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REVIEWS

Development of Vaccine Delivery Vehicles Based on Lactic Acid Bacteria

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Abstract Live recombinant bacteria represent attractive antigen delivery systems able to induce both mucosal and systemic immune responses against heterologous antigens. The first live recombinant bacterial vectors developed were derived from attenuated pathogenic microorganisms. In addition to the difficulties often encountered in the construction of stable attenuated mutants of pathogenic organisms, attenuated pathogens may retain a residual virulence level that renders them unsuitable for the vaccination of partially immunocompetent individuals such as infants, the elderly or immunocompromised patients. As an alternative to this strategy, non-pathogenic food-grade lactic acid bacteria (LAB) maybe used as live antigen carriers. This article reviews LAB vaccines constructed using antigens other than tetanus toxin fragment C, against bacterial, viral, and parasitic infective agents, for which protection studies have been performed. The antigens utilized for the development of LAB vaccines are briefly described, along with the efficiency of these systems in protection studies. Moreover, the key factors affecting the performance of these systems are highlighted.

Keywords Antigen · Immunization route · Lactic acid bacteria · Live vaccine · Vaccine delivery

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Introduction

Vaccination represents one of the most effective public health strategies to combat infectious diseases [1]. One of the technologies being developed for vaccine production is the use of bacteria as live vectors for the delivery of recombinant vaccine antigens to the immune system. Such vaccines have the potential for the production of protective antigens in vivo and are inexpensive to manufacture. Moreover, the possibility for simultaneous expression of multiple antigens in bacteria can result in development of multivalent vaccines, which require reduced number of administrations [2].

Most infections affect or initiate infectious processes at mucosal surfaces and mucosal local immune responses can block pathogens at the portal of entry. Live bacterial vaccines can induce mucosal, as well as systemic, immune responses when delivered via mucosal routes, such as oral or intranasal administration [1]. The mucosal, needle-free, administration of vaccines can significantly decrease the need for syringes with their inherent added cost and risk of disease transmission, and it can increase compliance, and consequently the coverage of vaccination programs [3].

The use of live bacterial vectors for vaccine delivery was first described in the 1980s [4]. The earliest live bacterial vectors were derived from pathogenic microorganisms such as *Shigella, Salmonella*, *Listeria*, and *Mycobacterium*. In these cases, stable attenuated mutants that are no longer pathogenic but remain immunogenic, must first be selected or constructed, a step which is tedious and time consuming in many instances. In addition, attenuated pathogenic strains that retain a residual virulence level are unlikely to be suitable for the vaccination of partially immunocompetent individuals, such as infants, the elderly or immunocompromised patients [5]. These problems can be addressed by the

application of safe organisms such as lactic acid bacteria (LAB) as antigen delivery vehicles. Since time immemorial, LAB have been used for the fermentation and preservation of food products, particularly dairy products, fermented meat, and vegetables. Consequently, they have a long record of safe association with humans and human foodstuffs [6–8]. In addition, they exhibit adjuvant properties, and are weakly immunogenic [9]. LAB therefore represent attractive alternatives as antigen carriers and their use has mainly been focused on the construction of mucosal vaccines.

This mini-review describes recent advances in the development of LAB vaccines against bacteria, viruses, and parasites. We only review LAB vaccines designed using antigens other than tetanus toxin fragment C (TTFC), for which protection studies have been carried out. The results that have been obtained with TTFC are conclusively reviewed elsewhere [10, 11]. Here, we do not describe studies devoted only to the expression of antigens in LAB and/or the analysis of immunological responses in the absence of functional assays. BalB/c mice were mainly used in studies explained in this article to evaluate immunological responses and protection levels unless otherwise stated. In addition, the control group of mice refers to mice vaccinated with control bacteria, which were parental bacterial hosts carrying expression vectors without antigen encoding genes. Methods used for the immunization of the control group of mice were identical to those of the test group of mice inoculated with LAB vaccines. Any deviations from these conditions were indicated in the text.

Bacterial Targets of LAB Vaccines

Streptococcal Infections

LAB have been used as carriers for antigens of several bacterial pathogens (Table 1). The M protein of *Streptococcus pyogenes* (Group A streptococci, GAS) is a virulence factor that facilitates the colonization of mucosal tissue by this organism, enabling it to evade host immune surveillance. The C-repeat region (CRR) of the M protein contains sequences which are conserved among many serotypes of GAS [12]. CD1 mice vaccinated nasally, or both nasally and subcutaneously, with a lactococcal vaccine that expressed the CRR of the M protein from *S. pyogenes* on the cell surface, were challenged nasally with *S. pyogenes* to evaluate their protection levels against pharyngeal infection. Twenty-five percent of mice vaccinated nasally with the recombinant *L. lactis* were infected with *S. pyogenes*, whereas 70% of mice in the control group were infected. Furthermore, 20% of mice immunized with the combined regimen acquired the infection, whereas 70% of mice in the control group were infected. However, mice vaccinated subcutaneously with the recombinant lactococci were not protected against pharyngeal infection. Analysis of the correlation between the antibody response and protection suggested that the CRR-specific salivary IgA antibodies induced in mice following nasal immunization were both necessary and sufficient to prevent pharyngeal infection [13].

Recently, it has been demonstrated that *Streptococcus agalactiae* (Group B streptococci, GBS) produces three types of pili encoded on three genomic islands [14], and the expression of pilin island 1 in *Lactococcus lactis* resulted in the assembly of the pilus on the cell surface. Immunization of CD1 female mice subcutaneously or intranasally with the recombinant *L. lactis* led to the protection of their offspring against a lethal intraperitoneal challenge with GBS harboring pilus 1. However, the level of protection following mucosal immunization (40%) was lower than that observed after systemic immunization (75%). Only about 3–6% of mice in control groups immunized with parental lactococci survived the challenge with GBS harboring pilus 1. Subcutaneous immunization of CD1 female mice with recombinant lactococci expressing a hybrid pilus consisting of GBS pilus 1 and GBS pilus 2 components, conferred protection to the pups against intraperitoneal challenge with GBS strains carrying either of pilus 1 or pilus 2, to 54 and 42%, respectively [15]. Some serotype-independent pneumococcal surface proteins represent promising candidates for the design of vaccines against *Streptococcus pneumoniae*. One of these candidates, pneumococcal surface protein A (PspA), is a choline binding protein, essential for the full virulence of pneumococci [16]. Nasal immunization of C57BL/6 mice with *Lactobacillus casei* expressing the N-terminal region of PspA intracellularly, resulted in significant induction of anti-PspA IgG in the sera. However, no anti-PspA IgA was detected in the pooled saliva or nasal washes. The elicited anti-PspA IgG was able to bind to native PspA on the cell surface of pneumococcal strains, inducing complement deposition on the surface of pneumococci to different extents depending on the pneumococcal species. No survival was observed in the control group of mice after systemic challenge with a virulent pneumococcal strain. However, 33% of mice immunized with *L. casei* producing PspA survived the pneumococcal challenge [17]. Intranasal vaccination of CBA/ca mice with *L. lactis* expressing the N-terminal region of PspA intracellularly afforded 40% protection against respiratory challenge with *S. pneumonia*, which was better than that elicited with the purified antigen administered intranasally (15%) or by injection with alum (20%). This finding was attributed to a shift towards a Th1 response. Survival of 20% of mice in the control group after respiratory challenge suggested that *L. lactis* alone may contribute to nonspecific host immunity. In intraperitoneal challenge with *S. pneumoniae*, the

