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Metabolic engineering of *Lactobacilli spp.* for disease treatment

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Abstract

Background A variety of probiotics have been utilized as chassis strains and engineered to develop the synthetic probiotics for disease treatment. Among these probiotics, *Lactobacilli*, which are generally viewed as safe and capable of colonizing the gastrointestinal tract effectively, are widely used.

Main body of abstract We review recent advancements in the engineering of *Lactobacilli* for disease treatment. Specifically, the *Lactobacilli* that are used for the construction of synthetic probiotics, the application of these engineered strains for diseases treatment, and the therapeutic outcomes of these engineered microbes are summarized in this review. Moreover, the applications of these engineered strains for disease treatment are categorized based on their engineering strategies. Of note, we compare the advantages and disadvantages of various engineering strategies and offer insights for the future development of genetically modified *Lactobacillus* strains with stable and safe properties.

Short conclusion Our study comprehensively reviews researches on engineering diverse *Lactobacillus* strains for disease treatment, categorized by their engineering strategies, and emphasizes the importance of developing synthetic probiotics with stable and safe characteristics to enhance their therapeutic applications.

Keywords Synthetic probiotics, *Lactobacillus*, Engineering strategies, Disease treatment

Background

Probiotics are live non-pathogenic microorganisms that can provide beneficial effects for the host when administered in proper amounts [1]. These microbes can deliver beneficial effects through multiple mechanisms, such as reducing intestinal pH, inhibiting the colonization and invasion of pathogenic organisms, and modulating host immune responses [2]. Based on these properties, numerous probiotics have been identified and utilized for the prevention and treatment of diseases [3]. However, the therapeutic application of many traditional probiotics is limited by several drawbacks, such as poor intestinal colonization, strain variability, and inadequate interaction with the host [4].

With advancements in synthetic biology tools and technologies, a wide range of probiotics have been

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Table 1 Plasmid-based surface display of functional elements in *Lactobacilli*

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
Displaying vaccines for treating virus infection							
L. plantarum CGMCC 1.557	Surface displaying the codon-optimized SARS-CoV-2 S protein	Plasmid-based	Antigen presentation for SARS-CoV-2	Against SARS-CoV-2			[11]
L. acidophilus NCFM	Surface display of HIV-1 Gag and *Salmonella enterica* Serovar Typhimurium FliC as adjuvant	Plasmid-based	Inducing the antigen-specific IgA production and stimulating the IFN-γ-producing cells	Against HIV		Female BALB/c mice	[12]
L. casei BLS	Surface displaying HPV type 16 E7 protein (HPV16 E7) with the poly-γ-glutamic acid synthetase complex A (PgsA) of *Bacillus subtilis* (*chungkookjang*) served as anchoring motif	Plasmid-based	Inducing the E7-specific serum IgG and mucosal IgA productions	Against HPV16 E7-based cervical cancer	For the C57BL/6 mice that immunized with HPV16 E7-displaying strain, the mean log titer of the serum IgG was increased from 1.24 ± 0.24 to 3.15 ± 0.02 after the first oral vaccination; The E7-specific lymphocyte proliferative response was increased from 7.8 ± 0.9 to 11.0 ± 1.4; The E7-specific cytotoxic T lymphocyte (CTL) response was increased from 21 ± 5 to 510 ± 36 spot-forming cells (SFC)/106 cells. For the TC-1 mouse tumor model, the survival rate of the recombinant HPV16 E7-displaying strain-immunized group was increased from 0–50%	Female C57BL/6 mice and the mice challenged with TC-1 cells	[13]
L. casei strain 525	Surface displaying HPV type 16 E7 protein (HPV16 E7)	Plasmid-based	Inducing E7-specific mucosal immunity	Against HPV16 E7-based cervical cancer		Female SPF C57BL/6 (H-2b) mice	[14]
L. casei strain 525	Surface displaying HPV type 16 E7 protein (HPV16 E7)	Plasmid-based	Inducing E7-specific mucosal immunity	Against HPV16 E7-based cervical cancer	70% of the CIN3 patients experienced a pathological down-grade to CIN2 at week 9	Cervical intraepithelial neoplasia grade 3 (CIN3) patients	[15]
L. plantarum CGMCC 1.557	Surface displaying the truncated and codon-optimized viral glycoprotein 5 (GP5) of PRRSV	Plasmid-based	Antigen presentation for PRRSV	Against PRRSV			[16]
L. plantarum NC8	Surface displaying the spike antigen of TGEV	Plasmid-based	Inducing cellular, mucosal, and humoral immunity	Against porcine TGEV	Inducing high expression levels of B7 molecules on DCs, as well as high levels of IgG, secretory IgA, and IFN-γ and IL-4 cytokines compared with the control group	SPF mice	[17]
L. casei ATCC 393	Surface displaying the core neutralizing epitope (COE) antigen of PEDV conjugated with M cell targeting peptide Co1 (adjuvant)	Plasmid-based	Inducing higher anti-PEDV serum IgG and mucosal SIgA antibody responses	Against PEDV	The mice that orally immunized the recombinant strain could induce the serum IgG antibody response to exhibit stronger PEDV-neutralizing activity (1:24) than and control groups (< 1:2). Moreover, this recombinant strain-induced SIgA antibody response elicited stronger anti-PEDV neutralizing activity (1:20) than the control group (< 1:2)	Female SPF BALB/c mice	[18]

Table 1 (continued)

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
L. casei ATCC 393	Surface displaying the core neutralizing epitope (COE) antigen of PEDV conjugated with the M cell-targeting peptide (Col) and dendritic cell-targeting peptide (DCpep)	Plasmid-based	Inducing the anti-PEDV mucosal, humoral, and cellular immune responses	Against PEDV	Providing stronger PEDV-neutralizing ability (1:36) than the control group (< 1:2)	Female SPF BALB/c mice	[19]
L. casei ATCC 393	Surface displaying the D antigenic site of the TGEV spike (S) protein and core neutralizing epitope of PEDV S protein	Plasmid-based	Increasing the levels of anti-PEDV and anti-TGEV serum immunoglobulin G (IgG) and mucosal secreted immunoglobulin A (SIgA) antibodies; strengthening the proliferation levels of lymphocytes	Against TGEV and PEDV		BALB/c mice	[20]
L. plantarum HA33-1	Surface displaying CSFV E2 protein in conjunction with thymosin α-1	Plasmid-based	Inducing protective immune responses by eliciting the IgA-based mucosal, IgG-based humoral, and CTL-based cellular immune responses	Against CSFV		CSFV infected pigs	[21]
L. plantarum ZN3	Surface displaying the H1N1 HA1 protein that fused to DCpep and the M cell-targeting peptide	Plasmid-based	Inducing mucosal, cellular and systemic immune responses	Against swIAV	For oral administration, the survival rate of H1N1 virus-challenged mice was increased from 0–60%; For intranasal administration, the survival rate of H1N1 virus-challenged mice was increased from 0–100%	BALB/c mice inoculated intranasally with H1N1 and H3N2	[22]
L. plantarum NC8	Surface displaying viral 3M2e-HA2	Plasmid-based	Increasing the mucosal and systemic immune responses	Against AIV	The survival rate of the H9N2-challenged mice that immunized with the recombinant strain were increased from 0–80%	BALB/c mice challenged with mouse-adapted H9N2 AIV or H1N1 influenza virus	[23]
L. casei L525	Surface displaying the hemagglutinin 1 (HA1) subunit of the A/Aquatic bird/Korea/W81/2005 (H5N2) that fused with the *Bacillus subtilis* poly γ-glutamic acid synthetase A (pgsA)	Plasmid-based	Increasing the HA1-specific serum IgG, mucosal IgA and neutralizing antibodies	Against AIV	For the oral and intranasal administration, the survival rate of H5N2 virus-challenged mice was increased from 0–100%	Mice challenged with homologous mouse-adapted H5N2 virus	[24]
L. plantarum	Surface displaying the VP2 protein of IBDV	Plasmid-based	Inducing humoral and cellular immune responses	Against vvIBDV	The survival rate of the vvIBDV-challenged chickens were increased from 0–100%	Chickens challenged with vvIBDV	[25]
L. plantarum NC8	Surface displaying the Gp85 protein of ALV-J	Plasmid-based	Inducing the cellular, humoral, and mucosal immunity responses	Against avian leukosis	The survival rate of the ALV-J-challenged chickens were increased significantly	Chickens that intramuscular injected with ALV-J HB2010001	[26]

Table 1 (continued)

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
L. plantarum HA33-1	Surface displaying the glycoprotein (G) of SVCV and ORF81 protein of KHV	Plasmid-based	Increasing the levels of immunoglobulin M (IgM)	Against SVCV and KHV	Providing effective protection to the vaccinated carps (71% protection) and koi (53% protection) at day 65 post challenge	*Cyprinus carpio* that oral administrated with SVCV; koi that oral administrated with KHV	[27]
Displaying vaccines for treating parasites infection							
L. plantarum NC8	Surface displaying EtMic2	Plasmid-based	Antigen presentation for *Eimeria tenella*	Against chicken coccidiosis	The lesion scores of cecum was decreased from 3.75 ± 0.520 to 2.30 ± 0.506; The OPG ($\times 10^6$) was decreased from 1.44 ± 0.02 to 0.71 ± 0.04; The ACI was increased from 74.93 to 145.15	Chickens challenged with *E. tenella* sporulated oocysts	[28]
L. plantarum NC8	Surface displaying EtMic2 and AMA1	Plasmid-based	Antigen presentation for *Eimeria tenella*	Against chicken coccidiosis	The BWG of *E. tenella*-challenged chicken was increased from 210.50 ± 16.16 g to 313.71 ± 6.60 g; The lesion scores in cecum were decreased from 3.83 ± 0.41 to 2.00 ± 0.63; The oocyst output ($\times 10^5$) was decreased from 9.50 ± 3.03 to 3.56 ± 1.30	Chickens challenged with *E. tenella* sporulated oocysts	[29]
L. Plantarum NC8	Surface displaying SO7 that fused to DCpep	Plasmid-based	Dendritic cell-targeting antigen presentation for *Eimeria tenella*	Against chicken coccidiosis	The body weight gains and serum antibody responses were increased in the *E. tenella*-challenged chicken, while the fecal oocyst shedding and pathological damage in cecum were decreased	Chickens challenged with *E. tenella* sporulated oocysts	[30]
L. plantarum NC8	Surface displaying eukaryotic initiation factor U6L5H2	Plasmid-based	Producing higher levels of specific cecal SIgA, serum IgG, transcription of cytokines IFN-γ and IL-2, and lymphocyte proliferation	Against chicken coccidiosis	The body weight gain of *E. tenella*-challenged chicken was increased from 83.32 ± 3.28 g to 101.57 ± 2.02 g; The average lesion score was decreased from 2.90 ± 0.42 to 1.79 ± 0.31; The oocyst output (×105) was decreased from 5.37 ± 0.43 to 1.35 ± 0.18; The ACI was increased from 109.90 to 168.28	Chickens challenged with *E. tenella* sporulated oocysts	[31]
***L. plantarum*NC8**	Surface displaying gp43 and nudix hydrolase (TsNd) of *Trichinella spiralis*	Plasmid-based	Inducing higher levels of specific humoral, mucosal, and cellular immune responses	Against trichinellosis	A 75.67% reduction of adult worms (AW) at 7 days post-infection (dpi) and 57.14% reduction of muscle larva (ML) at 42 dpi were observed in the larval-challenged mice	BALB/c mice challenged with infectious *T. spiralis*	[32]
Displaying vaccines for treating pathogens infection							
L. plantarum WCFS1	Surface displaying Ag85B and ESAT-6 (AgE6)	Plasmid-based	Inducing specific immune responses	Against tuberculosis (TB)	Inducing antigen-specific proliferative responses in lymphocytes purified from TB-positive donors; Inducing immune responses in mice after nasal or oral immunization	C57BL/6 BomTac mice	[33]
L. casei ATCC 393	Surface displaying the toxoid of *C. perfringens* α-toxin	Plasmid-based	Eliciting mucosal, humoral, and cellular immunity to neutralize the natural α-toxin of *C. perfringens*	Against *C. perfringens* infection	Improve the survival rates of *C. perfringens*-challenged mice from 0–90%	SPF BALB/c mice challenged with *C. perfringens* natural α-toxin and *C. perfringens* type A	[34]

