well as in the lymphoid follicles of nasopharynx-associated lymphoid tissues (NALT) and conjunctival tissues, M cells possess a high transcytotic capacity and can transport a broad range of materials to the lymphoid tissue in the intestinal mucosa[101]. Therefore, designing an oral probiotic vector vaccine targeting M cells based on simulated gut pathogen entry pathways is also a good choice for improving antigen presentation efficiency[102]. For antigen preparation, the application of prokaryotic expression of antigen proteins for transfection of LAB not only greatly reduces vaccine production costs but also avoids the problem of bacterial drug resistance[103].

6. COVID–19 vaccines applications

It's interesting to note that researchers have successfully developed a vaccine based on probiotic surface display with the spike protein of SARS-CoV, a member of the Coronaviridae family, to induce mucosal immune response[104]. Although researchers have used probiotic outer membrane vesicles (OMVs) to deliver two different antigens of SARS-CoV-2 and observed strong specific humoral immune responses in mice, this is not literally an application of probiotic vector vaccines for COVID-19 because it didn't involve live bacteria[105]. In recent years, membrane vesicles (MVs) from gram-positive bacteria have been described. Due to their function of targeting proteins via signal peptides of the secretory pathway and their safe and beneficial characteristics, MVs produced from probiotic bacteria, most of which belong to gram-positive bacteria, would be more promising as antigen delivery vehicles[106]. A more interesting study demonstrated that LPG (a unique strain of probiotic *Lactobacillus plantarum* GUANKE) could be applied in combination with SARS-CoV-2 vaccines to promote specific immune responses in both effective and memory phases through enhancing interferon signaling and suppressing apoptotic and inflammatory pathways[107]. In addition, the use of cell extract substitution for mouse immunization in one study due to the failure to successfully secrete SARS-CoV-2 antigenic proteins in *Lactococcus lactis* remains to be considered. Because two independent studies have shown that antigen surface presentation provides better immunogenicity than intracellular accumulation[108,109]. However, it was demonstrated that immunization with this cell extract of LAB containing the recombinant antigen did not overall alter the intestinal microbial community in mice[50]. Probiotic vector vaccines applied to COVID-19 are still in the laboratory stage (Table 3). Li Letian *et al*. have successfully, efficiently, and stably displayed the codon-optimized COVID-19 spike protein on the surface of *Lactobacillus plantarum* and exhibited high antigenicity[110]. Subsequently, *Lactobacillus plantarum* was successfully applied for the first time to display the SARS-CoV-2 RBD protein on the surface and elicit specific IgA antibodies by intranasal immunization of mice, however, it was rather disappointing that no specific IgG antibody production was observed[100]. Alexander Suvorov et al. inserted the gene fragment of the S1 SARS-Cov-2 gene into the *Enterococcal* genome to obtain strains with surface expression-specific epitopes. This modified strain has the ability to react specifically with human IgG from COVID-19 patients and is capable of binding to monoclonal antibodies against S-protein epitopes[111]. There is a temporary lack of clinical and real-world data on oral or intranasal immunization with COVID-19 probiotic vector vaccines. The world's first clinical trial of a probiotic vector vaccine for human use has been completed, but the results have not yet been announced[112].

Table 3. Development and immunogenicity of probiotic vector vaccine against COVID-19.

Vector	Ankyrin	Anchor site	Antigen	Immunization way	Immunogenicity	References
Lactobacillus plantarum (WCFS1)	Endogenous signal peptide 1320 (*Lactobacillus plantarum*)	Lipoprotein	SARS-Cov-2 Receptor binding domain	Mice INA: first dose on day1,2,3; boost dose on day15,16,17	Specific IgA and CD3 + CD4+ T cells increased significantly	[100]
Lactococcus lactis IL1403	USP45 (*Lactococcus lactis*)	Lipoprotein	SARS-Cov-2 Receptor binding domain	Mice OA: two consecutive days at week 1,2,3 respectively	IgA, IgG increased significantly	[50]
Escherichia coli Nissle 1917 (EcN)	Adhesin involved in diffuse adherence 1(AIDA1) (*Escherichia coli* strain 2787)	Outer membrane	SARS-CoV-2 spike protein	Mice OA: from weeks 0 to 4 and from weeks 8 to 12	Increasing IgG antibodies in the sera and significant titers of specific anti-SARS-CoV-2 SP IgA	[113]
Saccharomyces cerevisiae ST1814G	Aga1-Aga2	Cell surface	SARS-Cov-2 Receptor binding domain	Mice OA: day 0,5,10,21	Upregulated spike-detectable IgG and IgA antibodies	[114]
Lactococcus lactis	-	Cytoplasm	SARS-Cov-2 highly conserved region (HCR)	Mice INA and OA: day 1-3,14-16,28-30	Increased IgG for INA; increased IgA and CD8+T cells for INA and OA	[115]
Lactococcus lactis	-	Cytoplasm	SaRNA	Mice OA: different dosage at week 1,3	IgA, IgG increased significantly; Wide Neutrality Capability of SARS-COV-2 variants	[116]
Enterococcal	-	Cytoplasm	SARS-Cov-2 S protein	Mice IM: two times at 3-week intervals Rabbits IM: three times at 3-week intervals	IgG and react with S1-specific monoclonal antibodies; specific immune response in animals	[111]