Table 1 Lactic acid bacteria vaccines

Target infective agent	Antigen	Antigen carrier (antigen cellular location)	Immunization route	Immune responses	Protection studies	Ref.
Bacterial targets						
Streptococcus pyogenes	CRR of M protein	*Lactococcus lactis* (cell surface associated)	Nasal, nasal and subcutaneous	Serum IgG, salivary IgA	Protection against pharyngeal infection	[13]
Streptococcus agalactiae	Pilin island 1	*L. lactis* (cell surface associated)	Nasal, subcutaneous	IgA in nasal/vaginal lavage (intranasal route), serum IgG	Protection of offspring of vaccinated mice after intraperitoneal lethal challenge with *S. agalactiae*	[15]
Streptococcus pneumoniae	PspA (N-terminal region)	*Lactobacillus casei* (cytoplasmic)	Nasal	Serum IgG	Protection against intraperitoneal inoculation of *S. pneumoniae*	[17]
S. pneumoniae	PspA (N-terminal region)	*L. lactis* (cytoplasmic)	Nasal	Serum IgG, IgA and IgG in lung lavage fluids	Protection against respiratory and intraperitoneal challenges with *S. pneumoniae*	[18]
S. pneumonia	PsaA	*L. lactis*, *L. casei*, *Lactobacillus plantarum, Lactobacillus helveticus* (cell surface associated)	Nasal	Serum IgG, IgA in saliva, nasal and bronchial washes	Reduction in *S. pneumoniae* recovery from nasal mucosa upon colonization challenge was only observed for *L. helveticus* expressing PsaA	[19]
S. pneumoniae	PppA	*L. lactis* (cell surface associated)	Nasal	IgG, IgA and IgM in serum and bronchoalveolar lavage fluid	Increased resistance to intraperitoneal and respiratory challenges with *S. pneumoniae*	[23]
Helicobacter pylori	UreB	*L. lactis* (secreted)	Oral	Serum IgG, fecal IgA	Protection against intragastric *H. pylori* challenge	[25]
H. pylori	UreB	*L. plantarum*, alanine racemase mutant of *L. plantarum* (cytoplasmic)	Oral	Serum IgG and IgA	Reduction in *Helicobacter felis* load following an intragastric *H. felis* challenge	[26]
Enterotoxigenic *Escherichia coli* (ETEC)	STLTB	*Lactobacillus reuteri* (secreted)	Oral	Serum IgG, fecal IgA	Protection against fluid influx response in gut following intragastric ETEC challenge	[28]
ETEC K99	K99	*L. casei* (cell surface associated)	Oral, nasal	Serum IgG, IgA in intestinal, vaginal, bronchoalveolar lavage fluids, fecal IgA	Protection against intragastric ETEC challenge	[30]
ETEC K99	K99	*L. acidophilus* (secreted and cell associated)	None performed	None	Inhibition and competition against adhesion of ETEC K99 to pig intestinal brush borders	[31]
ETEC	F41	*L. casei* (cell surface associated)	Oral	Serum IgG, IgA in intestinal and bronchoalveolar lavage fluids, fecal IgA	Protection against intragastric ETEC challenge	[32]
ETEC K88	F4	*L. lactis* (secreted and cell associated)	Oral	Serum IgG	Protection against intragastric ETEC K88 challenge	[33]
F18 fimbrial *E. coli*	FedF	*L. lactis* (secreted, cell surface associated)	None performed	None	Binding of recombinant *L. lactis* cells to porcine intestinal epithelial cells	[35]

Table 1 continued

Target infective agent	Antigen	Antigen carrier (antigen cellular location)	Immunization route	Immune responses	Protection studies	Ref.
Salmonella enteritidis	Flagellin	*L. casei* (cell surface associated)	Oral	Cellular immune responses	Protection against intragastric *S. enteritidis* challenge	[38]
Listeria monocytogenes	LLO	*L. lactis* (secreted)	Intraperitoneal, Oral	Serum IgG, cytotoxic T-lymphocyte (intraperitoneal route)	Protection against intraperitoneal *L. monocytogenes* challenge	[41]
Yersinia pseudotuberculosis	LcrV	*L. lactis* (secreted)	Nasal, oral	Serum IgG, IgA in bronchoalveolar lavages, cellular immune responses (intranasal route)	Protection against intravenous and intragastric *Y. pseudotuberculosis* challenge (intranasal route)	[44]
Bacillus anthracis	PA	*Lactobacillus acidophilus* (secreted)	Oral	Serum IgG, IgA in small intestine, T-cell immune responses	Protection against intraperitoneal lethal challenge with *B. anthracis* strain Sterne	[46]
Proteus mirabilis	MrpA	*L. lactis* (secreted, cell surface associated)	Nasal	Serum IgG and cellular immune responses (secreted form), serum IgA (cell surface associated form)	Reduction of *P. mirabilis* kidney colonization after *P. mirabilis* challenge using an ascending urinary tract infection mouse model	[48]
Bordetella pertussis	S1 subunit of PT (N-terminal region)	*Streptococcus gordonii* (cell surface associated)	Subcutaneous, intraperitoneal	Serum IgG	Neutralization of cytotoxic effect of PT on CHO cells, protection from the toxic effect of PT in leukocytosis- promoting and histamine sensitization assays	[50]
Borrelia burgdorferi	OspA, OspAα	*L. plantarum* (cell associated)	Oral	Serum IgG, IgA in gut luminal material	Absence of *B. burgdorferi* dissemination after challenge by infestation with *Ixodes* scapularis nymphs carrying *B. burgdorferi*	[52]
Erysipelothrix rhusiopathia	SpaA	*L. lactis* (secreted)	Oral, nasal	Serum IgG, fecal IgA	Protection against inoculation of *E. rhusiopathia* in inner thigh	[54]
Viral targets						
Human immunodeficiency virus (HIV)	V2–V4 loop of HIV envelop protein	*L. lactis* (cell surface associated)	Oral	Serum IgG, fecal IgA, cellular immune responses	Reduction in viral load after intraperitoneal challenge with vaccinia virus expressing HIV envelope protein	[56]
Severe acute respiratory syndrome (SARS)	S protein (B-cell epitope and N-terminal receptor binding domain)	*L. casei* (cell surface associated)	Nasal, oral	Serum IgG, IgA in intestinal and bronchoalveolar fluids	SARS-pseudovirus neutralizing antibodies elicited	[9]
Human apillomavirus (HPV-16)	L1	*L. casei* (cytoplasmic)	Subcutaneous	Serum IgG	Recognition of both assembled VLPs and disassembled VLPs by elicited antibodies	[60]

Table 1 continued

Target infective agent	Antigen	Antigen carrier (antigen cellular location)	Immunization route	Immune responses	Protection studies	Ref.
HPV-16	E7	*L. casei* (cell surface associated)	Oral	Serum IgG, IgA in intestinal lavages and vaginal secretions, cellular immune responses in vagina	Reduction in tumor size and increase in survival rate following injection of E7-expressing tumor cell line	[62]
HPV-16	E7	*L. lactis* (cell surface associated)	Nasal	Serum IgG, IgA in bronchoalveolar lavage fluids, cellular immune responses	Protection against tumor development and reduction in tumor size following injection of E7-expressing tumor cell line	[63]
Dengue virus	EDIII	*L. lactis* (cytoplasmic)	Oral, nasal	Serum IgG	Viral neutralizing antibodies elicited	[65]
Rotavirus	VP7	*L. lactis* (secreted)	Oral	Serum IgG	Viral neutralizing antibodies elicited	[67]
Rotavirus	VP4	*L. lactis* (cell surface associated)	Oral	Serum IgG, IgA in ophthalmic and vaginal washes, fecal IgA, cellular immune responses	Viral neutralizing antibodies elicited	[68]
Rotavirus	VP4, VP4-LTB	*L. casei* (cell surface associated)	Oral	Serum IgG, IgA in ophthalmic and vaginal washes, fecal IgA		[69]
Transmissible gastroenteritis virus (TGEV)	S protein (N-terminal globular domain)	*L. lactis* (cell surface associated)	Oral	Serum IgG, intestinal IgA	Viral neutralizing antibodies elicited	[71]
TGEV	S protein	*L. casei* strain Shirota (secreted)	Oral	Serum IgG, IgA in intestinal washes	Viral neutralizing antibodies elicited	[72]
Porcine epidemic diarrheal virus (PEDV)	Nucleocapsid protein of PEDV	*L. casei* (cell surfaced associated)	Oral, nasal	Serum IgG, colostrum IgG, IgA in intestinal and bronchoalveolar lavage fluids, colostrum IgA	Viral neutralizing antibodies elicited	[73]
Porcine parvovirus (PPV)	VP2	*L. casei* (secreted, cell surfaced associated)	Oral	Serum IgG, IgA in intestinal lavage	Viral neutralizing antibodies elicited	[75]
Parasitic targets						
Rodent malaria	Msp1 (C-terminal fragment)	*L. lactis* (cytoplasmic)	Oral	Serum IgG	Reduction in parasitemia after challenge with asexual blood stage *Plasmodium yoelli* parasites	[79]
Giardia lamblia	CWP2	*L. lactis* (cell surface associated)	Oral	IgA in intestinal lavage fluids	Reduction in cyst output following a challenge with *Giardia muris*	[81]

