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Lactococcus lactis as a Live-Vector Vaccine Platform for Parasitic Diseases

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ABSTRACT

The development of expression systems using non-pathogenic microorganisms has enabled efficient and safe platforms for medical and food applications, including functional foods with therapeutic benefits. Among non-pathogenic bacteria, *Lactococcus lactis* (*L. lactis*) is widely used in biotechnology for various applications, such as vaccine development and protein expression. *L. lactis* serves as an effective *in vivo* expression system for developing vaccines and therapeutics in medical research, particularly for parasitic diseases. This review highlights examples of *L. lactis*-based vaccines for parasitic diseases, demonstrating their ability to elicit protective cellular and humoral immune responses.

INTRODUCTION: *L. lactis* from the past to the modern biotechnology

Over 2000 years ago, lactic acid bacteria (LAB) were used to produce fermented dairy products such as yogurt and cheese [1]. However, the modern use of lactic acid bacteria in industrial fermentation began in the mid-19th century, following their characterization by Louis Pasteur [2]. LAB encompass a diverse group with applications ranging from traditional fermentation to advanced gene expression systems in modern biotechnology [3]. *Lactococcus lactis* (*L. lactis*), a well-studied member of LAB [4, 5], was first genetically engineered in the 1990s to express recombinant proteins, enabling its use in biotechnology applications. Advancements in genetic engineering have enabled *L. lactis* to become an effective *in vitro* and *in vivo* protein expression system for developing vaccines and therapeutics, particularly for parasitic diseases [6].

General properties of *L. lactis* expression system

As a Generally Recognized as Safe (GRAS) host for producing heterologous recombinant proteins, *L. lactis* is widely used in biotechnology [7]. *L. lactis* (*subsp. lactis*) is a homofermentative, microaerophilic, non-sporulating Gram-positive bacterium of the order *Lactobacillales*, typically growing at 20–30°C. Its genome, approximately 2.5 Mbp with a 35.1% GC content, encodes around 2,400 proteins in commonly studied strains [8]. Advances in genomic sequencing, microbiology, bacterial physiology, and genetic engineering have established *L. lactis* as an effective host for recombinant protein expression [9]. Key features distinguishing *L. lactis* from other expression systems include the absence of lipopolysaccharides (LPS) and reduced protease activity, which enhances recombinant protein production efficiency. Unlike other engineered bacteria such as *Escherichia coli*, *L. lactis* has only two identified extracellular proteases, contributing to higher recombinant protein stability [10].

Auxotrophic *L. lactis* strains have recently been developed to minimize the risk of antibiotic resistance transfer to other bacteria by eliminating the need for antibiotic resistance markers. Antibiotic-free expression systems in auxotrophic *L. lactis* enhance its safety as a host/vector for food-grade technologies. These systems also prevent the transfer of antibiotic resistance genes to environmental microflora [11, 12]. These features, combined with the ability to lyophilize and reconstitute *L. lactis*, facilitate its use in research and development.

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Another key advantage is *L. lactis*'s ability to form multiple intramolecular disulfide bonds in proteins, enhancing their stability and functionality [13]. Additionally, *L. lactis* supports a range of constitutive and inducible promoters, enabling flexible gene expression as discussed in subsequent sections.

Despite its advantages, the *L. lactis* expression system has several limitations. First, a key limitation of *L. lactis*, like other bacterial expression systems, is its limited capacity for post-translational modifications such as glycosylation, which are critical for the function of many eukaryotic proteins. However, it can form intramolecular disulfide bonds, unlike some bacterial systems. Second, low protein yields in *L. lactis* result from low-copy-number plasmids and *HtrA* protease activity, a genome-encoded membrane-bound protease [14]. The Disrupting the *HtrA* gene can increase secreted protein yields in *L. lactis* [14, 15]. However, some studies indicate that low-level *HtrA* expression enhances overall recombinant protein yields compared to *HtrA*-null mutants, suggesting a balance between protease activity and protein stability [16]. The preference for AT-rich codons in *L. lactis* requires codon optimization for GC-rich target genes to enhance expression efficiency [17]. This codon preference makes *L. lactis* expression efficiency dependent on the target gene's GC content. In other words, codon optimization is often required for target genes from distantly related organisms, such as eukaryotes, to enhance expression efficiency in *L. lactis*. This is less critical for bacteria with similar GC content, such as certain *Streptococcus* or *Lactobacillus* species, facilitating efficient gene expression without extensive codon optimization [12]. Additionally, *L. lactis* has a lower transformation efficiency compared to *E. coli* expression systems, limiting its genetic manipulation [17]. The thick peptidoglycan layer of *L. lactis* requires specialized methods for cell lysis, complicating protein extraction [17-19].