Table 1 (continued)

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
L. casei ATCC 393	Surface displaying the NetB toxin of *C. perfringens*	Plasmid-based	Inducing high anti-toxin antibody response	Against *C. perfringens* infection		Chickens that orally inoculated with virulent *C. perfringens*	[35]
L. casei ATCC 393	Surface displaying the C-terminal domain of α-toxin of *C. perfringens*	Plasmid-based	Inducing specific serum anti-α antibodies	Against *C. perfringens* infection	The mean body weight changes of the recombinant strain-immunized chickens (35.61%) were higher than that of the non-vaccinated chickens (24.13%)	Ross 308 broiler chickens challenged with *C. perfringens* CP58	[36]
L. crispatus N-11	Surface displaying the α-β2-ε-β1 toxoid of *C. perfringens*	Plasmid-based	Stimulating the mucosal, cellular, and humoral immunity	Against the toxins of *C. perfringens*	The specific secretory IgA (SIgA) and IgY antibodies in the serum and intestinal mucus and the serum concentration of IFN-γ, IL-2, IL-4, IL-10, IL-12, and IL-17 were increased significantly in the recombinant strain-immunized group	Chickens challenged with the natural α-β2-ε-β1 toxin combined with *C. perfringens* type A and type B pathogenic bacteria	[37]
L. gasseri NM713	Surface displaying the conserved region of streptococcal M6 protein (CRR6)	Plasmid-based	Inducing specific systemic (IgG) and mucosal (IgA) immune responses against the streptococcal M6 antigen	Against the *S. pyogenes* infection	The mice that orally administered with the recombinant strain showed lower streptococcal infection (10%) and mortality (3.3%) rate as compared to the control group	Seven-weeks old mice that challenged with *S. pyogenes*	[38]
L. casei CC16	Surface displaying the Aha1 of *A. veronii* fused the cholera toxin B subunit (CTB) as adjuvant	Plasmid-based	Stimulating the humoral and cellular immunity	Against the *A. veronii* infection	The survival rate of *A. veronii*-challenged carp was increased from 0–64.29%	*Cyprinus carpio* that intraperitoneally injected with *A. veronii*	[39]
L. casei CC16	Surface displaying the Aha1 of *A. veronii* fused the *E. coli* intolerant enterotoxin B subunit (LTB)	Plasmid-based	Inducing the expression of various immune enzymes in the humoral immunity of carp and increasing the cytokine levels	Against the *A. veronii* infection	The survival rate of *A. veronii*-challenged carp was increased from 0–60.71%	*Common carp* that intraperitoneally injected with *A. veronii* TH0426	[40]
L. casei CC16	Surface displaying the SH type VI pili B (MshB) from *A. veronii* as an antigen and cholera toxin B subunit (CTB) as a molecular adjuvant	Plasmid-based	Stimulating the production of high levels of serum-specific immunoglobulin M (IgM) and enhancing the non-specific immunity	Against the *A. veronii* infection	The survival rate of *A. veronii*-challenged carp was increased from 0–60%	*Crucian carp* that intraperitoneally injected with *A. veronii*	[41]
L. casei ATCC 393	Surface displaying the outer membrane protein K (OmpK) of *V. mimicus* as an antigen, and cholera toxin B subunit (CTB) as a molecular adjuvant	Plasmid-based	Inducing the humoral and cellular immunity	Against the *V. mimicus* infection	The survival rate of the recombinant strain-immunized *Carassius auratus* was higher than the control group	*C. auratus* challenged with *V. mimicus*	[42]
L. plantarum NC8	Surface displaying the FomA protein of *F. nucleatum*	Plasmid-based	Increasing the mouse-specific humoral immunity and eliciting the mucosal and T cell-mediated immune responses	Against IBD	Decreasing the mortality rate and body weight loss	*F. nucleatum*-and DSS-induced IBD mice	[43]

Table 1 (continued)

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
L. reuteri WXD171	Surface displaying the iron-regulated surface determinant protein B (IsdB) of *S. aureus*	Plasmid-based	Inducing the mucosal responses in gut-associated lymphoid tissues	Against the *S. aureus* infection	Improving the survival rate of *S. aureus*-challenged mice from 10–70%	Mouse model of *S. aureus*-induced pulmonary, skin, and systemic infection.	[44]
L. casei ATCC 393	Surface displaying the outer membrane protein OMP19 of *Brucella* species	Plasmid-based	Providing a very good general and mucosal immune responses	Against brucellosis	The mice that orally immunized with OMP19-displaying strain showed higher degrees of protection (15-fold reduction of *B. abortus* 544 in spleen) as compared to the control group	BALB/c mice challenged intraperitoneally with the virulent *B. abortus* 544	[45]
Displaying functional elements for the intestinal exclusion of viruses and pathogens							
L. rhamnosus GG	Surface displaying IgG-binding domain of protein G	Plasmid-based	Capture rotavirus via hyperimmune bovine colostrum antibodies (HBC-IgG)	Against rotavirus (RRV)	The combination usage of HBC antibodies and this engineered strain was more effective (10 to 100-fold increase) in reducing the prevalence, severity, and duration of diarrhea	Mouse RRV infection model	[46]
L. paracasei BL23	Surface displaying rotavirus proteins 1 and 3 (ARP1 and ARP3)	Plasmid-based	Capture rotavirus by anti-rotavirus proteins	Against rotavirus			[47]
L. casei ATCC 334	Surface displaying the *Listeria* adhesion protein (LAP) from a non-pathogenic *Listeria* (*L. innocua*) and a pathogenic *Listeria* (*L. monocytogenes*)	Plasmid-based	Excluding *L. monocytogenes* competitively by occupying the surface presented LAP receptor, heat shock protein 60	Against the *L. monocytogenes* infection	The number *L. monocytogenes* cells that adhered to the intestine were 100-fold lower in the mice that treated with the recombinant strain; At 10 days post the *L. monocytogenes* challenge, the surviving rate of the recombinant strain-treated mice (~ 92%) was higher than the control group (60%)	Female mice (A/J: 6–8 weeks of age) challenged with *Listeria monocytogenes* F4244	[48]
L. casei ATCC 344	Surface displaying internalins A and B (*inlAB*) of *L. monocytogenes*	Plasmid-based	Inhibiting the adhesion, invasion and transcellular passage of *L. monocytogenes*	Against *L. monocytogenes* infection	Reducing the adhesion of *L. monocytogenes* by 50-53.6% at 16 and 24 h, far more than that of the control group (8%)	Caco-2 cells	[49]
Displaying pharmaceutical compounds and enzyme							
L. plantarum NC8	Surface displaying murine IL-10	Plasmid-based	Anti-inflammation	Against Th1 Responses of RAW264.7 Cells Stimulated with Poly(I: C) or LPS	Reducing the Poly(I: C)- or LPS-induced Th1 responses in RAW264.7 cells and decreasing the expression of TNF-α, IFN-γ, IL-1β, and IL-6	RAW264.7 cells stimulated with Poly(I: C) or LPS	[50]
L. plantarum NC8	Surface displaying the porcine IFN-λ3	Plasmid-based	Inhibiting the replication of PEDV and TGEV	Against TGEV and PEDV	Reducing the prevalence of PEDV and TGEV viruses by 53% and 59%, respectively	Intestinal porcine epithelial cell line J2 (IPEC-J2) that inoculated with PEDV strain CV777 or TGEV strain SY	[51]
L. reuteri CGMCC1.3264	Surface displaying lactonohydrolase	Plasmid-based	Degrading zearalenone	Against fungal mycotoxins zearalenone	This engineered strain was capable of hydrolyzing 2.5 mg/kg of ZEN-contaminated corn within 4 h		[52]

employed as chassis strains and specifically engineered to enhance their therapeutic efficacy, e.g., *Escherichia coli* Nissle 1917, *Clostridium butyricum*, *Saccharomyces boulardii*, and the microbes belonging to genera *Lactococcus*, *Lactobacillus*, *Bifidobacterium*, and *Bacteroides* [5–8]. Among these probiotics, *Lactobacillus* strains, which are generally viewed as safe according to the U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA), have been extensively utilized in the food and medical industries [9]. Given the fact that *Lactobacilli* can colonize the intestine effectively to facilitate the mucosal targeting [10], a wide range of *Lactobacillus* strains have been employed as functional chassis for the development of synthetic probiotics, e.g., *Lactobacillus plantarum* (*L. plantarum*), *Lactobacillus gasseri* (*L. gasseri*), *Lactobacillus johnsonii* (*L. johnsonii*), *Lactobacillus reuteri* (*L. reuteri*), *Lactobacillus paracasei* (*L. paracasei*), *Lactobacillus rhamnosus* (*L. rhamnosus*), *Lactobacillus jensenii* (*L. jensenii*), *Lactobacillus salivarius* (*L. salivarius*), and *Lactobacillus casei* (*L. casei*), etc. Here, we summarize researches focused on engineering *Lactobacilli* and categorize the applications of these engineered strains for disease treatment based on their respective engineering strategies.

Surface displaying functional elements in *Lactobacilli*

Plasmid-based surface display

To develop effective vaccines for treating various diseases, numerous antigens or functional genes have been expressed using plasmid-based strategies and displayed on the surface of *Lactobacilli.* (Table 1; Fig. 1A).