Note: The receptor binding domain of the COVID-19 spike protein is the most ideal antigen to display on the surface of bacteria, which can induce strong cellular and humoral immune responses in immunized mice, rabbits, and non-human primates. The immunization methods of probiotic-based COVID-19 vaccines are usually administered orally or nasally, and a continuous booster is required to achieve the best immune effect. Some of the current vaccines do not apply surface display schemes but instead directly serve as carriers to deliver antigens, achieving relatively good results. INA: intranasal administration; OA: oral administration; IM: intramuscular injection.

7. Discussion

The evaluation of vaccines, particularly those developed for COVID-19, mainly focuses on their ability to prevent infection, severe illness, death, etc[117]. The emergence of mutant strains as a result of virus evolution poses a great challenge for vaccines, as the strong immune evasion ability of omicron reduces the effectiveness of vaccines to a greater degree[118]. The level and duration of specific antibodies induced by the vaccines are undoubtedly key factors affecting the protection efficacy. However, studies on real-world conventional injectable vaccines and patients after COVID-19 infection have shown that the neutralizing antibody activity against mutants, particularly Omicron, is significantly reduced[119]. Multiple dosing strategies can be designed for probiotic vector vaccines because of their safe and beneficial characteristics, their ability to modulate the immune system, and the fact that the responses are dose-dependent[120]. Probiotics have been increasingly incorporated into foods in recent years to improve intestinal health by maintaining gastrointestinal microbial balance, and their application in preventing infections has also been reported[121]. Although probiotic-based vaccines have been extensively developed in the non-COVID-19 field, few vaccines available specifically for COVID-19. To date, no clearly defined probiotic vector vaccine has been marketed for large-scale use. This may stem from several shortcomings of this type of vaccine vector: 1) While various anchoring methods have existed for the display of heterologous proteins on the surface of bacteria, no research has shown which is most suitable for anchoring of COVID-19 antigens due to the diverse combinations of vectors and cargoes. However, it has been suggested that the pgsA transmembrane anchoring mode of *Bacillus subtilis* may be more conducive to antigen stability and accessibility[73,104]. *Lactobacillus plantarum* and *Lactobacillus lactis* may be the most commonly used strains for probiotic vectors, but the vaccine immunogenicity based on them cannot be directly quantitatively compared. A previous comparative study specifically on Lactobacillus vectors found that *Lactobacillus plantarum* and *Lactobacillus brevis* may have relatively high immunogenic character as they evoked antigen-specific IFN-γ and IL-17 secretion in splenocytes from immunized mice for cellular immune response and significant antigen-specific mucosal IgA for humoral immune response[122]. Therefore, *Lactobacillus plantarum* is expected to be the best candidate for the COVID-19 probiotic-based vaccines[123]. 2) The inevitable challenge of probiotic-based

vaccines by oral administration lies in the acidic environment of the gastrointestinal tract. Although the capability of probiotics to resist gastrointestinal conditions such as acid and salty environments has been taken into consideration to a certain extent, the actual delivery efficiency of CW anchoring-related antigens is not optimistic and may even lose surface antigens, partly due to the length difference of ankyrin or the variation in host systems[124–126]. Therefore, nasal or oral administration seems to be a crucial factor affecting the induction of the immune response, and nasal spray could be superior to oral administration. In contrast, it is interesting to note that in a previous study, oral administration of recombinant lactobacillus significantly increased DC activation, specific IgA production, CD8 T cell induction, and cross-protection against viral attacks in vivo compared to the nasal route[127]. At the same time, the encouraging news is that even if the COVID-19 antigens are not specifically anchored to the cell walls, researchers have also observed considerable cellular and humoral immune responses in vivo[115]. In a novel study, a saRNA (self-amplifying RNA) construct with the sequence of Norovirus GI, which can bind to different cells of the gut, and the sequence of the spike protein of SARS-CoV-2 was transfected into *Lactobacillus plantarum*. Increased antigen retention time in vivo, bacterial intracellular SARS-CoV-2 S protein production, and unexpected and dose-dependent SARS-CoV-2 specific antibody titers during immunization in mice were observed when applying this oral vaccine based on self-amplifying RNA lipid nanoparticles (saRNA LNPs) and probiotic transfection[116]. 3) Mucosal immunity mainly induces the production of secretory IgA, a problem also faced by probiotic vector vaccines correspondingly, it is difficult to observe high levels of specific IgG. The level of anti-Spike or anti-RBD IgG after vaccination is highly negatively correlated with the occurrence or severity of the disease, which is a direct response to the efficacy of the vaccine[128].