Additional concerns include the potential risk of transgene transfer to the environment or other bacterial apecies, and the use of antibiotic resistance markers. These issues can be overcome by using auxotrophic or inactivated *L. lactis* strains or food-grade, antibiotic-free plasmids [20-22].

Types of applicable promoters in *L. lactis* expression system

Various *L. lactis* strains have been developed with either constitutive or inducible promoters for recombinant protein expression. Constitutive promoters enable consistent recombinant protein production without the need for inducers, simplifying expression systems. Several constitutive promoters, ranging from weak (*e.g.*, P32, P44, P45) to strong (*e.g.*, P2, P3, P5, P8, P21, P23, P59) in transcription strength, are used to express recombinant proteins [23-26]. Continuous expression of certain recombinant proteins can cause cellular stress, protein misfolding, or aggregation, imposing a metabolic burden on the host cell. For instance, expressing membrane-bound or aggregation-prone proteins, such as enzymes or antibodies, can overwhelm protein folding machinery, and disrupting normal cellular processes. By contrast, inducible promoters mitigate toxicity and enable control of gene expression and enhancing recombinant protein production [27].

L. lactis expression systems include inducible promoters, which require specific inducers, and those regulated by environmental factors such as pH, temperature (*e.g.*, dnaJ promoter), or ionic concentrations (Table 1, Fig. 1) [28, 29]. Zinc-regulated promoters, such as Pzn/zitR and Zirex, are commonly used inducible systems controlled by zinc levels in the medium.

Table 1. Common constitutive and inducible promoters in the *L. lactis* expression system

Promoter Type	Promoter Name	Inducer	Description	References
Constitutive	P32, P44 and P45	None	Weak activity results in low transcription and expression levels	[23, 25]
	P2, P3, P5, P8, P21, P23 and P59	None	Strong activity results in high transcription and expression levels	[23, 108]
Inducible	Pzn/zitR	Zinc	Repressor-controlled promoter, zinc-repressed expression system	[23, 30-32]
	Zirex	Zinc	Activator-controlled promoter, expressed in the presence of zinc	
	dnaJ	Heat	Activated by heat shock (30°C to 42°C)	[28, 29]
	PA170	pH	Activated by pH changes and other environmental stressors	[28, 34]
	PxylT	Xylose	Activated by xylose, suitable for food-grade protein production	[33, 109]
	Lac operon	Lactose	Activate in presence of lactose (or lactose analogs).	[35, 36, 28, 37]
	PnisA	Nisin	Part of the NICE system; NisK detects nisin, activating NisR to initiate PnisA-driven transcription	[28, 33, 40]

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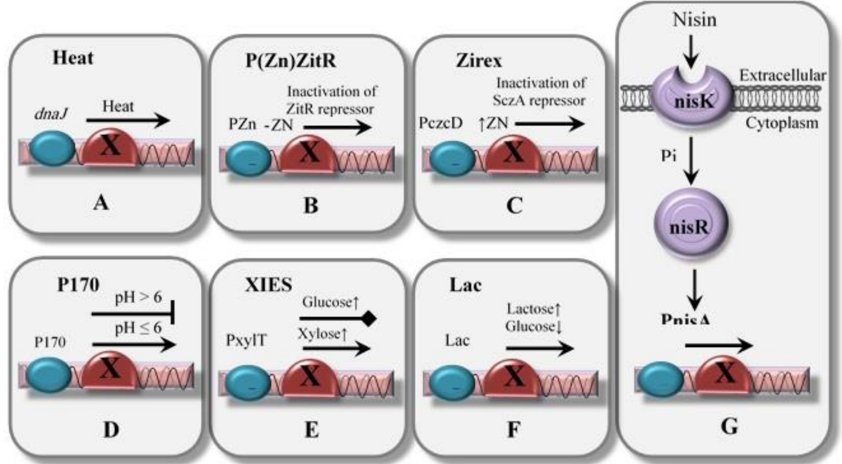