Plasmid-based surface displaying antigens for treating virus infection-associated diseases

To treat human virus infection-related diseases, the spike protein (S protein) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [11], the major structural protein Gag of human immunodeficiency virus (HIV) [12], and the E7 protein of human papillomavirus (HPV) type 16 (HPV16 E7) [13–15] had been selected as the functional antigens and displayed on the surface of different *Lactobacill*. These engineered microbes demonstrated high antigenicity and elicited robust antigen-specific immune responses, thereby enhancing the clearance of the aforementioned harmful viruses. Specifically, the engineered strain expressing Gag as a functional element induced antigen-specific IgA production and stimulated IFN-γ-producing cells via oral immunization [12]. For the HPV16 E7-displaying strain, it could increase the mean log titer of the serum IgG from 1.24 ± 0.24 to 3.15 ± 0.02, improve the E7-specific lymphocyte proliferative response (from 7.8 ± 0.9 to 11.0 ± 1.4), and enhance the E7-specific cytotoxic T lymphocyte (CTL) response (from 21 ± 5 to 510 ± 36 spot-forming cells (SFC)/10^6 cells) in C57BL/6 mice [13]. Moreover, immunization of TC-1 mouse tumor model with the HPV16 E7-expressing strain resulted in a substantial improvement in survival outcomes, elevating the survival rate from 0 to 50% [13]. Cervical intraepithelial neoplasia grade 3 (CIN3) is a mucosal precancerous lesion caused by high-risk human papillomavirus (HPV). Kei Kawana et al. evaluated the safety and clinical efficacy of an attenuated *Lactobacillus casei* strain 525 that expressed HPV16 E7 protein in patients with HPV16-associated CIN3 during a 9-week trial. It was noted that patients using 4–6 capsules/day showed increased E7-cell mediated immune response and exhibited pathological down-grade from CIN3 to CIN2 [15].

Similar engineering strategies have been successfully implemented in the development of recombinant *Lactobacilli* for the treatment of porcine viral infection-associated diseases. Among these porcine viruses, the viral structural glycoprotein 5 (GP5) of porcine reproductive and respiratory syndrome virus (PRRSV) [16], the spike antigen of porcine transmissible gastroenteritis virus (TGEV) [17], the core neutralizing epitope (COE) antigen of porcine epidemic diarrhea virus (PEDV) [18–20], the E2 protein of classical swine fever virus (CSFV) [21], and the HINI HA1 protein of the swine infuenza A virus (swIAV) [22] were proved to be the functional antigens and displayed on the surface of *Lactobacilli* for disease treatment. The *L. plantarum* NC8-derived recombinant strain expressing the spike antigen of TGEV on its surface significantly enhanced B7 molecule expression on dendritic cells (DCs) and elicited robust immune responses, as demonstrated by significantly increased levels of IgG, secretory IgA, and the cytokines IFN-γ and IL-4 [17]. When the COE antigen of PEDV was displayed as functional element, the engineered strain elicited a potent serum IgG antibody response, resulting significantly enhanced PEDV-neutralizing activity (1:24) compared to control groups (< 1:2) following oral administration. Furthermore, the secretory IgA induced by this *L. casei* ATCC 393-derived recombinant strain elicited stronger neutralizing activity against PEDV (1:20 titer) compared to the control group (< 1:2) [18]. By displaying the core COE antigen of PEDV that conjugated with the M cell-targeting peptide (Col) and dendritic cell-targeting peptide (DCpep) on the surface of *L. casei* ATCC 393, the engineered strain provided stronger PEDV-neutralizing ability (1:36) than the control group (< 1:2) after oral administration [19]. To fight against TGEV and PEDV infection simultaneously, a *L. casei* ATCC 393-derived recombinant strain was constructed by displaying the D antigenic site of the TGEV spike (S) protein and the COE of the PEDV S protein on surface. Oral administration of the engineered strain significantly enhanced systemic

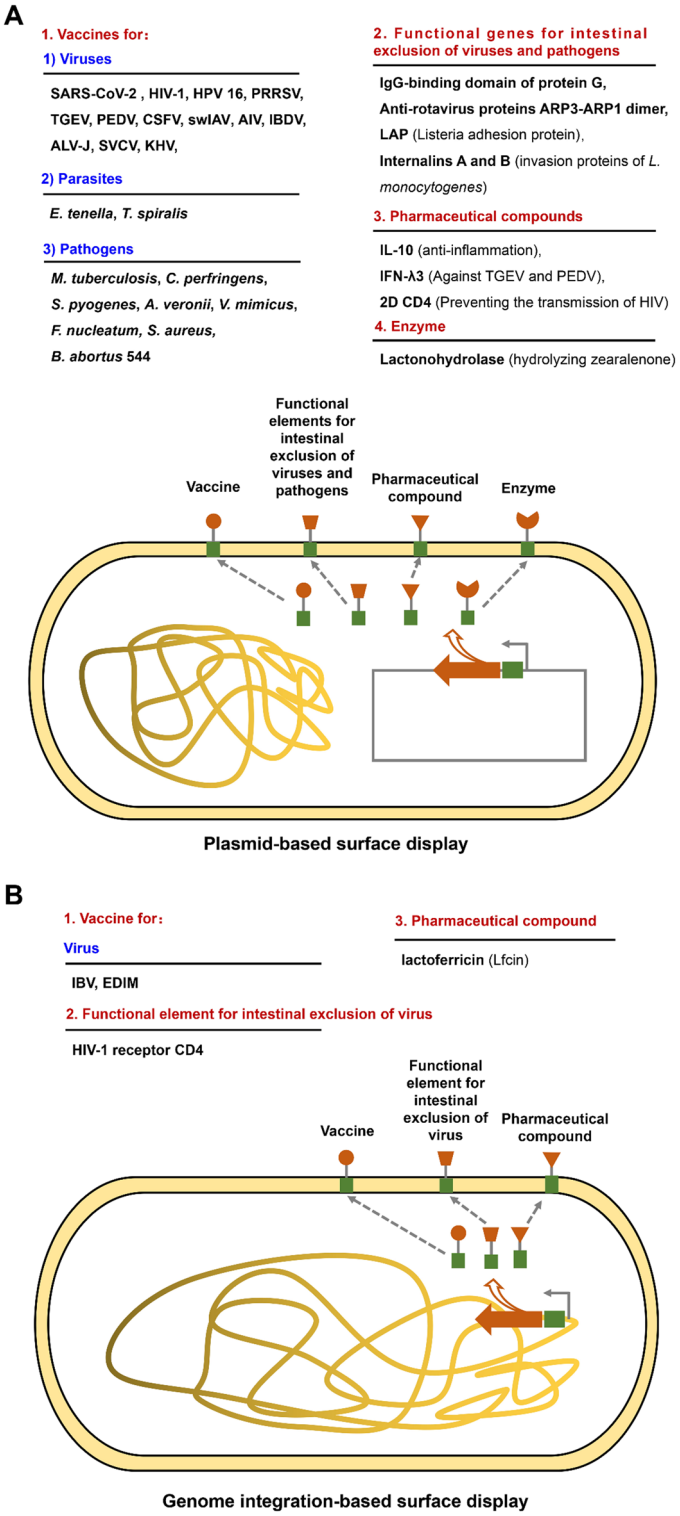

Fig. 1 Summary of the strategies used to display the functional elements on the cell surface of *Lactobacilli*. (**A**) Plasmid-based surface display of vaccines, functional elements for intestinal exclusion of viruses and pathogens, pharmaceutical compounds, and enzymes. (**B**) Genome integration-based surface display of vaccines, functional element for intestinal exclusion of virus, and pharmaceutical compounds

immunity, as evidenced by elevated serum levels of anti-PEDV and anti-TGEV IgG antibodies, increased mucosal secretion of SIgA, and enhanced lymphocyte proliferation capacity [20]. Furthermore, oral immunization of pig with the *L. plantarum* HA33-1-derived recombinant strain that displaying the CSFV E2 protein in conjunction with thymosin α-1 could help it to fight against CSFV infection by eliciting the IgA-based mucosal, IgG-based humoral, and CTL-based cellular immune responses [21]. To fight against H1N1 virus infection, *L. plantarum* ZN3 was genetically engineered to express a surface-displayed fusion protein containing the H1N1 HA1 protein, DC-targeting peptide (DCpep), and M cell-targeting peptide, which was subsequently administered via oral gavage to mice challenged with H1N1 virus. This engineered strain could induce effective mucosal, cellular, and systemic immune responses in the intestine and upper respiratory airways, thus increasing the survival rate of mice from 0 to 60% [22]. Notably, intranasal immunization with the recombinant strain conferred complete protection, with all immunized mice surviving (100% survival rate), whereas the control mice succumbed to infection within 10 days post-challenge (0% survival) [22].

Furthermore, a series of *Lactobacilli spp.* have been engineered to provide vaccines for the avian viruses-related diseases. For example, the virial proteins 3M2e and HA2 of avian influenza virus (AIV) were fused together and displaced on the surface of *L. plantarum* NC8 [23]; the hemagglutinin 1 (HA1) subunit of the A/Aquatic bird/Korea/W81/2005 (H5N2) that fused with the *Bacillus subtilis* poly γ-glutamic acid synthetase A (pgsA) was surface displayed on *L. casei* [24]; the VP2 protein of infectious bursal disease virus (IBDV) and the Gp85 protein of J subgroup avian leukosis virus (ALV) were displayed on the surface of *L. plantarum* [25, 26]. The 3M2e-HA2 display strategy demonstrated remarkable efficacy, with immunization using the recombinant strain increasing survival rates in H9N2-challenged mice from 0 to 80% [23]. Utilizing the HA1-pgsA display strategy, the engineered strain administered through either oral or intranasal routes provided full protection against H5N2 infection (100% survival). In contrast, all control animals succumbed to infection between 8 and 9 days post-challenge [24]. For the VP2 displaying strategy, the survival rates of the vvIBDV-challenged chickens were increased from 0 to 100% [25]. The Gp85 display strategy significantly enhanced survival rates in ALV-J-challenged chickens, demonstrating marked protective efficacy against viral infection [26].

Apart from the above-mentioned diseases, the glycoprotein (G) of spring viremia of carp virus (SVCV) and ORF81 protein of koi herpesvirus (KHV) have been proved to be functional antigens and co-expressed on the surface of *L. plantarum* HA33-1 to provide protective immunity for cyprinid fish [27]. Compared to the control group, oral administration of the engineered strain elicited robust IgM production, conferring effective protection against viral challenge with 71% and 53% survival rates in vaccinated common carp and koi at 65 days post-infection [27].