CRR C-repeat region, *PspA* pnemococcal surface protein A, *PsaA* pneumococcal surface antigen A, *PppA* pnemococcal protective protein A, *PT* pertussis toxin, *PA* protective antigen, *LcrV* low calcium response V antigen, *UreB* urease subunit B, $STLT_B$ fusion protein consists of heat stable enterotoxin and B subunit of heat labile enterotoxin, *K99, F41, F4, FedF* fimbrial adhesins, *LLO* listeriolysin O, *MrpA* structural protein of mannose-resistant proteus-like fimbriae, *OspA* outer surface protein A, *OspA*α mutated outer surface protein A, *SpaA* surface protective antigen, *L1* major capsid protein, *E7* oncoprotein, *EDIII* domain III of flaviviral E protein, *VP7* cell attachment protein, *VP4* hemagglutinin, *VP4-*LT_B fusion protein consists of VP4 and LT_B, *VP2* structural capsid protein, *Msp1* merozoite surface protein 1, *CWP2* major component of cyst wall structure

lactococcal nasal vaccine afforded resistance to the infection similar to that obtained with the injected purified antigen and both vaccines produced equivalent mean survival times (78 h), whereas the survival time of mice in the control group was 56 h. The survival time of the control mice after respiratory pnemococcal challenge was 57 h, whereas that of mice immunized nasally with the lactococcal vaccine was 90 h. When inactivated, the lactococcal vaccine still afforded protection against respiratory challenge with *S. pneumoniae* and the survival time of mice vaccinated with mitomycin C-inactivated lactococcal vaccine following respiratory pnemococcal challenge was 76 h. The reduced efficacy of the inactivated vaccine against respiratory challenge maybe explained by a shift towards a Th2 response [18]. In another study, pneumococcal surface antigen A (PsaA), a conserved membrane-anchored virulence factor [16], was expressed in different strains of LAB in a cell wall associated form. *Lactobacillus plantarum* and *Lactobacillus helveticus* were found to be more effective at inducing mucosal and systemic anti-PsaA immune responses than *L. casei* following intranasal vaccination of C57BL/6 mice. Because all three *Lactobacillus* strains expressed almost the same amount of PsaA and were also recovered from mice nasal mucosa in the same period (3 days), the observed differences among their respective antibody responses may reflect their different intrinsic adjuvant properties. PsaA expressed by *L. lactis* at 2×10^{-8} ng/colony forming unit (CFU), which was about 10% of PsaA amount produced by the *Lactobacillus* strains. The recombinant *L. lactis* remained in the nasal mucosa only 1 day after the inoculation. Therefore, the inability of *L. lactis* expressing PsaA to significantly induce serum IgG or secreted IgA in C57BL/6 mice can be explained by the low level of antigen production in this bacterium compared with the *Lactobacillus* strains and also its shorter persistence in the nasal mucosa. Intranasal inoculation of the mice with *L. lactis* expressing PsaA did not exert any effect on *S. pneumonia* recovery from the nasal mucosa upon colonization challenge, in comparison with inoculation of saline or the control bacterium. On the other hand, all the recombinant lactobacillus strains showed a significant reduction of *S. pneumonia* colonization when compared with the saline group ($10^{0.6}$–$10^{1.35}$ CFU). However, only *L. helveticus* expressing PsaA showed a significant reduction of *S. pneumoniae* colonization in relation to control *L. helveticus* (10 CFU) [19]. Among LAB, *L. lactis* and *Lactobacillus* strains have been mostly used for the construction of LAB vaccines. Selection of LAB strains for use as antigen carriers depends on their persistence in the host, capacity to express foreign antigens, and intrinsic adjuvanticity. *L. lactis* does not colonize the internal cavities of man or animals. Therefore, the use of lactobacilli which are able to colonize the cavities such as the gastrointestinal tract (GIT) transiently seems more advantageous than that of *L. lactis* for developing LAB vaccines because the longer persistence of lactobacilli in the host body may enhance immunological responses. On the other hand, the progress in the genetics of lactobacilli is more recent than that of lactococci. Furthermore, the availability of a commercial powerful gene expression system for *L. lactis*, nisin inducible gene expression system, urged many researchers to establish LAB vaccines based on lactococci. It has been reported that several LAB strains particularly strains from the genera *Lactobacillus* are able to act as adjuvants. This aspect should be considered when selecting a vaccine strain as it is a natural way to potentiate the immune reaction against heterologous antigens produced by recombinant LAB. It might be speculated that a high level of the antigen expression will not be necessary when using immunostimulatory LAB strains. However, studies have not yet been reported for comparison of the adjuvanticity of *L. lactis* and different lactobacilli. *L. casei* and *L. plantarum* are among lactobacillus strains which can colonize the GIT of human and mice and they show immunostimulatory properties [20, 21].

A pneumococcal surface protein, identified as pneumococcal protective protein A (PppA), is antigenically conserved among different serotype strains of *S. pneumoniae* [22]**.** Nasal immunization of Swiss albino mice with recombinant lactococci expressing PppA on the cell surface (*L. lactis* $PppA^+$) induced strong mucosal and systemic immune responses, which were significantly higher in young mice than in adult mice. Vaccination with *L. lactis* $PppA^+$ increased resistance to both systemic and respiratory infections with different pneumococcal serotypes. Adult mice immunized with *L. lactis* $PppA^+$ showed a survival percentage of 60% on day 21 after intraperitoneal challenge with a serotype 14 strain of *S. pneumoniae*. However, 100% of mice immunized with *L. lactis* without induction of PppA expression (*L. lactis* PppA) died between days 4 and 7 postchallenge. Vaccination of young mice with *L. lactis* $PppA^+$ enabled 70% of the animals to survive up to day 21, while 100% of young mice vaccinated with *L. lactis* PppA died on days 3–5 postchallenge. Furthermore, lung bacterial cell counts were negative after respiratory challenge with either serotype 14 or 23F pneumococcal strains in both adult and young mice vaccinated with *L. lactis* $PppA^+$. In contrast, in control animals, *S. pneumonia* serotypes used in the challenge were detected in lung cultures [23].