Fig. 1. Schematic representation of promoters in *L. lactis* expression systems. A) The dnaJ promoter is activated by heat shock at temperatures above 30°C (typically 30–42°C), inducing transcription of downstream genes. This activation is reversible, with gene expression returning to baseline levels upon restoration of normal temperatures. B) The Pzn/zitR system is regulated by zinc levels. In the absence of zinc, the ZitR repressor is inactivated, leading to upregulation of the Pzn promoter. This mechanism likely involves reduced DNA-binding affinity of ZitR under low zinc conditions. C)The Zirex system is activated by high zinc concentrations, which inactivate the SczA repressor, upregulating the PczcD promoter. This process is likely mediated by zinc binding to SczA, altering its DNA-binding affinity and relieving repression. D) The PA170 promoter is pH-sensitive, activated under acidic conditions (low pH) and repressed under alkaline conditions (high pH), enabling pH-dependent control of gene expression. E) The Xylose-Inducible Expression System (XIES) is activated by xylose addition to the culture medium, inducing the PxylT promoter. Conversely, glucose represses PxylT, providing a switchable expression system. F) The lac operon is regulated by glucose and lactose availability. In the absence of glucose and presence of lactose, allolactose (a lactose isomer) binds the lac repressor, preventing its interaction with the operator and enabling transcription. Glucose inhibits the operon by lowering cAMP levels, which reduces cAMP-CAP (Catabolite Activator Protein) complex formation and operon activation. G) The NICE system is activated by nisin in the culture medium, which binds to NisK, triggering its autophosphorylation. The phosphate is transferred to NisR, activating the PnisA promoter and driving transcription of the target gene, such as a reporter or functional protein.

The Pzn/zitR system, based on the zit operon, uses the zinc-responsive repressor zitR, which is inactivated at low zinc levels, activating the Pzn promoter (Fig. 1B). In contrast, the Zirex system relies on the pneumococcal repressor S_{czA}, which, at high zinc levels, activates the P_{czcD} promoter (Fig. 1C) [23, 30-33]. The pH-sensitive PA170 promoter (Fig. 1D) is activated by lactic acid accumulation in the stationary phase, enabling controlled protein expression [34, 28]. The Xylose-Inducible Expression System (XIES) uses the PxylT promoter, which is activated by xylose addition and repressed by glucose (Fig. 1E) [33]. The lac operon, activated by lactose, drives transcription of genes encoding lactose-metabolizing enzymes via the Plac promoter (Fig. 1F) [28, 35-37]. Additional inducible promoters, such as those activated by chloride ions (*e.g*., Pcl), expand the versatility of *L. lactis* expression systems [38].

The NICE system, an inducer-based system using nisin, facilitates recombinant protein production in *L. lactis*. The NICE system offers advantages such as precise expression control, high protein yields, and scalability for industrial applications [39-41]. The NICE system uses regulatory elements of the nisin operon to control gene expression via nisin induction (Fig. 1G) [28, 33]. The nisin operon, present in certain *L. lactis* strains, contains 11 genes (designated nisABTCIPRKEFG) responsible for nisin production, supporting the NICE system. Nisin, a 34-amino-acid antimicrobial peptide, binds to lipid II in the cytoplasmic membrane, serves as a food preservative, and induces the NICE system. Three elements of the nisin operon (PnisA, nisK, nisR) play key roles in regulating gene expression in the NICE system. The PnisA promoter, a key inducible promoter, drives recombinant protein production in the NICE system. NisK, a histidine protein kinase in the cytoplasmic membrane, phosphorylates in the presence of nisin and transfers the phosphate to nisR [28, 33]. NisR then activates transcription via the PnisA promoter (Fig. 1G).