Plasmid-based surface displaying vaccines for treating the parasites infection-associated diseases

For the parasites infection-associated diseases, *L. plantarum* NC8 was engineered to display the *Eimeria tenella* (*E. tenella*) -derived proteins (SO7, EtMic2, AMA1, and U6L5H2) as antigens on surface [28–31]. Immunizing chickens with these *L. plantarum* NC8-derived engineered strains could protect them from *E. tenella* challenge efficiently. For the EtMic2-displaying strain, the *E. tenella* infection-induced lesion scores of cecum was decreased from 3.75 ± 0.520 to 2.30 ± 0.506, the oocysts per gram of droppings ($\times 10^6$) was decreased from 1.44 ± 0.02 to 0.71 ± 0.04, while the anticoccidial index (ACI) was increased from 74.93 to 145.15 [28]. For the EtMic2 and AMA1-displaying strain, the body weight gain (BWG) of *E. tenella*-challenged chicken was increased from 210.50 ± 16.16 g to 313.71 ± 6.60 g, the lesion score in cecum was decreased from 3.83 ± 0.41 to 2.00 ± 0.63, the oocyst output ($\times 10^5$) was decreased from 9.50 ± 3.03 to 3.56 ± 1.30 [29]. For the recombinant strain that displaying SO7 that fused to DCpep, the body weight gain and serum antibody responses were increased in the *E. tenella*-challenged chicken, while the fecal oocyst shedding and pathological damage in cecum were decreased [30]. For the *L. plantarum* NC8-derived recombinant strain that displaying the eukaryotic initiation factor U6L5H2, the body weight gain of *E. tenella*-challenged chicken was increased from 83.32 ± 3.28 g to 101.57 ± 2.02 g, the average lesion score was decreased from 2.90 ± 0.42 to 1.79 ± 0.31, the oocyst output ($\times 10^5$) was decreased from 5.37 ± 0.43 to 1.35 ± 0.18, the ACI was increased from 109.90 to 168.28 [31]. Apart from *Eimeria tenella* infection-associated disease, the gp43 and nudix hydrolase (TsNd) of *Trichinella spiralis* (*T. spiralis*) were displayed on the surface of *L. plantarum* NC8 to provide effective vaccines against trichinellosis [32]. Immunizing the larval-challenged mice with the recombinant strain brought a 75.67% reduction of adult worms (AW) at 7 days post-infection (dpi) and 57.14% reduction of muscle larva (ML) at 42 dpi [32].

Plasmid-based surface displaying vaccines for treating the pathogen infection-associated diseases

To treat the pathogen infection-associated diseases, a recombinant *L. plantarum* strain that displaying the fusion antigen AgE6 (comprising Ag85B and ESAT-6) of *Mycobacterium tuberculosis* on surface was constructed

and used for the treatment of tuberculosis [33]. The AgE6-displaying *L. plantarum* strain could not only induce antigen-specific proliferative responses in lymphocytes that purified from tuberculosis-positive donors, but also induce immune responses in mice after nasal or oral immunization [33]. As for the *Clostridium perfringens* (*C. perfringens*) infection-associated disease, a genetically engineered *L. casei* 393 was constructed by displaying the toxoid of *C. perfringens* α-toxin on surface. Oral administration of this engineered strain could improve the survival rates of *C. perfringens*-challenged mice (from 0 to 90%) by eliciting mucosal, humoral, and cellular immunity to neutralize the natural α-toxin of *C. perfringens* [34]. Besides, oral immunization of broiler chickens with the *L casei*a ATCC 393-derived recombinant strain that displaying the NetB toxin or the C-terminal domain of α-toxin from *C. perfringens* on surface could protect the chickens from *C. perfringens*-induced necrotic enteritis [35, 36]. In this strategy, the mean body weight change of the recombinant strain-immunized chickens (35.61%) were higher than that of the non-vaccinated chickens (24.13%) [36].

Apart from the strategies mentioned above, the α-β2-ε-β1 toxoid protein of *C. perfringens* had also been used as functional antigen and displayed on the surface of *L. crispatus* N-11 [37]. After booster immunization, the recombinant strain-immunized group showed higher levels of specific secretory IgA (SIgA) and IgY antibodies in the serum and intestinal mucus. Besides, the serum concentration of IFN-γ, lL-2, IL-4, IL-10, IL-12, and IL-17 were increased significantly in the same group [37]. To fight against *S. pyogenes* infection-associated diseases, the conserved region of streptococcal M6 protein (CRR6) was displayed on the surface of *L. gasseri* NM713. Oral administration of this engineered strain could induce systemic and mucosal immune responses to protect the host from *S. pyogenes* infection [38]. Specifically, after the nasal challenge of *S. pyogenes*, the mice that orally administered with the recombinant strain showed lower streptococcal infection (10%) and mortality (3.3%) rate as compared to the control group [38]. To prevent *Aeromonas veronii* (*A. veronii*) infection, *L. casei* was used as antigen deliver carrier and engineered to display the Aha1 of *A. veronii* that fused with the cholera toxin B subunit (CTB) or *E. coli* intolerant enterotoxin B subunit (LTB) as adjuvant on surface. Oral immunization of these engineered strains to carp protected them from *A. veronii* infection by stimulating the humoral and cellular immunity [39, 40]. For the Aha1-CTB displaying strain, it improved the survival rate of *A. veronii*-challenged carp from 0 to 64.29% [39]. Similarly, the Aha1-LTB displaying strain could increase the survival rate of *A. veronii*-challenged carp from 0 to 60.71% [40]. Furthermore, an engineered *L. casei* CC16 was constructed to surface display the MSH type VI pili B (MshB) of *A. veronii* as an antigen and cholera toxin B subunit (CTB) as a molecular adjuvant. Oral immunization of crucian carp with this *L. casei* CC16-derived engineered strain could protect it from *A. veronii* infection by improving the immune response [41]. Compared with the control group (about 10%), the survival rate of the recombinant strain-immunized crucian carp was increased to 60% [41]. To provide efficient vaccine for *Vibrio mimicus* (*V. mimicus*) infection, *L. casei* ATCC 393 was engineered to display the outer membrane protein K (OmpK) of *V. mimicus* as an antigen and cholera toxin B subunit (CTB) as the molecular adjuvant on surface. Oral administration of this engineered strain could protect *Carassius auratus* from *V. mimicus* infection by inducing humoral and cellular immunity [42]. At 10 days post *V. mimicus* challenge, the survival rate of the recombinant strain-immunized *Carassius auratus* was higher than that of the control group. As for the treatment of *Fusobacterium nucleatum* (*F. nucleatum*) infection associated inflammatory bowel disease (IBD), the FomA of *F. nucleatum* was surface-displayed on *L. plantarum* NC8. Oral immunization of mice with this engineered strain could decrease their mortality rate and body weight loss by inducing various immune responses to relieve *F. nucleatum*- or DSS-induced IBD [43]. To provide protective vaccine for *Staphylococcus aureus* (*S. aureus*) infection, the iron-regulated surface determinant protein B (IsdB) of *S. aureus* was displayed on the surface of *L. reuteri* WXD171. This engineered strain could induce mucosal responses in gut-associated lymphoid tissues and improve the survival rate of *S. aureus*-challenged mice from 10 to 70% [44]. To treat brucellosis, *L. casei* ATCC 393 was engineered to display the outer membrane protein OMP19 of *Brucella* species on surface. Oral administration of this engineered strain to mice could provide them with sufficient mucosal immune responses to resist the challenge of *Brucella abortus* 544 [45]. By assaying the CFU numbers of *B. abortus* 544 in spleen, the mice that orally immunized with OMP19-displaying strain showed higher degrees of protection (15-fold reduction of *B. abortus* 544 in spleen) as compared to the control group [45].

Plasmid-based surface displaying functional genes to facilitate the intestinal exclusion of viruses and pathogens

To treat rotavirus, an important pediatric pathogen for severe diarrhea, *L. rhamnosus* GG had been engineered to display the IgG-binding domain of protein G on surface. This engineered strain could fight against the rotavirus infection-induced diarrhea in mice by capturing rotavirus (simian strain RRV) via hyperimmune bovine colostrum antibodies (HBC-IgG) [46]. Compared with the usage of HBC alone, the combination usage of HBC antibodies and this engineered strain was more effective

(10 to 100-fold increase) to reduce the prevalence, severity, and duration of diarrhea, thus decreasing the treatment costs considerably [46]. Similarly, displaying the two VHH fragments ARP1 and ARP3 on the cell surface of *L. paracasei* BL23 could facilitate the capture of rotavirus, thus reducing the diarrhea rate of rotavirus infection-induced mouse model [47]. To prevent *L. monocytogenes* infection-associated disease, *L. casei* ATCC 334 had been engineered to display the *Listeria* adhesion protein (LAP) on surface. This engineered strain could prevent the intestinal colonization of *L. monocytogenes* by occupying the surface presented LAP receptor Hsp 60 [48]. Compared with the control group, the number *L. monocytogenes* cells that adhered to the intestine were 100-fold lower in the mice that treated with the recombinant strain [48]. At 10 days post *L. monocytogenes* challenge, the surviving rate of the recombinant strain-treated mice (~92%) was higher than that of the control group (60%) [48]. Besides, displaying the invasion proteins internalins A and B (*inlAB*) of *L. monocytogenes* on the cell surface of *L. casei* ATCC 334 could protect Caco-2 cell from adhesion, invasion, and transcellular passage of *L. monocytogenes* [49]. In the adhesion assay, the recombinant strain reduced the adhesion of *L. monocytogenes* by 50% and 53.6% at 16 and 24 h, respectively, far more than that of the control group (8%) [49].

Plasmid-based surface displaying pharmaceutical compounds and enzymes

To endow the *Lactobacilli* with anti-inflammation ability, *L. plantarum* NC8 had been engineered to display murine IL-10 on the cell surface. This engineered strain could reduce the Poly(I: C)- or LPS-induced Th1 responses in RAW264.7 cells and decrease the expression of TNF-α, IFN-γ, IL-1β, and IL-6 [50]. Moreover, displaying the porcine IFN-λ3 on the cell surface of *L. plantarum* NC8 could inhibit the replication of TGEV and PEDV, thus reducing the prevalence of PEDV and TGEV viruses by 53% and 59%, respectively [51]. As to the functional enzyme, *L. reuteri* had been engineered to detoxify the fungal mycotoxins zearalenone (ZEN) by displaying the ZEN hydrolyzing enzyme lactonohydrolase on surface. This engineered strain was capable of hydrolyzing 2.5 mg/kg of ZEN-contaminated corn within 4 h [52].

Genome integration-based surface displaying vaccines, functional elements, and the pharmaceutical compound for the intestinal exclusion of virus

To guarantee the stable inheritance of functional genes, many researchers had tried to integrate the functional genes into the genome of *Lactobacilli* and then displayed these elements on cell surface (Table 2; Fig. 1B). For example, to provide effective vaccine for infectious bronchitis virus (IBV), the UTEpi C-A expression cassette containing the EpiC of IBV was integrated into the genome of *L. salivarius* TCMM17. This engineered strain could display EpiC on surface and served as a stable oral vaccine for the treatment of IBV [53]. To treat the rotavirus infection-induced illness, *L. acidophilus* NCFM was engineered to display the VP8* domain of the rotavirus EDIM VP4 capsid along with the adjuvants FimH and FliC on surface. Gavaging this engineered strain to BALB/cJ mice could reduce the fecal shedding of rotavirus antigen (4-fold) by inducing the immune responses [54]. To treat HIV infection, *L. acidophilus* ATCC 4356 was engineered to display the HIV-1 receptor CD4 on surface. This engineered strain could decrease the infection rate (57% reduction) of the HIV-1-challenged mice by adsorbing HIV-1 particles directly [55]. To construct a synthetic probiotic with antimicrobial activity, *L. casei* ATCC 393 was engineered to insert the lactoferricin (Lfcin) expression cassette at the *thyA* (thymidylate synthase) site. This engineered strain displayed Lactoferricin on surface, showed good antibacterial activity against *Escherichia coli* (40.05% inhibition) and *Staphylococcus aureus* (42.22% inhibition), and exhibited antiviral activity against PEDV (2-fold suppression of viral replication) [56].