Gastrointestinal Infections

Gastric Helicobacter Infections

Almost all *Helicobacter pylori* strains produce a special urease that decomposes urea and forms ammonia, which is harmful to the gastric mucosa. Urease is composed of four

subunits and among them, subunit B (UreB) shows the strongest antigenicity among all *H. pylori* proteins [24]. The UreB antigen was expressed extracellularly in *L. lactis*. Oral inoculation of mice with the recombinant lactococci elicited an UreB-specific serum IgG response. Furthermore, a specific anti-UreB IgA response could also be detected in the feces of the immunized mice. Following intragastric challenge with *H. pylori*, *H. pylori* count in the stomachs of mice vaccinated with UreB producing lactococci was from 2×10^4 to 3×10^5 CFU/g stomach, whereas that of the control mice was from 3×10^4 to 4×10^5 CFU/g stomach [25]. In another study, the potential of both wild type *L. plantarum* and a mutant of *L. plantarum* affected in its cell-wall composition as a result of inactivation of its alanine racemase gene was evaluated for delivering UreB antigen to the GIT in a mouse model of *Helicobacter felis* infection. Mice vaccinated intragastrically with each of the recombinant lactobacillus strains and also a mixture of recombinant UreB and cholera toxin (rUreB/CT), were orally challenged with *H. felis*. *H. felis* load in the stomachs of mice immunized with UreB producing *L. plantarum* (4.9×10^4 CFU/g of gastric tissue) was slightly lower than that of the control mice (5.1×10^4 CFU/g of gastric tissue). *H. felis* load in mice immunized with UreB producing mutant *L. plantarum* was 2.9×10^4 CFU/g of gastric tissue. These results indicated that the alanine racemase mutant of *L. plantarum* promoted a more pronounced reduction in *H. felis* load in the stomachs of the immunized mice than its wild type counterpart. *H. felis* load in mice immunized with rUreB/CT was 7.2×10^3 CFU/g of gastric tissue. Therefore, the highest degree of protection against *H. felis* was achieved with rUreB/CT [26].

Escherichia coli Induced Diarrhea

The enterotoxins of enterotoxigenic *Escherichia coli* (ETEC) are classified as heat labile (LT) or heat stable (ST) and they induce diarrhea in the host by a mechanism of villous hypersecretion. LT consists of a single A domain and five B subunits. The B subunit (LT_B) binds predominantly to the GM1 ganglioside acting as a receptor on eukaryotic cell surfaces [27]. A fusion protein consisting of green fluorescent protein (GFP), ST, and LT_B (GFP–ST–LT_B) was expressed extracellularly in *Lactobacillus reuteri*. Oral inoculation of the recombinant lactobacilli in mice elicited significant induction of serum IgG and mucosal IgA against ST-LT_B. When mice vaccinated with the recombinant *L. reuteri* were intragastrically challenged with ETEC, they were fully protected against the fluid influx response in the gut. In contrast, mice in the control group displayed massive fluid accumulation after the challenge [28].

K99, K88 (F4), 987P, FY, and F41 are the fimbrial adhesins that are important in ETEC infection of neonatal animals. F41 is less prevalent than K99, F4 or 987P and is usually accompanied by K99. The K99 fimbrial antigen is one of the major adherence factors found on ETEC of neonatal calves and pigs [29]. Immunization of mice intranasally or orally with recombinant *L. casei* cells expressing the K99 fimbrial antigen on the cell surface resulted in high levels of serum IgG and mucosal IgA against ETEC K99. More than 80% of mice immunized with the recombinant lactobacilli were protected against ETEC following oral challenge with the standard-type ETEC C83912, regardless of the immunization route. However, 100% mortality was observed in the control mice after the challenge [30]. Moreover, *L. acidophilus* expressing the K99 fimbrial adhesin in secreted and cell-associated forms showed inhibition and competition with ETEC K99 adhesion to the intestinal brush borders of pigs in a dose-dependent manner [31].

Oral inoculation of mice with *L. casei* expressing the F41 fimbrial adhesin on the cell surface elicited mucosal IgA at the site of inoculation, as well as remote mucosal sites. High levels of systemic specific IgG were also induced. While more than 80% of mice vaccinated with the recombinant *L. casei* were protected against oral challenge with the standard-type ETEC C83919, 100% of the control mice died after the challenge [32].

ETEC strains that produce F4 fimbriae on their surfaces commonly induce diarrhea in piglets. FaeG is the structural subunit of F4 fimbriae [29]. It was shown that oral administration of recombinant *L. lactis* cells expressing F4 fimbrial adhesin in secreted and cell-associated forms in ICR mice could successfully induce the secretion of F4-specific IgG antibodies. Following intragastric challenge with the standard-type ETEC C83549, mortality rate of mice vaccinated with the recombinant lactococci was 10%, while that of the control group of mice was 50% [33].

F18 fimbrial *E. coli* strains are associated with porcine postweaning diarrhea and pig edema disease. Adherence of F18 fimbrial *E. coli* to porcine intestinal epithelial cells is mediated by the FedF adhesin of F18 fimbriae [34]. For the development of a mucosal vaccine against porcine postweaning diarrhea and edema disease, different expression cassettes for the display of FedF on the cell surface of *L. lactis* were constructed. Preliminary attempts to express the entire FedF protein as a fusion protein in *L. lactis* resulted in inefficient secretion and degradation of the adhesin. Therefore, only those regions of FedF required for binding specificity to porcine intestinal epithelial cells, were used in the construction of cell surface display systems. Initially, recombinant *L. lactis* clones secreting the partially overlapping receptor binding domains of FedF (42 and 62 amino acid residues) as fusions with the H domain of *L. lactis* proteinase P (PrtP) protein were prepared using two different signal peptides. Substantially higher levels of

the fusion proteins (four- to six-fold) were secreted by the clones possessing *Lactobacillus brevis* SlpA signal peptide than by those possessing *L. lactis* Usp45 signal peptide. For the construction of surface display systems, the secreted proteins were anchored to the cell wall of *L. lactis* via the cell wall anchoring region of the lactococcal acetyl muramidase A (AcmA) protein or that of the lactococcal PrtP protein. Three groups of expression vectors with *prtP* spacer sequences of 0.6, 0.8, and 1.5 kb were also designed. Whole-cell ELISA for the detection of cell surface exposure of the FedF receptor binding regions showed that the AcmA anchor performed significantly better than the PrtP anchor, particularly in a *L. lactis* mutant devoid of the extracellular housekeeping protease, HtrA. Among the cell surface display systems possessing the AcmA anchor, only those with the longest PrtP spacer resulted in efficient binding of the recombinant *L. lactis* cells to porcine intestinal epithelial cells [35].

Salmonellosis, Listeriosis, and Yersiniosis

It has been reported that the flagellin of *Salmonella* has significant vaccine potential because it is the only surface antigen of *Salmonella* detected to have a mitogenic stimulatory effect on lymphocytes [36]. Bacterial flagellins can also induce innate immune responses through their interaction with Toll-like receptor 5 (TLR5) [37]. Recombinant *L. casei* cells expressing the flagellin of *Salmonella enterica* serovar *enteritidis* on their cell surface (LCF) were constructed. *S. enteritidis* count in the spleen from C3H-HeJ mice immunized with the recombinant *L. casei* was 10^{-1} CFU less than that from the control mice after intragastric challenge with *S. enteritidis*. There was no significant difference in the level of protection after immunization with the recombinant lactobacilli compared with the free flagellin isolated from *S. enteritidis*, although the amount of flagellin carried by LCF was less than that of the free flagellin. The immunization of mice with the recombinant lactobacilli did not result in antigen-specific antibody responses in either feces or sera but did induce the release of interferon (IFN)-γ on restimulation of primed lymphocytes ex vivo. These results suggested that the protective efficacy provided by flagellin-expressing *L. casei* was mainly attributable to cell-mediated immune responses. When the levels of IFN-γ produced by primed and flagellin-restimulated lymphocytes were compared between the recombinant *L. casei* cells expressing flagellin on their cell surface, and a mixture of the purified flagellin and normal *L. casei*, the results indicated that the *Lactobacillus* strain functions as an adjuvant only when the flagellin is expressed on the cell surface [38].