Subcellular localization of expressed heterologous protein in *L. lactis*

A key feature of *L. lactis* is its ability to express recombinant proteins in various subcellular locations (cytoplasmic, cell wall-attached, or secreted) using diverse signal peptides (Table 2). Cytoplasmic proteins are often stable against degradation, but their extraction requires cell lysis, complicating purification. However, the secretory pathway is often preferred over cytoplasmic

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expression for easier protein purification, increased yields due to reduced cellular stress, and improved interactions with target molecules [42, 43]. Additionally, signal peptides and propeptides enhance secretion efficiency and reduce degradation of secreted proteins [44]. Signal peptides, typically located at the protein's N-terminus, direct the secretion of recombinant proteins, with USP45 being a commonly used example in *L. lactis* [45-47]. For example, the USP45 signal peptide has been used to secrete proteins like green fluorescent protein (GFP) and enzymes in *L. lactis* [45]. Recently, the SPK1 signal peptide from *Pediococcus pentosaceus* has been shown to outperform USP45 in secretion efficiency in *L. lactis* [48]. Adding the LEISSTCDA propeptide sequence to the N-terminus of the signal peptide enhances secretion efficiency by stabilizing recombinant proteins [10, 49]. In addition, three propeptides, DTNSDIAKQD, DTTTDIAKQE, and DTSAIANQE, naturally occurring in *L. lactis*, enhance the secretion yields of heterologous recombinant proteins [50]. Concomitant expression of target proteins with the PrsA protein from *Bacillus subtilis* has been shown to increase protein secretion in *L. lactis*. This effect is attributed to PrsA, a secretory protein with chaperone activity, which reduces the degradation of secreted proteins [51, 52]. Each protein requires evaluation with various signal peptides to optimize expression and secretion, as secretion efficiency depends on protein type, secondary structure, codon optimization, signal peptide characteristics, and host microorganism [53, 54].

Table 2. Signal peptides, propeptides, and proteins for secretion or cell wall anchoring in *L. lactis* for gene expression and vaccine development.

Name	Function	Origin	References
USP45	Secretory signal peptide for heterologous protein secretion	*L. lactis*	[110, 111]
SPK1	Efficient signal peptide for protein secretion	*Pediococcus pentosaceus*	[112]
LEISSTCDA	Propeptide enhancing protein secretion efficiency	Synthetic peptide	[10, 49, 113]
DTNSDIAKQD	Synthetic propeptide increasing secretion yield	*L. lactis*	[50]
PrtP	Signal peptide for cell wall anchoring	*L. lactis*	[80, 114]
WxL domain	Facilitates non-covalent cell wall attachment and peptidoglycan binding	*Enterococcus faecium*	[115]
M6	Cell wall anchoring domain for lactic acid bacteria	*Strep. pyogenes*	[116, 117]
PrsA	Chaperone protein reducing secreted protein degradation	*Bacillus subtilis*	[51, 52, 118]
LPXTG	Motif for covalent peptidoglycan binding	*Staph. aureus* and *Strep. pyogenes*	[51, 57, 80]
LysM	Motif for non-covalent peptidoglycan binding	In multiple Gram-positive bacterial	[51, 119, 120]
AcmA	Autolysin with LysM motifs for non-covalent peptidoglycan binding	*L. lactis*	[80, 121]

To target proteins to the cell wall, multiple strategies are available, including signal peptide-based targeting, anchor sequence attachment, covalent linkage to cell wall components, non-covalent binding, and fusion with cell surface proteins. For example, the target gene can be cloned downstream of the PrtP signal peptide [55] or the M6 protein from *Streptococcus pyogenes* [56]. Alternatively, proteins can be anchored to the cytoplasmic membrane using a lipoprotein or intracellular protein. The most common non-genetic method for cell wall attachment involves the LPXTG (Leu-Pro-X-Thr-Gly) motif, which enables covalent binding to the cell wall and is recognized by the sortase enzyme [51, 57]. Non-covalent surface display can be achieved by expressing the target protein fused to binding domains, such as the LysM motif or the WxL domain from *Enterococcus faecalis*, which interacts with the AcmA autolysin in *L. lactis* [51, 58]. In this approach, the protein can be expressed in an alternative system and non-covalently attached to the *L. lactis* cell wall by mixing with its culture medium [59]. This non-genetic approach is suitable for expressing eukaryotic proteins requiring post-translational modifications. Thus, the bacterium serves solely as a carrier, with no role in antigen expression [60-62].