Lactobacilli-based secretion of functional elements

Plasmid-based secretion

Apart from displaying the functional elements on the surface of *Lactobacilli*, many functional elements were engineered to be secreted out of the cells for disease treatment (Table 3; Fig. 2A).

Plasmid-based secretion of vaccines for treating virus infection

As reported, hemagglutinin (HA) had been proved to be an effective vaccine antigen against avian influenza virus (AIV). Thus, to provide effective vaccine for AIV, HA was co-expressed with the dendritic cell-targeting peptide (DCpep) in *L. plantarum* NC8. This engineered strain could improve the survival rate of AIV-infected mouse and chicken models by inducing robust immune responses [57]. To provide improved vaccines for classical swine fever virus (CSFV) and porcine parvovirus (PPV), *L. casei* ATCC 393 were engineered to co-express the CSFV-specific cytotoxic T lymphocyte (CTL) epitope 290 and the VP2 antigen of PPV. This engineered strain showed 86.7% effective protection for the CSFV-challenge pig, whereas pigs in the control group developed severe clinical signs of CSF [58]. To treat the disease caused by the infectious pancreatic necrosis virus (IPNV), *L. casei* ATCC 393 was engineered to secret the VP2 protein of IPNV as antigen. Oral administration of this engineered strain to juvenile rainbow trouts could prevent IPNV infection by inducing local and systemic

Table 2 Genome Integration-based surface display of functional elements in *Lactobacilli*

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
Displaying vaccines for treating viruses infection							
L. salivarius TCMM17	Surface displaying the EpiC of IBV	Genome integration	Stimulating immunity responses	Against IBV			[53]
L. acidophilus NCFM	Surface displaying the VP8* domain of the rotavirus EDIM VP4 capsid protein along with the adjuvants FimH and FliC	Genome integration	Inducing the anti-rotavirus serum IgG and antigen-specific antibody-secreting cell responses	Against rotavirus diarrhea-associated illness	Reducing the fecal shedding of rotavirus antigen (4-fold)	BALB/cJ mice challenged with murine rotavirus strain EC_{WT}	[54]
Displaying functional element for the intestinal exclusion of virus							
L. acidophilus ATCC 4356	Surface displaying the HIV-1 receptor CD4 of human beings	Genome integration	Adsorbing HIV-1 particles to block intrarectal HIV-1 infection	Against human HIV-1 infection	Decreasing the infection rate (57% reduction) of the HIV-1-challenged mice	Bone marrow, liver, and thymus (BLT) humanized mice challenged with HIV-1 JR-CSF	[55]
Displaying pharmaceutical compound							
L. casei ATCC 393	Surface displaying the lactoferricin (Lfcin) polypeptide	Genome integration	Modulating host immune responses; Inducing autolysis death of bacteria cell by increasing its membrane permeability; Blocking the iron intake of microorganisms to act antimicrobial activity	Against *Escherichia coli*, *Staphylococcus aureus*, and PEDV	This engineered strain showed good antibacterial activity against *E. coli* (40.05% inhibition) and *S. aureus* (42.22% inhibition) and antiviral activity against PEDV (2-fold suppression of viral replication)	VERO cells infected with PEDV	[56]

immune responses. Compared to the control group, this recombinant strain showed more than 3-fold reduction in viral load [59].

Plasmid-based secretion of vaccines for treating parasite infection

To treat the *Cryptosporidium parvum* (*C. parvum*) infection-related disease, the P23 immunodominant surface protein of *C. parvum* was expressed stably in *L. casei* Zhang. Oral immunization of this engineered strain to mice could promote the clearance of *C. parvum* by inducing mucosal immune system and increasing the secretion of immunity factors, such as IgA, IL6, and IFN-γ [60].

Plasmid-based secretion of vaccines for treating pathogens infection

To provide effective oral vaccine against F4 + enterotoxigenic *Escherichia coli* (ETEC) infection, *L. casei* ATCC 393 was engineered to express FaeG, the main subunit of F4 (K88) fimbrial adhesin. Using the heat-labile enterotoxin A (LTAK63) and heat-labile enterotoxin B (LTB) as oral adjuvant, this engineered strain exhibited 100% protection against ETEC challenge and developed mild diarrhea for 2–3 days, whereas 80% of mice in the control group succumbed to the infection following viral challenge, exhibiting severe diarrhea that persisted for more than 12 days prior to mortality [61]. To provide protective immunity against *Bacillus anthracis* (*B. anthracis*), *L. acidophilus* NCFM was engineered to secret the protective antigen (PA) of *B. anthracis* that fused to a DC-binding peptide (DCpep). Oral administration of this genetically engineered strain conferred protection against *B. anthracis* infection (100% survival) in mice through the induction of both protective antigen (PA)-neutralizing antibodies and T cell-mediated immune responses [62]. To fight against the *C. perfringens*-derived necrotic enteritis, *L. reuteri* was engineered to secret nanobodies against NetB and α-toxin of *C. perfringens*. Oral administration of the recombinant strain to chickens demonstrated protective efficacy against necrotic enteritis, reducing both mortality rates (1.7- to 2.6-fold reduction) and pathological scores (2.5- to 3.6-fold reduction) [63].

Plasmid-based secretion of vaccines for treating neurodegeneration disease

Alzheimer's disease (AD), one of the neurodegenerative disorder diseases, is a global health concern with huge implications for both the individuals and whole society. Gut microbiota contains the largest number of microbes

Table 3 Plasmid-based secretion of functional elements in *Lactobacilli*

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
Secreting vaccines for treating virus infection							
L. plantarum NC8	Co-expressing hemagglutinin (HA) with the dendritic cell-targeting peptide (DCpep)	Plasmid-based	Inducing avian influenza virus-specific cell-mediated and humoral immune responses	Against H9N2 AIV	Improving the survival rate of AIV-infected mouse and chicken models by inducing robust immune responses	Mice and chicken challenged with H9N2 virus	[57]
L. casei ATCC 393	Co-expressing CSFV-specific cytotoxic T lymphocyte (CTL) epitope 290 and the VP2 antigen of PPV	Plasmid-based	Increasing the mucosal and systemic immune responses	Against CSFV and PPV	This engineered strain showed 86.7% effective protection for the CSFV-challenged pig	Pig challenged with CSFV strain Shimen	[58]
L. casei ATCC 393	Secreting the VP2 protein of infectious pancreatic necrosis virus (IPNV)	Plasmid-based	Inducing local mucosal and systemic immune responses	Against IPNV	This recombinant strain induced more than 3-fold reduction in viral load compared to control group	Rainbow trout (*Oncorhynchus mykiss*) that intraperitoneally injected with IPNV	[59]
Secreting vaccine for treating parasite infection							
L. casei Zhang	Expressing immunodominant surface protein P23 of *C. parvum* sporozoites	Plasmid-based	Inducing mucosal immune system to elicit serum immunoglobulin G (IgG) and mucosal IgA	Against *C. parvum*	Increasing the secretion of immunity factors such as IgA, IL6, and IFN-γ	BALB/c mice	[60]
Secreting vaccines for treating pathogens infection							
L. casei ATCC 393	Expressing F4 (K88) fimbrial adhesin FaeG	Plasmid-based	Inducing effective fimbriae-specific mucosal and systemic immune responses	Against enterotoxigenic *Escherichia coli* (ETEC) infection	Using LTAK63 and LTB as oral adjuvant, this engineered strain exhibited 100% protection against ETEC challenge and developed mild diarrhea for 2–3 days	SPF BALB/c mice challenged with F4 + ETEC strain CVCC 230	[61]
L. acidophilus NCFM	Secreting the protective antigen (PA) of *B. anthracis* that genetically fused to a DC-binding peptide (DCpep)	Plasmid-based	Inducing PA-neutralizing antibody and T-cell mediated immune responses	Against *B. anthracis* infection	Increasing the survival rate of *B. anthracis*-infected mice from 0–100%	A/J mice challenged with *B. anthracis* Sterne $pXO1^{+}$/$pXO2^{-}$	[62]
L. reuteri 3630 and 3632	Secreting the nanobodies against NetB and α toxin of *C. perfringens*	Plasmid-based	Neutralizing NetB and α toxin of *C. perfringens*	Against necrotic enteritis	Protecting the chickens from necrotic enteritis-associated mortality (1.7- to 2.6-fold reduction) and reducing the pathological scores by 2.5- to 3.6-fold	Chickens challenged with *Eimeria maxima* and *C. perfringens*	[63]
Secreting vaccines for treating neurodegeneration disease							
L. lactis subsp. cremoris MG1363	Expressing human p62 protein	Plasmid-based	Improving memory function, modulating of neuronal proteolysis, and decreasing AD typical signs	Against Alzheimer's disease (AD)	The Aβ(1–42) peptide level was decreased by 42%; The activities of proteasome T-L and branched-chain amino acid preferring (BrAAP) were inhibited by 70% and 50%, respectively; The levels of protein oxidation products (3-NT and carbocyanine) were decreased by 1.37- to 1.78-fold; The levels of lipid peroxidation product (4-HNE) was decreased by 65%; The expression of anti-inflammatory cytokine (IL-10) was upregulated by 3-fold; The expression of pro-inflammatory cytokines (e.g., INF-γ, IL-1β, TNF-α, IL-2) were decreased by 2.0- to 3.25-fold	Triple transgenic mice 3xTg-AD	[65]

Table 3 (continued)