Listeriolysin O (LLO) is the major virulence factor of *Listeria monocytogenes*. Upon phagocytosis, secreted LLO forms pores in the phagosomal membrane enabling access of the bacterium to the cytoplasm of the host cell [39]. In addition to the role of LLO in the intracellular pathogenesis of *L. monocytogenes*, it is also a major immunodominant listerial antigen and anti-listerial protective immunity is dependent on cytotoxic $CD8^+$ cell-mediated immunity against LLO [40]. To evaluate the potential of *L. lactis* as a vaccine platform against listeriosis, LLO was expressed in this bacterium using either a constitutive promoter (P44) or an inducible promoter (PnisA) in intracellular and secreted forms. LLO was not expressed from the constitutive intracellular cassette. However, all other constructs were capable of efficient expression of LLO in an active form with hemolytic activity. In contrast to *L. monocytogenes* cells, the recombinant lactococcal strains were unable to grow in J774 macrophage-like cells. The recombinant strains were tested in a murine model to evaluate the vaccination efficacy against *L. monocytogenes* infection. $CD8^+$ T-lymphocytes specific for the $LLO_{91–99}$ epitope were detected when strains were administered via the intraperitoneal, but not the oral route. *L. lactis* cells expressing LLO intracellularly from the inducible promoter were not able to elicit serum antibody responses. However, the two other lactococcal strains secreting LLO were able to induce significant serum antibody responses when administered by the intraperitoneal route, but not the oral route. Following intraperitoneal challenge with *L. monocytogenes*, the greatest protection against listerial organ colonization was attained in mice immunized with the inducible LLO-secreting *L. lactis* cells via the intraperitoneal route and this correlated with the highest LLO expression level in this bacterial strain. Listerial count in livers and spleens of mice immunized with *L. lactis* was $10^{4.5}$ and 10^5 CFU/organ, respectively, while that of mice immunized with the inducible LLO-secreting *L. lactis* was 10^2 CFU/organ [41]. The cellular location of antigens can influence the strength of elicited immunological responses. Exported antigens (secreted into the medium or cell surface anchored) are better recognized by the immune system than those produced intracellularly. Furthermore, intracellular production of antigens may limit their in vivo release. Halling-Brown et al. showed that several vaccine antigens experimentally known to confer immune protection against bacterial pathogens in at least one animal model of human diseases were either secreted from the bacterium or located on the cell surface. This confirms the validity of the assumption that the most immunogenic proteins will be those that are most quickly and readily visible to the host immune system. However, compared with intracellular antigens, the exported antigens are more susceptible to the proteolytic degradation when they are exposed to the host body fluids [42].

A promising candidate for vaccination against *Yersinia* is low calcium response V (LcrV) antigen. LcrV circumvents

host innate immunity by inducing the production of anti-inflammatory cytokine interleukin (IL)-10 and by participating in the secretion and translocation of *Yersinia* outer protein toxins into phagocytes [43]. Following intranasal delivery, recombinant *L. lactis* cells secreting the *Yersinia pseudotuberculosis* LcrV (LL-LcrV) antigen induced specific systemic and mucosal antibody responses and cellular immunity in vaccinated mice. Ninety percent of animals vaccinated with LL-LcrV were protected against oral *Y. pseudotuberculosis* challenge, whereas only 10% of the control mice survived the challenge. Furthermore, mice immunized with LL-LcrV survived completely following intravenous challenge of *Y. pseudotuberculosis*, which killed 100% of the control mice. However, no specific systemic or mucosal immune responses were triggered by oral immunization of mice with the recombinant *L. lactis* and this route did not confer any protection against oral *Y. pseudotuberculosis* challenge. Intranasal vaccination of mice with the recombinant lactococci did not show any protection against intradermal *Yersinia pestis* challenge and the application of LcrV as an antigen did not confer any cross protection between closely related *Yersinia* species [44].

Other Bacterial Infections

Bacillus anthracis produces two exotoxins, which share a common cell receptor binding protein, protective antigen (PA), originally identified as a protein providing protection against infection in experimental animals [45]. A vaccine strategy was established utilizing *Lactobacillus acidophilus* to deliver PA to dendritic cells (DCs) via the use of a specific DC-targeting peptide. Oral vaccination of A/J mice with *L. acidophilus* expressing the fusion of PA to the N-terminus of a DC-targeting peptide in its secreted form, activated mucosal antigen-presenting cells, which in turn induced anti-PA neutralizing antibodies, IgA secretion, and T-cell immunity against *B. anthracis*. Seventy-five percent of the immunized mice were protected against intraperitoneal challenge with *B. anthracis* strain Sterne. By contrast, *L. acidophilus* expressing PA fused to a control peptide, which does not improve DC binding, showed a lower level of protection (25%). The current anthrax vaccine, rPA adsorbed to alhydrogel, given in a single subcutaneous injection, protected 80% of immunized mice from a lethal challenge with *B. anthracis* strain Sterne. These results highlighted the efficacy of employing LAB in vaccine platforms, whereby microbial immunogens can be delivered to mucosal DCs using small DC-targeting peptides, which may enhance antigen uptake by DCs [46].

Several potential virulence factors have been suggested for uropathogenic *Proteus mirabilis* including mannose-resistant proteus-like fimbriae (MR/P). MR/P fimbriae may contribute to colonization of the urinary tract by *P. mirabilis* [47]. The structural protein of MR/P, MrpA, represents a promising candidate antigen for mucosal vaccination. MrpA was efficiently expressed in *L. lactis* in cell wall-anchored or secreted forms. CD-1 mice were used for immunological and protection studies. Significant induction of the serum IgG response was only observed in mice intranasally immunized with the recombinant lactococci secreting MrpA and significant induction of the serum IgA response was only observed in mice intranasally immunized with the recombinant lactococci expressing MrpA in its cell wall-anchored form. However, induction of the mucosal antibody response was not observed for any of these strains. In addition, only *L. lactis* secreting MrpA was able to induce significant IFN-γ production. Protection assays showed that *P. mirabilis* kidney colonization in mice immunized with each of the recombinant *L. lactis* cells was about 30% less than that of mice treated with phosphate buffer saline (PBS) [48]. Although this study indicated the influence of the antigen cellular location on the type of induced immunological responses, more extensive studies are required to elucidate the correlation between the antigen cellular localization and type of immunological responses.

Pertussis toxin (PT) is an AB toxin consisting of an A-protomer (S1 subunit), as the toxic subunit, and a B-oligomer, as the pentamer that binds to the surface receptors on eukaryotic cells and translocates the toxic subunit across the cell membrane [49]. The S1 subunit is considered as the major immunogen for eliciting neutralizing antibodies against PT. *Streptococcus gordonii* cells expressing the N-terminal 179 amino acids of the S1 subunit of PT from *Bordetella pertussis* on the cell surface, were capable of eliciting serum immune response in New Zealand white rabbits when injected subcutaneously. However, the induced anti-S1 antibody showed a weak ability to neutralize the cytotoxic effect of PT on Chinese hamster ovary (CHO) cells. Mice immunized intraperitoneally with the recombinant heat-killed *S. gordonii* were fully protected from the toxic effect of PT injected intraperitoneally in leukocytosis-promoting and histamine sensitization assays, whereas only 17% of the nonimmunized mice survived the challenge [50].

It has been shown that high titers of antibodies to outer surface protein A (OspA) of the tick-borne spirochete *Borrelia burgdorferi* prevented *Borrelia* infection in mice [51]. A mutant of OspA was constructed in which the autoantigen sequence of OspA was replaced with the analogous region from a non-arthritogenic European species (*Borrelia afzelii*) that possesses these compensatory changes. The C-terminal of the mutant OspA was further stabilized by replacing it with the analogous region from *B. afzelii*. Significant mucosal IgA and serum IgG responses were induced in C3H-HeJ mice vaccinated orally

with *L. plantarum* expressing either the wild type (LpA) or mutant OspA (LpAα). Protection of the vaccinated mice against infection was determined by the absence of *B. burgdorferi* dissemination after challenge with *Ixodes scapularis* nymphs carrying *B. burgdorferi*. Mice immunized with either LpA or LpAα were free of the spirochete, whereas the control mice were infected with *B. burgdorferi* [52].