Multiple studies have shown that cell-wall-anchored recombinant proteins elicit stronger immune responses and significantly higher specific antibody levels compared to secreted or cytoplasmic forms [56, 63, 64].

L. lactis in clinical applications

L. lactis is a probiotic that plays a significant role in mucosal health and disease prevention [65]. Numerous studies and clinical reports demonstrate that *L. lactis* can be used in clinical research for non-infectious diseases (*e.g*., diabetes, cancer, and respiratory disease) and infectious diseases (*e.g*., bacterial, viral, and parasitic). Multiple studies indicate that *L. lactis* has diverse applications in the prevention and treatment of intestinal and gastrointestinal diseases. The survival and stability of *L. lactis* post-administration are critical for its therapeutic efficacy [65-69]. Although *L. lactis* typically survives for 1–2 days in the digestive tract [66, 67], nanoparticle encapsulation can extend its stability to 24 weeks, suggesting potential for sustained therapeutic effects [68]. Leveraging these advantages, recombinant *L. lactis* was initially developed to treat gastrointestinal and

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metabolic diseases, such as type 1 diabetes, by regulating inflammation and inducing immunomodulatory responses [69]. Recent studies report that probiotic bacteria, including *L. lactis*, exhibit anti-inflammatory properties and potential for treating skin diseases [70]. *L. lactis* serves as an expression system for heterologous antigens *in vitro* and *in vivo*, a live-vectored vaccine using a non-pathogenic organism, and a delivery vehicle for pathogen-derived antigens [20-22, 71-73] (Fig. 2). Given the widespread use of *L. lactis*, this review primarily focuses on its applications in parasitic disease vaccines.