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
Secreting allergens for treating allergy							
L. plantarum NCIMB8826 Int-1	Secreting the major birch pollen allergen Bet v 1	Plasmid-based	Decreasing allergen-specific IgE and increasing allergen-specific IgA at the mucosae	Against birch pollen allergy	This engineered strain could activate the Th1-type immune responses effectively in mice and reduce the production of IL-5 significantly	Aerosol challenges with a 1% birch pollen solution in female BALB/c mice	[66]
L. plantarum NCL21	Secreting the Japanese cedar pollen allergen Cry j 1 (Cry j 1-LAB)	Plasmid-based	Suppressing the allergen-specific IgE response and nasal symptoms	Against cedar pollinosis	Bringing 2-fold reduction for the allergen-specific IgE response	Female BALB/c mice immunized with Cry j 1/alum	[67]
Secreting antibodies							
L. paracasei	Secreting the 3D8 single-chain variable fragment (scFv)	Plasmid-based	Hydrolyzing the nucleic acids of virus	Against avian influenza virus (AIV)	Decreasing virus shedding to protect the chickens from H9N2 infection	SPF chickens challenged with AIV	[68]
L. paracasei BL23	Secretion and surface display of TcdB-neutralizing antibody	Plasmid-based	Neutralizing the cytotoxic effect of the toxin B	Against *C. difficile*	Improving the survival rate of *C. difficile* spore-challenged hamsters from 0–50%	Hamsters challenged with spores of a $TcdA^{-}TcdB^{+}$ strain of *C. difficile*	[69]
Secreting pharmaceutical compounds							
L. johnsonii FI9785	Exportation of bacteriophage endolysin CP25L	Plasmid-based	Lysing C. perfringens	Against *C. perfringens*	2- to 2.6-log less *C. perfringens* was observed in the coculture experiment		[70]
L. Reuteri 6475	Secreting murine interleukin-22 (IL-22)	Plasmid-based	Modulating the cytokines level in the serum and intestine	Against total body irradiation (TBI)	The survival rate of irradiation-treated mice was improved from 10–60% at day 30	C57BL/6NTac mice irradiated to 8.75 and 9.25 Gy	[71]
L reuteri 6475	Secreting murine interleukin-22 (IL-22)	Plasmid-based	Reducing the liver weight and triglycerides	Against fatty liver disease	22.3% decrease for liver weight ratio; 4.6-fold decrease for liver triglyceride	Male C57BL/6J mice with high-fat diet-induced obesity	[72]
L. reuteri 6475	Secreting murine interleukin-22 (IL-22)	Plasmid-based	Irradiation protection	Against alcoholic liver disease (ALD)	Increasing the expression level of Reg3g in small intestine, decreasing the level of Cxcl1 and Cxcl2 mRNAs, and reducing the bacteria translocation to liver	C57BL/6 mice with chronic and binge alcohol feeding (NIAAA)	[73]
L. plantarum NC8	Expressing angiotensin converting enzyme inhibitory peptide (ACEIPs)	Plasmid-based	Inhibiting angiotensin-converting enzyme (ACE)	Against hypertension	The SBP was decreased from 184.810 ± 4.305 mmHg to 167.111 ± 3.418 mmHg at day 15; The serum triglyceride was decreased from 1.213 ± 0.176 mM to 0.750 ± 0.181 mM	The spontaneously hypertensive rats (SHR)	[74]
L. reuteri 647	Secreting the Kv1.3 potassium blocker ShK-235	Plasmid-based	Blocking the Kv1.3 currents	Against rheumatoid arthritis	Reducing the mean score of arthritis from 25 ± 2 to 4 ± 1 (84% reduction)	Rat model of rheumatoid arthritis	[75]
Secreting functional enzymes							
L. plantarum WCFS1	Secreting the hydrolase domain of glycoside hydrolase PelA (PelAh) from *P. aeruginosa*	Plasmid-based	Degrading the biofilm of *P. aeruginosa*	Against *P. aeruginosa*	The cultures and supernatants of this engineered strain exhibited 80% and 85% reduction in biofilm biomass of *P. aeruginosa*		[76]

Table 3 (continued)

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
L. paracasei F19	Expressing human N-acyl-phosphatidylethanolamine-specific phospholipase D (NAPE-PLD)	Plasmid-based	Anti-inflammation	Against ulcerative colitis (UC)	Decreasing the DAI score (71% reduction), spleen weight (62% reduction), colitis histopathological score (47% reduction), MPO activity (56% reduction), the colonic level of iNOS (80% reduction), COX-2 (75% reduction) and IL-1β (63% reduction), the plasma level of NO (79% reduction), PGE2 (74% reduction), IL-1β (81% reduction) and TNF-α (86% reduction); Increasing the colon length (1.13-fold increment) and colonic expression of zonula occludens (ZO-1) (5.43-fold increment) and occluding (3.97-fold increment)	DSS-induced colitis mouse model	[77]
L. paracasei ATCC 27092	Secreting angiotensin converting enzyme 2 (ACE2) that fused with the non-toxic subunit B of cholera toxin	Plasmid-based	Reducing inflammation and oxidative stress by degrading Angiotensin II	Against diabetic retinopathy	Reducing the number of acellular capillaries, blocking the 20% retinal ganglion cell loss, and decreasing the expression of retinal inflammatory cytokines	STZ-induced diabetic $eNOS^{-/-}$ mice and Akita mice	[78]
L. plantarum WCFS1	Secreting oxalate decarboxylase (OxdC)	Plasmid-based	Increased intestinal oxalate degradation	Against hyperoxaluria	Reducing serum uric acid (34% reduction), urinary oxalate excretion (40% reduction) and CaOx crystal deposition	Male wistar albino rats with hyperoxaluria	[79]
L. reuteri 100–23 C	Expressing phenylalanine lyase	Plasmid-based	Decreasing blood Phe concentrations	Against phenylketonuria (PKU)		PHA^{enu2} mouse model of PKU	[80]
L. plantarum CM_PUJ411	Secreting the human phenylalanine hydroxylase (PAH)	Plasmid-based	Secreting PAH to transport through the cell monolayer of Caco-2 cells and decrease phenylalanine (Phe)	Against phenylketonuria (PKU)	Decreasing the Phe levels (28% reduction)	Caco-2 cells	[81]

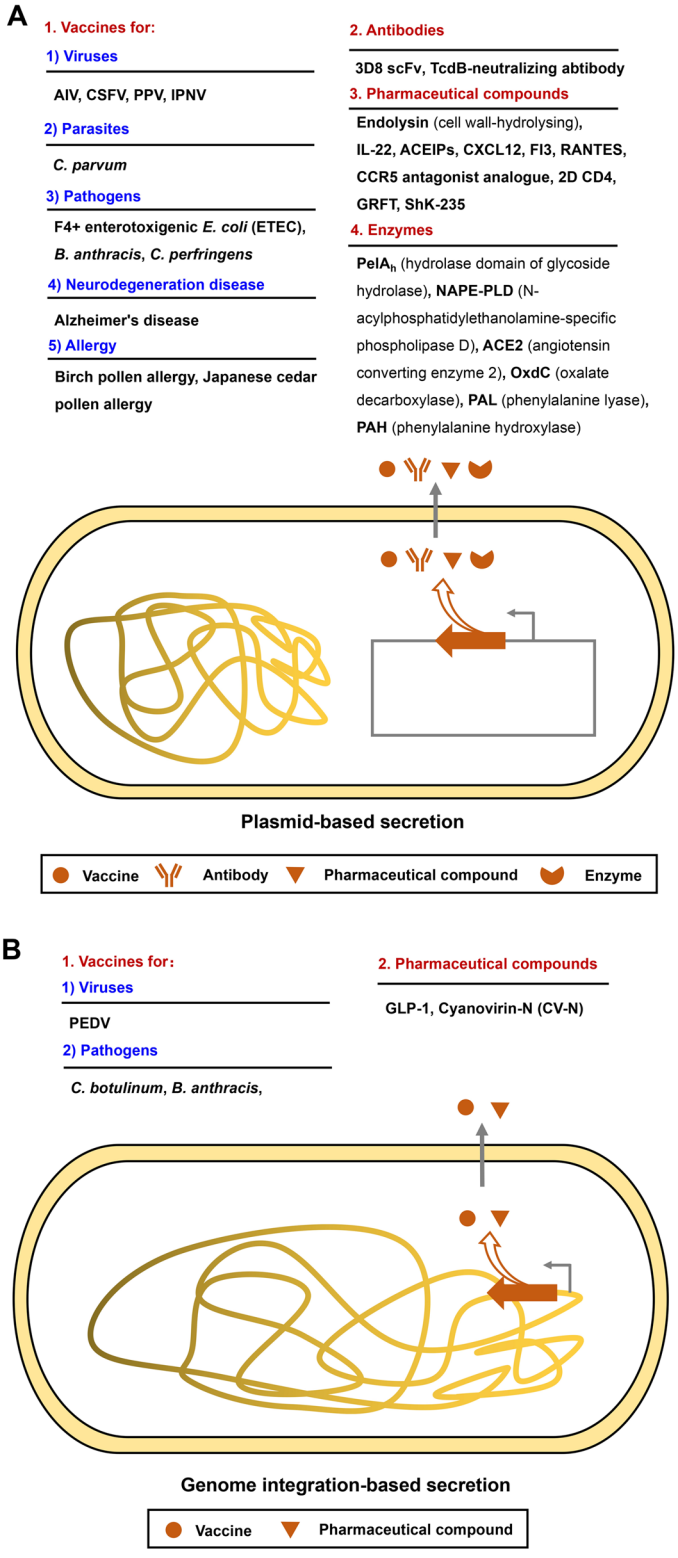

Fig. 2 Summary of the strategies used to secret functional elements in *Lactobacilli*. (**A**) Plasmid-based secretion of vaccines, antibodies, pharmaceutical compounds, and enzymes. (**B**) Genome integration-based secretion of vaccines and pharmaceutical compounds

in the intestine and has the potential to influence the host metabolism greatly. Thus, a healthy, balanced, and diverse gut microbiome is closely associated with the overall health of host. Recently, a plenty of results indicate that the dysbiosis of gut microbiome exerts a profound effect on the progression of neurological diseases, indicating the potential of treating these brain diseases through the gut-brain axis [64]. To alleviate AD, *L. lactis subsp. cremoris* MG1363 was engineered to express the p62 protein of human beings. Oral administration of this engineered strain to the 3xTg-AD mice could benefit them by improving the memory function, modulating the neuronal proteolysis, and decreasing the AD typical signs [65]. For the recombinant strain gavaged group, the Aβ(1–42) peptide level was decreased by 42%; The activities of proteasome T-L and branched-chain amino acid preferring (BrAAP) were inhibited by 70% and 50%, respectively; The levels of protein oxidation products (3-NT and carbocyanine) were decreased by 1.37- to 1.78-fold; The levels of lipid peroxidation product (4-HNE) was decreased by 65%; The expression of anti-inflammatory cytokine (IL-10) was upregulated by 3-fold; The expression of pro-inflammatory cytokines (e.g., INF-γ, IL-1β, TNF-α, IL-2) were decreased by 2.0- to 3.25-fold [65].

Plasmid-based secretion of allergens for treating allergy

To prevent birch pollen allergy, *L. plantarum* NCIMB8826 Int-1 was engineered to produce the major birch pollen allergen Bet v 1. Systemic immunization of mice with this engineered strain could induce lower allergen-specific IgE and higher allergen-specific IgA. Besides, this engineered strain could activate the Th1-type immune responses effectively in mice and reduce the production of IL-5 significantly [66]. Furthermore, a recombinant *L. plantarum* NCL21 strain was constructed to express a major Japanese cedar pollen allergen Cry j 1 (Cry j 1-LAB). This engineered strain could provide prophylactic effect for the murine model of cedar pollinosis by suppressing the allergen-specific IgE response (2-fold reduction) [67].