Surface protective antigen (SpaA) of *Erysipelothrix rhusiopathia* is secreted in its mature form and then binds to the bacterial cell surface via its C-terminal region which comprises tandem GW repeats. The N-terminal region of SpaA is responsible for eliciting protective immunity against *E. rhusiopathia* [53]. Oral or nasal vaccination of dd-Y mice with recombinant lactococci producing SpaA in a secreted form elicited antigen-specific serum IgG and fecal IgA responses. The immunized mice were fully protected against challenge with *E. rhusiopathia* in the inner thigh. In contrast, none of the mice in the control group survived the challenge [54].

Viral Targets of LAB Vaccines

The development of safe, effective, and inexpensive vaccines capable of inducing both mucosal and systemic immunity is highly desirable to prevent the spread of human immunodeficiency virus (HIV). The HIV type 1(HIV-1) envelope protein mediates sequential binding to CD4 and a coreceptor, with these interactions triggering conformational changes in the envelop protein that result in fusion between the viral and cellular membranes [55]. A recombinant *L. lactis* vector expressing the V2–V4 loop of the envelope protein of HIV-1 on the cell surface (IL1403-pHIV) was constructed. The V2–V4 loop is the most immunogenic epitope of the envelope protein. Oral immunization of mice with IL1403-pHIV, using cholera toxin as an adjuvant, induced high levels of HIV-specific serum IgG and fecal IgA antibodies. Furthermore, vaccination induced strong HIV-specific cell-mediated immunity. The number of IFN-γ-secreting cells was significantly greater in mice vaccinated with IL1403-pHIV than in the control mice. The absolute number of HIV-specific IFN-γ-secreting lymphocytes was higher in the intestinal lymph nodes than in the spleen of IL1403-pHIV-immunized mice, suggesting that oral immunization was particularly effective at inducing mucosal immunity. IL1403-pHIV cells were able to infect immature DCs to induce HIV-specific cytotoxic T-lymphocyte responses. In an intraperitoneal challenge with vaccinia virus expressing the envelope protein, viral loads in the IL1403-pHIV-immunized mice were 350-fold lower than those in the control mice [56].

The spike (S) protein of severe acute respiratory syndrome-associated coronavirus (SARS-CoV), a type I envelope glycoprotein, is not only responsible for receptor binding and virus fusion, but is also considered a major target for vaccine development against SARS because of its ability to elicit virus neutralizing antibodies [57]. Two segments of the SARS-CoV S protein, including B-cell epitopes and the receptor binding domain located at the N-terminus, were expressed on the surface of *L. casei* cells. C57BL/6 mice were immunized either orally or intranasally with the recombinant *L. casei*. For the oral route, 5×10^9 recombinant *L. casei* cells in 100 μl suspension were administered daily via intragastric lavage on days 0–4, 7–11, 21–25, and 49–53. A lighter schedule was employed for nasal immunization and 2×10^9 recombinant *L. casei* cells in a 20 μl suspension were inoculated daily into the nostrils of lightly anesthetized mice on days 0–2, 7–9, 21, and 49. Oral or intranasal inoculation of the mice with the recombinant *L. casei* cells resulted in high levels of serum IgG and mucosal IgA responses as detected in the intestinal and bronchoalveolar lavage fluids. The induced antibodies mounted potent neutralizing activities against SARS-pseudovirus. Antiviral activity of serum IgG in orally immunized mice was 72%, which was about 17% more than that in intranasally immunized mice. No antiviral activity was observed in the serum IgG of mice in the control groups. Neutralizing activity of anti-SARS IgA in intestinal lavage fluids of mice immunized orally was 63%. In contrast only a low level of neutralizing activity was observed in bronchoalveolar lavage fluids (8%). In intranasally immunized mice, antiviral activity of anti-SARS IgA in bronchoalveolar lavage fluids was at a moderate level (20%). However, very little, if any, was detected in intestinal lavage fluids. In the control groups, no antiviral activity was detected in bronchoalveolar lavage fluids and a low level of neutralizing activity was observed in intestinal lavage fluids (9%) [9].

More than 50% of cases of human papillomavirus (HPV)-related cervical cancer have been associated with HPV type 16. The HPV-16 L1 major capsid protein can spontaneously self assemble into virus-like particles (VLPs), which are structures that are morphologically similar to native papillomavirus [58]. HPV-16 L1 expressed in the cytoplasm of *L. casei* (*L. casei*/L1), was able to self-assemble into VLPs intracellularly. However, the *L. casei*/L1 cells exhibited different VLP production patterns. While a few cells exhibited large amounts of VLPs and electron-dense bodies containing VLP aggregates, other cells exhibited reduced production of VLPs. Moreover, the sizes of the *L. casei*-produced VLPs were heterogeneous, ranging from 30 to 60 nm in diameter, which were in the same size range with HPV-16 L1 VLPs used in the commercial vaccines [59]. Interestingly, some *L. casei*/L1 cells were up to 10 μm in length, whereas normal *L. casei* cells range from 2 to 4 μm in length. Furthermore, the growth rate of *L. casei*/L1 cells was lower than that of

the wild type strain. VLPs expressed by *L. casei* were recognized by the anti-HPV-16 VLP conformational antibody (H16.V5), which suggested that the L1 protein produced by *L. casei* self-assembled into VLP-like structures, which displayed the HPV-16 VLP-specific conformational epitopes. These epitopes are important for eliciting virus neutralizing antibodies. Anti-L1 IgG was detected in the sera of mice immunized subcutaneously with *L. casei*/L1. These sera recognized both assembled VLPs and disassembled VLPs, indicating that L1 expressed in *L. casei* existed within VLPs, as well as unfolded L1 protein. However, nasal inoculation of mice with *L. casei*/L1 did not produce any mucosal or systemic humoral responses against L1, which can be attributed to insufficient levels of the L1 protein presented by the recombinant lactobacilli at mucosal sites [60].

Vaccines based on virion capsid proteins, such as L1, are unlikely to be applicable therapeutically in infected patients since the capsid proteins are absent in cervical cancer (CxCa) cells. By contrast, the HPV-16 E7 oncoprotein is constitutively expressed in CxCa cells during malignant progression of HPV-16-induced cervical lesions, and may therefore be a more effective target for cancer immunotherapy [61]. The HPV-16 E7 antigen was expressed on the surface of *L. casei* cells by employing poly-γ-glutamic acid synthetase complex A (PgsA) as a cell surface anchoring motif (*L. casei*-PgsA-E7). Oral immunization of C57BL/6 mice with *L. casei*-PgsA-E7 induced both systemic and mucosal (intestinal/vaginal IgA) HPV-16 E7-specific humoral immune responses, which were significantly enhanced after boosting. In addition, both systemic and local cell-mediated immune responses were elicited in the mouse vagina. In an E7-based mouse tumor model, the animals receiving *L. casei*-PgsA-E7 orally generated antitumor effects against HPV-16 E7 expressing murine tumors and the average tumor size in the vaccinated animals was 54% smaller than that in the control mice. In addition, the survival rate of mice immunized with the recombinant lactobacilli was 50%, whereas all of the control mice died. However, the antitumor effect of *L. casei*-PgsA-E7 was not very high, which maybe caused by the low immunogenicity of mucosal immunization and the low level of E7 antigen displayed on *L. casei* cells (100–300 ng/10^9 cells) [62]. *L. lactis* was also engineered to express HPV-16 E7 on the cell surface (LL-E7) and its coadministration with another lactococci secreting IL-12 (LL-IL-12) was investigated for the immunization and immunotherapy of HPV-related CxCa. IL-12 is a heterodimeric cytokine that induces Th1 responses, enhances cytotoxic T-lymphocyte (CTL) maturation, promotes natural killer (NK) cell activity, and induces IFN-γ production. C57BL/6 mice were vaccinated intranasally with LL-E7 and were then challenged by injection of tissue culture number 1 (TC-1) tumor cells. All mice vaccinated with *L. lactis* alone (LL) died following the development of aggressive tumors. In contrast, 35% of mice vaccinated with LL-E7 remained tumor free and the tumor median size in the remaining 65% tumor-bearing mice was 2-fold lower than that measured in LL immunized mice. Coadministration of LL-IL-12 with LL-E7 resulted in significantly higher antitumor activities as 50% of the inoculated mice remained tumor free and the tumor median size in the remaining tumor-bearing animals was 30% of that in LL-E7 immunized mice. Antitumoral activity elicited by covaccination with LL-E7 and LL-IL-12 appeared to be long-lasting as when the tumor free animals were rechallenged 3 months later with TC-1 cells, they remained tumor free for up to 6 months. The cell-mediated response elicited by mucosal covaccination with LL-E7 and LL-IL-12 was $CD4^+$- and $CD8^+$-dependent and it was likely responsible for tumor protection. However, these experiments were only performed on mice sacrificed 7 days after the last injection and not on long-term protected mice. To investigate the therapeutic effects of the coadministration of LL-E7 and LL-IL-12, mice were first challenged with the TC-1 tumor cell line before the initiation of immunotherapy. Once 100% of the mice had palpable tumor, the immunotherapy was started. Only LL-E7/LL-IL-12 treatment resulted in total tumor regression in 35% of the immunized animals. Moreover, the tumor median size in the remaining tumor-bearing mice was 2- and 4-fold lower than that measured in mice treated with LL-E7 and LL, respectively. In contrast, no tumor regression was observed in mice treated with LL or LL-E7 alone. Mice immunized with LL-E7/LL-IL-12 also exhibited both systemic and mucosal humoral responses, which were induced at higher levels than those in mice vaccinated with LL-E7 or LL [63].