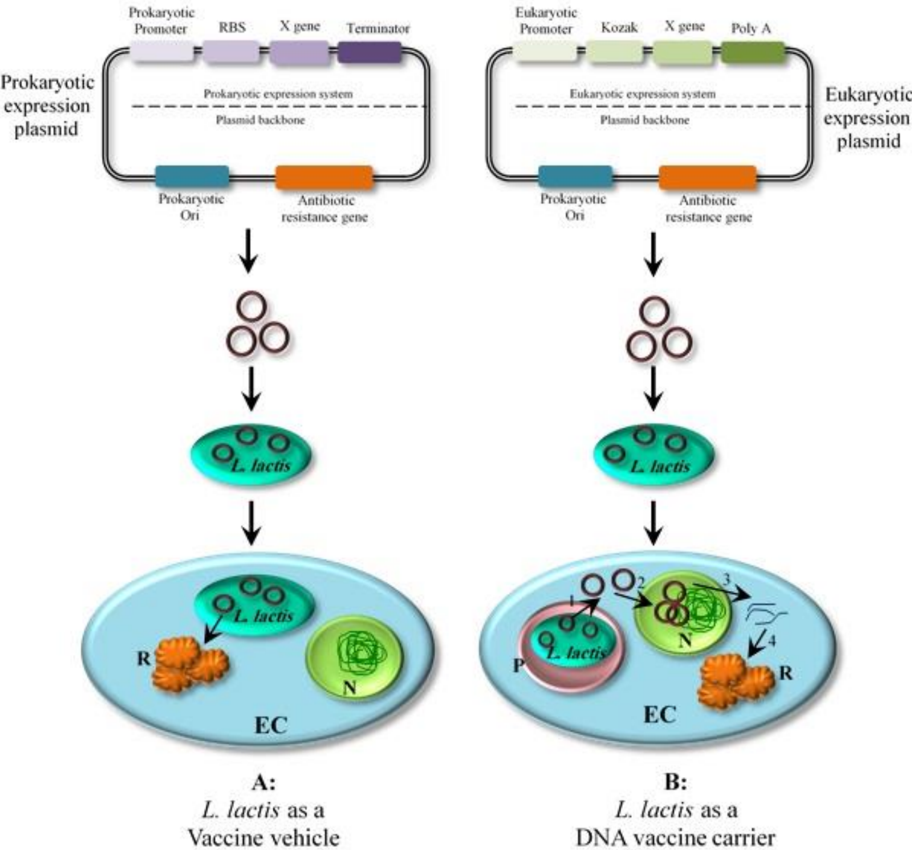

Fig. 2. Schematic illustration of *L. lactis* as a live vaccine vehicle and DNA vaccine carrier. A) Live vaccine vehicle: The gene of interest (X) is cloned into a prokaryotic expression plasmid downstream of a promoter (P), between a ribosome-binding site (RBS) and a stop codon, as provided by standard prokaryotic vectors. The recombinant plasmid is transformed into *L. lactis*. Depending on the immunization route, transgenic *L. lactis* interacts with specific eukaryotic cells (EC). For example, oral administration typically confines *L. lactis* to the gut lumen, where it interacts with epithelial cells without internalization. Parenteral routes (*e.g*., intramuscular or subcutaneous) may lead to uptake by antigen-presenting cells (APCs), such as macrophages or dendritic cells. The bacteria produce and release recombinant proteins (R), which are processed and presented via MHC class I (for intracellular antigens) or MHC class II (for extracellular antigens) pathways. B) DNA vaccine carrier: The plasmid includes elements for bacterial replication (origin of replication, Ori), selection (*e.g*., antibiotic resistance gene), and eukaryotic expression, such as a eukaryotic promoter (P), Kozak sequence, and polyadenylation (poly-A) signal to ensure efficient translation and mRNA stability. The recombinant plasmid is transformed into *L. lactis*. The transgenic bacteria deliver the plasmid to eukaryotic cells (EC), either through intestinal epithelial cells (IECs) or directly to dendritic cells (DCs). Following phagocytosis, *L. lactis* is lysed in the phagolysosome (P), releasing plasmids into the cytoplasm. Plasmids may utilize nuclear localization signals (NLS) to facilitate transport across the nuclear envelope into the nucleus (N), where eukaryotic transcription and translation machinery initiate antigen expression.

L. lactis as a live-vectored vaccine against parasitic diseases

Live-vectored vaccines leverage the potential of non-pathogenic microorganisms, particularly probiotic bacteria, as vectors. Genes of interest can be cloned into suitable vectors to produce recombinant proteins under the control of appropriate promoters, targeting specific subcellular compartments (Fig. 2A). The subcellular localization of proteins determines the immune presentation pathways for antigens [20, 71].

Multiple studies demonstrate that *in vivo* administration of recombinant *L. lactis* stimulates mucosal, humoral, and cellular immunity against infectious diseases in experimental animal models [55, 74, 75]. *L. lactis* can survive multiple passages through the gastrointestinal tract of animals and humans for 2–3 days post-administration without colonizing the host [20].

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Table 3 summarizes studies demonstrating that intrinsically non-pathogenic *L. lactis* serves as a live-vectored vaccine against parasitic diseases caused by protozoans (*Toxoplasma* and *Trypanosoma*), insect-vector-borne parasites (*Plasmodium* and *Leishmania*), and intestinal parasites (*Giardia* and *Eimeria*). Examples for each parasite are provided in the following sections.

Table 3. Studies using recombinant *L. lactis* as a live vaccine against protozoan and intestinal parasites