The necessity and advantages of unconventional vaccine strategies are well demonstrated by several mucosal immunization vaccines that have been developed and marketed. The application and enhancement of probiotic vectors, which are a novel form of antigen delivery, can be based on several aspects. First, more researched should be implemented to apprehend the mechanisms of nasal and gastrointestinal tract immune action, especially the effects of probiotic interactions in the internal environment to maximize the first-site-response effect of mucosal immunity. Evidence has shown that probiotics can reduce the incidence and severity of diseases, suggesting their promise for treating or preventing COVID-19, even though more studies are required to examine the conclusion and the potential vaccine-boosting effect[129]. Obviously, immunization through the gastrointestinal tract will face greater difficulties due to its special acidic environment and complex digestive mechanism. Therefore, one of the possible reasons for the superiority of nasal administration over oral administration in previous studies is that the delivery technique in the complex environment of the GIT has not been fully addressed. Given this consideration, the strategy of supplementing an alkalescent buffer like bicarbonate to neutralize the acidic environment of pepsin, which poses a threat to antigens anchored to the surface of bacteria, provides a more interesting idea. It's no denying that orally administered vaccines are forward-looking and novel, the health effects of probiotics and the high efficiency of antigen delivery, however, provide us with an ideal way to figure out the vaccine dilemma. Secondly, the ability to induce the production of specific IgG and IgA in sufficient titers at the mucosal level, which can act as the first immune barrier against the entry of SARS-CoV-2 into the body through the upper respiratory tract or the GIT, is a challenge that needs to be addressed for the future application of probiotic vector vaccines and is also their greatest application superiority. Numerous studies have shown that higher antibody titers or antibody concentrations are negatively associated with a higher viral load and severe illness and death, indicating a promising application for predicting or preventing the pandemic[130,131]. A comparatively ideal solution is to include specific targeting sequences, such as the DC targeting peptide sequence, as well as the human norovirus GI sequence in the expressed sequence. There is also a reference to the application of the new generation of mRNA liposome encapsulation technology, which is crucial for enhancing antigen presentation and inducing ideal immune responses. The problem that needs to be solved for probiotic vector vaccines will be the screening of antigenic epitopes and the rationalization of immunization protocols. In contrast to the peak anti-Spike IgG titers that reach two weeks after the infection and remain high for 115 days, anti-Spike and anti-RBD IgA and IgM levels are only about 70%-80% and 60%-70% of their maximum levels, respectively, in the third to fourth months[132]. Therefore, probiotic-based vaccines focusing on mucosal immunity need to take into account the background of rapid and substantial decay of IgA in serum and saliva and adjust immunization strategies accordingly. It

is also important to note that the concept of hybrid immune damping has been proposed in recent years, i.e., multiple immunizations with the same antigen may reduce the immune effect of novel variants[133]. Finally, given that the human immune function against SARS-CoV-2 is a mixed mode of action of humoral and cellular immunity[134], coordinated CD4+ T-cell, CD8+ T-cell, and antibody responses are protective against severe respiratory syndrome caused by SARS-CoV-2[135,136]. Consequently, based on improving the level of humoral immunity, for which antibody titers might be the main measure, the goal of enhancing cellular immunity by probiotic vectors should be considered as well.

8. Conclusions

In the post-COVID-19 era, we need to adequately prepare for ongoing defense against COVID-19 and its variants in the post-pandemic world. The technical shortcomings of probiotic antigen delivery are not sufficient to negate its promising application in the COVID-19 pandemic. Considering the global pandemic of COVID-19 and the immune escape dilemma due to the continued emergence of mutant strains, it is promising that implementing oral probiotics and applying multiple dosing strategies to generate long-lasting and high levels of antibodies will provide a solution to the frequent emergency of SARS-CoV-2 variants or we have to coexist with the virus for long periods.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

Zha RY and Yi BY completed the literature review, analyzed the data and wrote the manuscript equally. Liao CH collected the epidemiological study data. Li XK contributed to literature review and data collection. Lu JH provided funding and coordinated all sectors to provide great supports to this research.

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