The flaviviral E protein is the major envelope protein of dengue virus. This protein is mostly glycosylated and is essential for viral infectivity via receptor-mediated endocytosis [64]. Domain III of the E protein (EDIII) functions as the cellular receptor binding domain for dengue virus in both human and mosquito cells. The EDIII domain from dengue virus serotype 2 was expressed in the cytoplasm of cells of *L. lactis* strain MG1363 (LL-EDIII). The antigen was produced in a soluble and non-degraded form up to late exponential phase, at which point the bacteria were used to immunize mice. Because dengue virus is a blood-borne pathogen, only the systemic antibody responses were determined in both Balb/c and C57BL/6 mice. Nasal or oral administration of LL-EDIII resulted in the induction of anti-EDIII antibodies in the sera of vaccinated mice. However, the antibody responses were dependent on the mouse strain and the route of administration. In addition, heterogeneity was observed in the level of antibody responses in the animals within the same group immunized

with LL-EDIII and protective immune responses were only elicited in up to 50% of the immunized animals. The protective potential of the immune sera was measured using a plaque reduction neutralization test. Pooled sera from Balb/c and C57BL/6 mice vaccinated with the heat inactivated virus were able to inhibit the viral replication by up to 62 and 70%, respectively, while pooled sera from the control animals exhibited no neutralizing activity against the virus. The immune sera from the mice inoculated orally with LL-EDIII showed higher neutralizing activities compared with the nasally-immunized animals. Antiviral activities of sera from Balb/c and C57BL/6 mice inoculated nasally with LL-EDIII were up to 20 and 43%, respectively. However, the viruses neutralizing activities of sera from Balb/c and C57BL/6 mice inoculated orally with LL-EDIII, were up to 45 and 78%, respectively. In addition, their results indicated that the level of anti-EDIII IgG antibody did not directly correlate with the plaque neutralizing activity [65].

Rotavirus particles possess outer capsids composed of VP7, the cell attachment protein, and VP4, the viral hemagglutinin. Both of these components are able to elicit virus neutralizing antibodies [66]. VP7 was expressed in *L. lactis* in secreted or cell wall-anchored forms. Intragastric immunization of mice with the lactococcal strains expressing VP7 resulted in the induction of antigen-specific antibody responses in some of the animals. The anchoring of VP7 to the cell wall of *L. lactis* resulted in a reduced ability to induce antibodies, and these antibodies did not show neutralizing activity against rotavirus infection of monkey kidney (MA104) cells. By contrast, the recombinant lactococci secreting VP7 were more immunogenic and sera from the immunized mice were able to neutralize the virus infectivity, whereas sera from mice inoculated with parental lactococci were not [67]. Oral immunization of mice with *L. lactis* cells expressing VP4 on the cell surface resulted in the induction of serum IgG and mucosal IgA. The induced antibodies inhibited the cytopathicity of porcine rotavirus infection on MA104 cells in a dose-dependent manner. In contrast, sera from the control mice could not inhibit the virus-induced cytopathocity. Splenic cells from the mice immunized with VP4-producing *L. lactis* cells significantly increased following specific stimulation with the purified porcine rotavirus VP4 protein compared with the proliferation of spleen cells harvested from the control mice [68]. Oral immunization of mice with *L. casei* cells expressing VP4 or a fusion of VP4 to LT_B (VP4-LT_B) on the cell surface also triggered induction of the serum IgG and mucosal IgA responses. The anti-VP4 IgG levels in the sera of mice immunized with each of the recombinant *L. casei* cells were similar. Reduction of the virus cytopathic effect observed using serum IgG of mice fed with *L. casei* expressing VP4 and *L. casei* expressing VP4-LT_B in relation to that of the control mice was 50 and 56%, respectively. In addition, the *L. casei* cells expressing VP4-LT_B induced a higher level of mucosal IgA responses in the vaccinated animals than *L. casei* cells expressing VP4 alone. This result demonstrated the specific mucosal adjuvanticity of LT_B [69].

The spike (S) protein of transmissible gastroenteritis virus (TGEV) is one of the main structural proteins of the virus, involved in attachment to host cells and the induction of fusion between viral and cellular membranes in the initial stage of infection. The S protein has been used mainly for the induction of protective immunity against TGEV and the relevant neutralization epitopes have been mapped to the N-terminal domain of this protein [70]. Recombinant *L. lactis* cells expressing the N-terminal globular domain of the S protein on the cell surface were constructed. Oral immunization of mice with the recombinant lactococci resulted in the production of intestinal mucosal IgA and serum IgG responses. The induced antibodies demonstrated neutralizing effects against TGEV infection in a plaque reduction assay. Reduction in the virus plaque number obtained using sera and intestinal lavages of mice immunized with the recombinant *L. lactis* in relation to that of the control mice was 22 and 36%, respectively [71]. *L. casei* strain Shirota expressing the S protein of TGEV in its secreted form was also examined as a mucosal vaccine against TGEV. Oral immunization of mice with recombinant lactobacilli that constitutively expressed the S protein induced both mucosal and systemic immune responses against TGEV and the induced antibodies exhibited neutralizing effects on virus infection in a plaque reduction assays. Reduction in the number of the virus plaques obtained using intestinal lavages or sera of mice fed with the recombinant *L. casei* compared with that of the control mice was about 15% [72].

Nucleocapsid proteins are important for inducing immune responses to porcine epidemic diarrheal virus (PEDV). Oral or intranasal inoculation of pregnant sows and C57BL/6 mice with *L. casei* cells expressing the nucleocapsid protein of PEDV on the cell surface elicited serum IgG and mucosal IgA responses. Only background levels of immunological responses were detected in control animals. Following oral vaccination, the level of IgA in the colostrum of pregnant sows was greater than that of IgG. After suckling the colostrum secreted from the sows previously inoculated with the recombinant *L. casei* cells, IgG levels in the piglets were increased slightly. However, the IgG purified from the sera of the immunized pregnant sows and the piglets after suckling the colostrum, did not inhibit plaque formation by PEDV. Similarly, the colostrum IgA did not inhibit plaque formation [73].