Plasmid-based secretion of antibodies

To treat AIV, *L. paracasei* was engineered to secret the 3D8 single-chain variable fragment (scFv), which could bind and hydrolyze nucleic acids. Gavaging this engineered strain to chickens could lower virus shedding significantly and protect them from H9N2 avian influenza virus [68]. To fight against *Clostridium difficile* (*C. difficile*) infection, an engineered *L. paracasei* BL23 was constructed by expressing two neutralizing anti-TcdB VHH fragments (VHH-B2 and VHH-G3). Gavaging this engineered strain to the *C. difficile* spore-challenged hamsters could improve their survival rate from 0 to 50% [69].

Plasmid-based secretion of pharmaceutical compounds

To facilitate the exclusion of *C. perfringens*, *L. johnsonii* FI9785 was engineered to export the cell wall-hydrolysing endolysin (CP25L) of phage. Compared with the wild-type *L. johnsonii*, the numbers of *C. perfringens* were 2- to 2.6-log less when it was co-cultured with this engineered strain [70]. To protect the intestine from irradiation, an engineered *L. reuteri* 6475 was constructed to release the murine IL-22, which was functioned as an irradiation protector. Oral administration of this engineered strain to irradiated mice could modulate the cytokines level in the serum and intestine, thus improving the survival rate of irradiation-treated mice from 10 to 60% at day 30 [71]. Apart from protecting the host intestine from irradiation, this IL-22-secreting strain could also reduce the fatty liver disease of high-fat diet-fed mice by reducing the liver weight ratio (22.3% decrease) and liver triglyceride (4.6-fold decrease) [72]. Additionally, oral administration of this recombinant strain could reduce the liver damage of the alcoholic liver disease mouse model by increasing the expression level of *Reg3g* in small intestine, decreasing the expression level of *Cxcl1* and *Cxcl2,* and reducing the number of the bacteria that translocated to liver [73]. To treat hypertension, the angiotensin converting enzyme inhibitory peptide (ACEIPs) was overexpressed in *L. plantarum* NC8. Oral administration of this recombinant strain could treat hypertension effectively in spontaneously hypertensive rats. Compared with the control group, the systolic blood pressure (SBP) of the engineered *L. plantarum*-treated group was decreased from 184.810 ± 4.305 mmHg to 167.111 ± 3.418 mmHg at day 15. Besides, the serum triglyceride was decreased from 1.213 ± 0.176 mM to 0.750 ± 0.181 mM [74]. To treat rheumatoid arthritis (RA), *L. reuteri* 647 was engineered to secret the Kv1.3 potassium blocker ShK-235 (LrS235). Gavaging this engineered strain to the rat model of rheumatoid arthritis could reduce the mean score of arthritis from 25 ± 2 to 4 ± 1(84% reduction) [75].

Plasmid-based secretion of functional enzymes

To suppress *Pseudomonas aeruginosa* (*P. aeruginosa*) infection, *L. plantarum* WCFS1 was engineered to secrete $PelA_h$, the hydrolase domain of glycoside hydrolase PelA from *P. aeruginosa*, to degrade the biofilm of *P. aeruginosa* [76]. The cultures and supernatants of this engineered strain exhibited 80% and 85% reduction in biofilm biomass of *P. aeruginosa*, respectively [76]. To provide safe and efficient strategy for the treatment of ulcerative colitis (UC), *L. paracasei* F19 was engineered to produce palmitoylethanolamide (PEA) by expressing the N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) of human beings. Co-administration of this engineered strain with palmitate to the DSS-induced colitis mice could decrease the

disease activity index (DAI) score (71% reduction), spleen weight (62% reduction), colitis histopathological score (47% reduction), and MPO activity (56% reduction), while increase the colon length (1.13-fold increment) and colonic expression of zonula occludens (ZO-1) (5.43-fold increment) and occluding (3.97-fold increment). Besides, the colonic level of iNOS (80% reduction), COX-2 (75% reduction), and IL-1β (63% reduction) and the plasma level of NO (79% reduction), PGE2 (74% reduction), IL-1β (81% reduction), and TNF-α (86% reduction) were decreased in colitis mice that were orally administrated with this engineered strain and palmitate [77]. To treat diabetic retinopathy, *L. paracasei* ATCC 27092 was engineered to secret the angiotensin converting enzyme 2 (ACE2), which was fused with the non-toxic subunit B of cholera toxin to facilitate transmucosal transportation. The secreted ACE2 could degrade angiotensin II and reduce inflammation and oxidative stress. Oral administration of this engineered strain could alleviate diabetic retinopathy by reducing the number of acellular capillaries, blocking the retinal ganglion cell loss, and decreasing the expression of retinal inflammatory cytokines [78]. To treat hyperoxaluria, *L. plantarum* WCFS1 was engineered to secrete the oxalate decarboxylase (OxdC) of *Bacillus subtilis* to degrade oxalate. Gavaging this engineered strain to male wistar albino rats with hyperoxaluria could reduce their serum uric acid (34% reduction), urinary oxalate excretion (40% reduction) and CaOx crystal deposition [79]. To treat phenylketonuria (PKU), the phenylalanine lyase gene of *Anabaena variabilis* (AvPAL) was codon-optimized and expressed in *L. reuteri* 100–23 C. Gavaging this engineered strain to PAH^{enu2} mouse model of PKU could reduce their blood Phe concentrations [80]. Besides, *L. plantarum* CM_PUJ411 was engineered to secret human phenylalanine hydroxylase (PAH), an enzyme that can metabolize Phe. Assisted with the signal peptide GI1 or GI2, the secreted PAH could transport through the cell monolayer of Caco-2 cells to reduce the Phe levels (28% reduction) [81].

Genome integration-based secretion of vaccines and pharmaceutical compounds

Until now, a series of functional elements had been integrated into the genome of *Lactobacilli* and secreted out of cell to treat diseases (Table 4; Fig. 2B). For example, to fight against PEDV, the PEDV S1 gene was integrated into the genome of alanine racemase-deficient *L. paracasei*△Alr HLJ-27 strain. Oral administration of this engineered strain could activate the mucosal, humoral, and cellular immune responses in mice and piglets effectively. The piglet challenging experiment results indicated that *L. paracasei*△Alr HLJ-27 administration could endow the piglets with resistance against PEDV LJB2019 infection by decreasing the PEDV copy number, maintaining intact villi structure, and relieving the inflammatory status [82]. To provide potent vaccine for

Table 4 Genome Integration-based secretion of functional elements in *Lactobacilli*

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
Secreting vaccine for treating virus infection							
L. paracasei△Alr HLJ-27	Expressing the S1 gene of PEDV	Genome integration	Activate the mucosal, humoral, and cellular immune responses	Against PEDV	*L. paracasei*△Alr HLJ-27 administration could endow the piglets with resistance against PEDV LJB2019 infection by decreasing the PEDV copy number, maintaining intact villi structure, and relieving the inflammatory status	SPF BALB/c mice and large landrace piglets	[82]
Secreting vaccine for treating pathogen infection							
L. acidophilus NCFM	Secreting the host receptor-binding domain of the heavy chain of *C. botulinum* serotype A and the anthrax protective antigen of *B. anthracis*	Genome integration	Inducing mucosal immune responses	Against *C. botulinum* and *B. anthracis* infection			[83]
Secreting pharmaceutical compounds							
L. gasseri ATCC 33323	Secreting the inactive full-length form of GLP-1(1–37)	Genome integration	Stimulating the conversion of intestinal epithelial cells into insulin-secreting cells; Increasing the insulin levels and glucose tolerant	Against diabetes	The recombinant microbe-fed diabetic rats showed high insulin levels (average 60% more total insulin (intestines and pancreases combined)) and glucose tolerance	STZ-induced diabetic rats	[85]

C. botulinum and *B. anthracis* infection simultaneously, *L. acidophilus* NCFM was engineered to secret the host receptor-binding domain of the heavy chain of *C. botulinum* serotype A and the anthrax protective antigen of *B. anthracis*. This engineered strain was a promising candidate for the development of mucosal vaccines against botulism and anthrax [83]. To treat diabetes, *L. gasseri* ATCC 33323 was engineered to secret the inactive full-length form of GLP-1(1–37), which could stimulate the conversion of intestinal epithelial cells to insulin-secreting cells [84]. The diabetic rats that fed with this engineered strain developed insulin-producing cells in the upper intestine and showed high insulin levels and glucose tolerance [85].

Engineering vaginal *Lactobacilli* for disease treatment

As reported, *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners* are the four main vaginal *Lactobacilli* and play important roles in sustaining the health of females. Although most of the vaginal *Lactobacilli* exhibit beneficial properties, the application of these microbes as probiotics is limited by the deficiency of appropriate delivery systems [86]. Here, the current strategies that used for engineering vaginal *Lactobacilli* for disease treatment are reviewed (Table 5).

Plasmid-based surface displaying functional element

To prevent the heterosexual transmission of HIV, *L. jensenii* 1153 was engineered to display the two-domain of high-affinity HIV-binding protein CD4 (2D CD4) on surface. The 2D CD4 molecules were distributed uniformly on the bacterial surfaces and could be recognized by the conformation-dependent anti-CD4 antibody [87]. Thus, this engineered strain might prevent the HIV transmission by adsorbing the HIV particles directly.

Plasmid-based secretion of functional elements

To treat intrauterine adhesions (IUA), *L. crispatus* MH175 was engineered to secret the murine CXCL12, which could recruit immune cells to promote the tissue regeneration and repair. Vaginal application of this engineered strain could decrease the levels of pro-inflammatory factors IL-1β and TNF-α in the serum and uterine tissues of IUA mice, inhibit the inflammatory and fibrotic signalling pathways in uterine tissue [88]. Besides, the engineered *L. crispatus*-treated mice could restore the vaginal microbiota composition of IUA mice by increasing the abundance of *Lactobacillus* and decreasing the abundance of *Klebsiella* microbes [88]. To treat human immunodeficiency virus (HIV) infection, *L. plantarum* ATCC 14917 *and L. gasseri* ATCC 9857 were engineered to secret the HIV-1 fusion inhibitors FI-3 to facilitate the neutralization of primary HIV-1 isolates and SHIV-162P3. For the HIV-1 isolates, the FI-3 expressing *L. plantarum* could reduce the viral infection of five HIV-1 isolates by 71–98%. Furthermore, the *Lactobacillus*-derived FI-3 could achieve 72% inhibition of SHIV-162P3 infection [89]. *L. jensenii* 1153 was engineered to secret the anti-HIV-1 chemokine RANTES and a CCR5 antagonist analogue as live HIV-1 blockers. The *L. jensenii*-derived wild-type RANTES could inhibit the acute HIV-1 infection, with IC_{50}s reached 0.54 nM against HIV-1_{BaL} and 1.14 nM against HIV-1_{SF162}. Differently, although the *Lactobacillus*-derived C1C5 RANTES was devoid of proinflammatory activity, it showed lower anti-HIV-1 activity (IC_{50}s of 5.00 nM for HIV-1_{BaL} and 4.8 nM for HIV-1_{SF162}) as compared to the wild-type RANTES [90]. Apart from the above-mentioned strategy, *L. jensenii* had been engineered to secrete the two-domain CD4 (2D CD4) proteins to inhibit HIV infection by blocking its entry into target cells. Single-cycle infection assay indicated that this engineered strain could inhibit the HIV-1_{HxB2} entry into HeLa cells by 95% and inhibit the HIV-1_{JR-FL} entry by 55% [91]. To inhibit the transmission of HIV, *L. rhamnosus* GG and GR-1 were engineered to express carbohydrate-binding agent griffithsin (GRFT). The cytosolic protein fractions of two engineered strain were able to inhibit the T-tropic (X4) HIV-1 NL4.3 infection with an EC_{50} value of 1/1710 and 1/3021 [92]. Besides, these two engineered strains showed significant activity against the M-tropic (R5) HIV-1 BaL strain, with a dilution factor of 1/605 and 1/1143 [92].