VP2 is one of the structural polypeptides of the porcine parvovirus (PPV) icosahedron protein coat, which encompasses the major antigenic domains of PPV and may induce

neutralizing antibodies for inhibition of PPV infection [74]. Recombinant strains of *L. casei* were constructed, which produced VP2 in two cellular locations, either in the external milieu or anchored to the cell surface. Following oral administration, both of these recombinant strains were able to elicit both PPV-specific systemic and mucosal antibody responses in mice. However, the highest antibody titers were obtained with *L. casei* producing the VP2 protein in the extracellular milieu. The induced antibodies demonstrated neutralization activity against PPV infection in a plaque reduction assay. Intestinal lavages and sera from parental *L. casei* were used as negative controls to determine the plaque reduction percentage. A 98% reduction in the number of plaques was observed using intestinal lavages or sera of mice vaccinated with *L. casei* expressing VP2 in external milieu. The plaque reduction percentage using intestinal lavages and sera of mice vaccinated with *L. casei* displaying VP2 on the cell surface was 48 and 80%, respectively [75].

Parasitic Targets of LAB Vaccines

Merozoite surface protein 1 (MSP1) is present in all species of *Plasmodium*, and has been widely studied as a major vaccine candidate against malaria. The carboxy terminal 19-kDa fragment of MSP1 ($MSP1_{19}$) has been shown to be immune-effective against challenge with the *Plasmodium* parasite [76–78]. $MSP1_{19}$ of *Plasmodium yoelii* was expressed intracellularly in *L. lactis*. Oral administration of the recombinant lactococci to BALB/c mice resulted in more than 10-fold reduced parasitemia compared with control mice inoculated with *L. lactis* cells after challenge with the asexual blood stage of the *P. yoelli* parasites. However, in the case of the C57BL/6 mice, all members of the control group of mice immunized with normal *L. lactis* cells, died after infection with *P. yoelii*, whereas all of the mice vaccinated with the recombinant *L. lactis* survived and the parasite was eradicated [79].

CWP2 is the major component of the cyst wall structure of *Giardia lamblia*. Newly expressed CWP2 has a molecular mass of 39 kDa and possesses a hydrophobic N-terminal signal peptide and a 13 kDa carboxy end tail region. However, mature CWP2, detected within the cyst wall, migrates at 26 kDa since it lacks the 13 kDa tail portion to the post translational processing. In addition, the mature form of CWP2 contains the relevant B-cell epitope [80]. Oral immunization of mice with recombinant lactococci expressing mature CWP2 on the cell surface resulted in the induction of CWP2-specific mucosal IgA antibodies in the intestine. In a challenge experiment with *Giardia muris*, mice immunized with the recombinant lactococcal cells showed 63% less cyst output than the non-immunized control mice [81].

Concluding Remarks

LAB appear to be attractive vehicles for vaccine delivery, able to induce both mucosal and systemic immune responses. However, it is important to increase the potency of these systems to obtain high levels of protective immune responses at lower doses. The ability of LAB carriers to induce protection against infective agents depends on sufficient antigen delivery in vivo, and optimization of LAB carriers requires selection of the most suitable LAB strains, mode of antigen presentation (cytoplasmic, secreted or cell surface exposed), and immunization regimen (route, dose and timing). The coadministration of immunoregulatory cytokines with LAB vehicles, in addition to the coexpression of antigens with adjuvants and antigen- presenting cells targeting peptides in LAB vehicles, represent valuable steps towards increasing the efficiency of these vaccines. Other developments involve the improvement of genetic tools. Although there are sequences currently available for the secretion and anchoring of proteins and peptides in LAB, these processes are often inefficient, resulting in reduced levels of secreted and surface-exposed antigens. The characterization of secretion, anchoring, and regulatory signals from genome sequences should improve these features. Furthermore, the low transformation efficiency of LAB is a major obstacle in preparing LAB vaccines and the establishment of efficient transformation protocols is therefore necessary. Methods should also be devised to analyze the in vivo antigen production level and the fate of the administered LAB vaccines. Ongoing studies into the probiotic effects of LAB and their interactions with the immune system may ultimately help to identify the strains of LAB that are most appropriate and beneficial for particular vaccine delivery systems.

DNA vaccination is an advanced vaccine methodology which involves the direct inoculation of plasmid vectors derived from bacteria containing a gene encoding an antigen of interest under the control of a eukaryotic promoter into a host, where it is taken up by cells and the antigen expressed, processed, and transported to the cell surface for recognition by immune cells [82]. Moreover, baculoviruses which are a group of insect viruses can be used as efficient expression vectors for the production of recombinant vaccines in insect and mammalian cells. These viruses possess a rod-shaped capsid in which a condensed DNA genome consisting of a double-stranded, covalently closed circular molecule ranging between 80 and 200 kbp in length is packaged [83]. LAB vaccines are produced by the bacterial fermentation so their

manufacturing cost is lower than that of either DNA vaccines (plasmids vectors) purified from bacterial hosts or vaccines derived from mammalian or insect cell cultures infected with baculoviruses. The administration of DNA vaccines is mostly carried out by invasive methods including intramuscular injection or intradermal gene gun delivery, which is cumbersome for mass vaccination. Immunity could also be induced upon mucosal administration. However, eliciting immunological responses using the oral route requires the encapsulation of DNA vaccines in biodegradable polymeric microspheres, which imposes an additional cost. This approach protects DNA against enzymatic degradation and acid hydrolysis as it passes through the upper gastrointestinal tract [84, 85]. In contrast, as described in this review, several LAB vaccines can elicit adequate protection levels against infectious agents without their encapsulation. Eukaryotic cells uptaking DNA vaccines in immunized hosts or those used in baculovirus based production systems are able to perform a range of post translational modifications for instance glycosylation, which are not common in LAB. As a result, in viral infections the use of DNA vaccines or baculovirus systems derived vaccines is more favorable than that of LAB vaccines because for viral antigens post translational modifications such as glycosylation should be carried out to obtain adequate immunological responses. In contrast, LAB systems are preferred for vaccine construction using antigens which are natively nonglycosylated such as bacterial antigens. In addition, the baculovirus infection ultimately results in cell death and lysis and the heterologous gene cannot be expressed continuously. Every round of the synthesis of the vaccine of interest requires the infection of new cells [83]. Therefore this system is inferior to LAB systems in terms of its capacity for continuous production of recombinant vaccines.

To date, the data that have been obtained regarding LAB vaccines are promising because they indicate that LAB are capable of delivering antigens to the mucosal and systemic immune systems to elicit protection against infective agents. However, no clinical trials have yet been reported for LAB vaccines. The release of genetically modified organisms through clinical use raises legitimate safety concerns about their survival and propagation in the environment and about the transfer of their genetic materials such as antibiotic selection markers and the antigen encoding gene to other microorganisms. Therefore, proper biologic containment strategies should be implemented before clinical applications of LAB vaccines to prevent escape into the environment of the genetically modified microorganisms and their genetic materials. The existing food-grade expression systems for LAB, which rely on food-grade markers for selection of recombinant bacteria instead of antibiotic resistance markers, should be further expanded to inhibit transfer of antibiotic resistance genes to other microorganisms. LAB maybe considered promising vehicles not only for antigens but also for biologically active compounds such as immunomodulators, antibodies, enzymes or peptides. Genetically modified *Lactococcus lactis* secreting IL-10 provides a therapeutic approach for inflammatory bowl disease. To address the safety concerns related to the release of this genetically modified organism through the clinical use, the chromosomal thymidylate synthase (*thyA*) gene was replaced by the *IL10* gene to generate a thymine auxotroph. Viability of the *thyA hIL10*$^{+}$ strain was reduced by several orders of magnitude in the absence of thymidine or thymine as is the case following shedding the bacterium in the environment and the containment was validated in vivo in pigs [86]. Notably, a small phase I human clinical trial conducted with the *thyA hIL10*$^{+}$ strain in patients with Crohn's disease showed not only that the containment strategy was effective but also the mucosal delivery of IL-10 by *L. lactis* is feasible in humans [87]. ActoGenix Company performed a clinical phase IIa study with the same IL-10 secreting *L. lactis* in human subjects with moderately active ulcerative colitis. The results of this study confirmed the suitability of the applied containment system in humans. These first clinical trials should pave the way for the development of other LAB-delivery applications in humans including vaccine delivery in the future.

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