Genome integration-based secretion of functional element

To fight against HIV infection, *L. jensenii* 1153–1666 was engineered to secret the HIV inhibitor cyanovirin-N (CV-N). This engineered strain could colonize the vagina and prevent the repeated vaginal simian-HIV ($SHIV_{SF162P3}$)-challenged Rhesus Macaque from HIV infection by remodeling the vaginal mucosal environment, e.g., lowering pH by an estimated 0.4 pH units and increasing the anti-inflammatory cytokine IL-1RA [93].

Future perspectives and challenges

Though probiotics inherently carry genetic signatures that promote the health of the host, the metabolically engineered *Lactobacilli* are further augmented with functional elements for the potential treatment of disease indications. The *Lactobacilli* have been engineered to secrete or surface display the functional elements to treat various diseases. As reported, the microbial cell surface display technology, which fused the heterologous protein to the anchor proteins, had been widely used in the application of biotechnology and biomedicine and vaccine delivery. Compared to the protein secretion system, the cell surface display strategy showed enhanced protein activity and stability [94]. However, there are

Table 5 Engineering vaginal *Lactobacilli* for disease treatment

Strains	Strategy	Expression mode	Mechanisms	Diseases	Treating effect	Experimental model	Reference
Plasmid-based surface displaying functional element for treating HIV							
L. jensenii 1153	Displaying the two-domain of high-affinity HIV-binding protein CD4 (2D CD4)	Plasmid-based	Absorbing the HIV particles directly	Against HIV			[87]
Plasmid-based secretion of functional elements							
L. crispatus MH175	Secreting the murine CXCL12	Plasmid-based	Decreasing the levels of IL-1β and TNF-α in serum and uterine tissues; Inhibiting the inflammatory and fibrotic signaling pathways in the uterine tissues; Restoring the vaginal microbiota composition of IUA mice	Against intrauterine adhesions (IUA)		Intrauterine adhesion mice with or without diabetes	[88]
L. plantarum ATCC 14917 and *L. gasseri* ATCC 9857	Secreting HIV-1 fusion inhibitors (FI3)	Plasmid-based	Neutralizing primary HIV-1 isolates and SHIV-162P3	Against HIV	Reducing the viral infection of five HIV-1 isolates by 71–98%; Achieving 72% inhibition of SHIV-162P3 infection	TZM-bl cells infected with infectious molecular clone HIV-$1_{NL4.3}$, single-cycle HIV-$1_{NL-Luc/VSV-G}$ reporter virus, primary clinical HIV-1 isolates and SHIV-162P3	[89]
L. jensenii 1153	Secreting RANTES and a CCR5 antagonist analogue	Plasmid-based	Blocking virus entry into target cells	Against HIV	RANTES inhibited the acute HIV-1 infection, with IC_{50}s reached 0.54 nM against HIV-1_{BaL} and 1.14 nM against HIV-1_{SF162}; C1C5 RANTES inhibited the acute HIV-1 infection, with IC_{50}s reached 5.00 nM against HIV-1_{BaL} and 4.80 nM against HIV-1_{SF162}.	HIV-1_{BaL} infected human monocyte-derived macrophages (MDM); R5 HIV-1 infected human $CD4^+$ T cell clone PM1	[90]
L. jensenii	Expressing two-domain CD4 (2D CD4) proteins	Plasmid-based	Binding gp120 to inhibit HIV-1 viral entry	Against HIV	This engineered strain could inhibit the HIV-1_{HxB2} entry into HeLa cells by 95% and inhibit the HIV-1_{JR-FL} entry by 55%	Env-pseudotyped HIV-1_{HxB2} infected HeLa-CD4-CXCR4 cells	[91]
L. rhamnosus GG and GR-1	Secreting HIV-inhibiting griffithsin (GRFT)	Plasmid-based	Blocking and inhibiting HIV transmission	Against HIV	The cytosolic protein fractions of two engineered strains were able to inhibit the T-tropic (X4) HIV-1 NL4.3 infection with an EC 50 value of 1/1710 and 1/3021; These two engineered strains showed significant activity against the M-tropic (R5) HIV-1 BaL strain, with a dilution factor of 1/605 and 1/1143.	The $CD4^+$, $CXCR4^+$, $CCR5^+$ TZM-bl cells infected with T-Tropic (X4) HIV-1 strain NL4.3 or the M-tropic (R5) HIV-1 BaL	[92]
Genome integration-based secretion of functional element							
L. jensenii 1153–1666	Secreting wild-type HIV inhibitor Cyanovirin-N	Genome integration	Remodeling the vaginal mucosal environment	Against HIV	Lowering pH by an estimated 0.4 pH units and increasing the anti-inflammatory cytokine IL-1RA	$SHIV_{SF162P3}$ challenged macaque	[93]

several unavoidable challenges for this strategy, e.g., the size limitation of the displayed protein, the low display efficiency, the lack of proper carrier protein on cell surface, and the insertion site of the heterologous protein into the carrier (the insertion site affects the stability and activity of the displayed protein) [94, 95]. Based on these limitations, the smaller proteins, the functional fragment of big proteins, peptides, and vaccines can be displayed on the cell surface to exert specific functions. However, if the desired protein is too big to be displayed on surface, the protein secretion system should be used.

Given that both plasmid-based gene expression systems and genome integration systems have been widely utilized in metabolic engineering of host cells, we conducted a comprehensive comparison of their advantages and disadvantages across several key aspects: Firstly, the simplicity for metabolic engineering. Compared to genome integration systems, plasmid-based expression systems offer greater convenience and flexibility for metabolic engineering of host cells. Secondly, the gene expression level. In the genome integration strategy, a single copy of the functional element was inserted into the host cell's genome, resulting in relatively low expression levels of the target element. In contrast, the use of high-copy-number plasmids enables significantly higher expression levels of the plasmid-encoded functional elements. Thirdly, the growth stress of host strain. Previous studies have reported that overexpression of heterologous proteins in bacterial systems can impose substantial metabolic burden on host cells, resulting in significantly reduced growth rates. Based on this conception, the plasmid-based high-level gene expression system may impose greater growth inhibition on host cells compared to the genome integration approach. Fourthly, the stable inheritance of the functional genes. For the plasmid-based gene expression system, the plasmids are unstable and can be lost after the long-term passage, especially under the antibiotic-free condition. In contrast, chromosomal integration of genes through genome-editing technologies ensures stable inheritance and maintenance of functional genes. Fifthly, the usage of antibiotic genes. The antibiotic genes on the plasmid can be diffused through transposition, transfer, or homologous recombination during operation, resulting the emergence of drug-resistant strains [96]. When functional elements were integrated into the host genome, the resulting strains did not require antibiotic resistance genes for maintenance. Lastly, the stability of genetic element. Replication of plasmids through the rolling circle mechanism generates unstable single-stranded DNA intermediates, which can result in deletions of genetic elements [97]. However, this disadvantage can be overcome by integrating the functional element in the genome of host cell.

Based on the abovementioned advantages of genome integration system, more and more researchers had focused on constructing the synthetic probiotics by editing the genome of *Lactobacilli*. Until now, several genome-editing technologies had been developed to facilitate the genome editing of *Lactobacilli*, e.g., the methods based on the insertion sequence (IS) elements [98], Cre-lox-based systems [99], and the clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas) systems [100]. However, compared with the high copy number of functional genes in the plasmid-based expression system, the genes that integrated into the genome has only one copy, which will limit the expression of antigens or functional genes. Thus, the expression of the genome-integrated element should be enhanced by optimizing the expression related elements, e.g., the optimization of promoters or ribosomal binding sites. Until now, the number of genome-edited *Lactobacilli* are much lower than that of the plasmid-containing microbes, thus, more efforts should be done to generate the genome engineered *Lactobacilli*.

Taking the biosafety into consideration, the leakage of engineered microbes to environment will bring the problem of bio-contamination. To provide biocontainment for engineered microbes, several useful strategies had been applied during the construction of engineered probiotics: (1) Generation of auxotrophs to prevent the engineered probiotics from escaping to the environment [101]; (2) Construction of orthogonalized genetic central dogma by using artificial elements or coding principles [102, 103]. Based on this strategy, the biocontainment can be achieved by inhibiting the survival of engineered probiotics under the condition without non-natural synthetic substances; (3) Application of suicide circuit. For the passive suicide circuit, the killing module is coupled with a sensing module which can detect environmental signals. The engineered bacteria that harbours the suicide circuit commit suicide when the living environment is changed [104].

Collectively, with the assistance of the efficient genome-editing technologies and the biocontainment strategies, further studies should focus on constructing the engineered *Lactobacilli* with stable and safe characteristics for disease treatment.

Conclusion

Engineered probiotics are promising avenues for the treatment of various diseases by expressing functional elements or delivering intestine-directed therapeutics. Among these probiotics, *Lactobacilli* is widely used as functional chassis. In this review, the research progresses on engineering *Lactobacilli* for disease treatment are summarized according to the different engineering strategies. By discussing the shortcomings of the

plasmid-based strategies that are used for the construction of synthetic microbes, our study stresses the importance of constructing synthetic probiotics with stable characteristics. Moreover, our study provides biocontainment strategies to improve the biosafety of the engineered microbes for the potential treatment of disease indications.

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Author contributions
"Y.P.Y. conceived the project. Y.P.Y., P.J.Y., Y.F.H., W.Y.Z., Y.H.N. and C.S.G. wrote the main manuscript text. Y.P.Y. prepared Figs. 1-2. Y.P.Y. and P.J.Y. prepared the Tables 1-5. All authors reviewed the manuscript."

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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