Genus	Parasite species	Delivery vehicle	Antigen	Subcellular localization/Vector	Animal model	Immunization route	Outcome	References
Plasmodium	*Plasmodium yoelii*	*L. lactis* (LM234, 5×10^9 CFU)	MSP1 C-terminal	Cytoplasmic/pTRKL2	BALB/c, C57BL/6	Oral	Reduced parasitemia and increased survival	[79]
Plasmodium	*P. falciparum*	*L. lactis* (NZ9000, NZ9700)	MSA2	Cell wall-anchored	Rabbits	Oral, nasal	Elicited systemic antibodies	[80]
Plasmodium	*P. falciparum*	*L. lactis* (NZ9000, NZ9700, 5×10^9 CFU)	MSA2	Cell wall-anchored	Mice	Oral, nasal	Elicited specific antibody response; covalently attached MSA2 (MSA2cP) outperformed non-covalently attached (MSA2cA)	[81]
Plasmodium	*P. falciparum*	*L. lactis*	MSA2	Cytoplasmic, cell wall-anchored	BALB/c	Oral, nasal	Induced IgG1, IgG2a, IgG2b in young mice and IgG3 in older mice for both cytoplasmic and cell wall-anchored forms	[82]
Toxoplasma	*T. gondii*	*L. lactis* (10×10^{10} CFU)	ROP1	Cytoplasmic, secreted, cell wall-anchored	BALB/c	Oral	Induced mucosal and humoral immune responses but not protective	[84]
Leishmania	*L. donovani*	*L. lactis* (NZ9000, ~2×10^9 CFU)	A2	Cytoplasmic, secreted, cell wall-anchored	BALB/c	Subcutaneous	Cell wall-anchored *A2* elicited high specific antibody levels, increased IFN-γ, and decreased IL-10	[56]
Leishmania	*L. major*	*L. lactis* (NZ9000, 0.5×10^9 CFU)	LACK+IL-12	Cytoplasmic, secreted, cell wall-anchored	BALB/c	Subcutaneous	Co-administration of *LACK* and *IL-12* induced *Leishmania*-specific Th1 response	[75]
Leishmania	*L. major*	*L. lactis* (PH3960, 4×10^9 CFU)	LACK+IL-12	Cytoplasmic, secreted, cell wall-anchored	BALB/c	Oral	Delayed footpad swelling and reduced parasite burden	[74]
Leishmania	*L. major*	*L. lactis* (NZ9000, ~2×10^9 CFU)	PpSP15	Cell wall-anchored	BALB/c	Subcutaneous	Conferred short- and long-term protection, reduced swelling, lowered parasite burden, and increased IFN-γ/IL-5, IFN-γ/IL-10, IL-17/IL-5, and IL-17/IL-10 ratios	[55]
Trypanosoma	*T. cruzi*	*L. lactis* (NZ9000, 1×10^9 CFU)	trans-sialidase (*TScf*) enzyme + c-di-AMP adjuvant	Cytoplasmic	BALB/c	Oral	Co-administration of *TScf* and c-di-AMP stimulated immune response	[83]
Giardia	*G. lamblia*	10×10^{10} CFU *L. lactis* (NZ9000)	CWP2	Cytoplasmic, secreted, cell wall-anchored	BALB/c	Oral	Elicited CWP2-specific IgA antibodies and reduced cyst formation by up to 63%	[85]
Eimeria	*E. tenella*	*L. lactis* (NZ9000)	3-1E	pTX8048	Chickens	Oral	Provided partial protection, controlled disease, and improved body weight	[87]

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Eimeria	*E. tenella*	5×10^{9} CFU *L. lactis*	AMA1	Cytoplasmic, secreted, cell wall-anchored	Chickens	Oral	Cell wall-anchored EtAMA1 induced higher IgG titers and CD4+ T cell proportions, enhancing protective immunity	[88]
Eimeria	*E. tenella*	1×10^{10} CFU *L. lactis* (NZ9000)	Dendritic cell-targeting peptide (DCpep) and 3-1E	pTX8048, *Usp45* cell wall-anchored	Chickens	Oral	Elicited higher 3-1E-specific serum IgG, secretory IgA, CD4+ and CD8α+ cells, and increased IL-2 and IFN-γ mRNA in spleen	[89]
Eimeria	*E. tenella*	*L. lactis*	IMP1	Cytoplasmic, secreted, cell wall-anchored	Chickens	Oral	Induced T lymphocyte proliferation, IL-2, IL-4, IL-10, and IFN-γ mRNA in spleen, and increased serum IgG and secretory IgA	[90]

Plasmodium

Plasmodium, a protozoan parasite, causes malaria in humans and other vertebrates. The *L. lactis* expression system was initially used to produce *Plasmodium falciparum* (*P. falciparum*) antigens of varying sizes with multiple intramolecular disulfide bonds, which were challenging to express in other systems [13]. For example, *L. lactis* successfully expressed chimeric proteins GLURP (Glutamate-rich protein) and MSP3 (Merozoite surface protein 3) [76], as well as Pfs48/45 and Pfs230 from *P. falciparum* [77]. These recombinant proteins elicited antibody production in immunized mice. High-yield production of soluble recombinant CSP (circumsporozoite protein) by *L. lactis* increased functional antibody levels in immunized mice [78].

One of the earliest studies using *L. lactis* as a live vaccine delivery system for malaria was conducted by Zhi-Hong Zhang *et al.* (2005) [79]. They expressed the C-terminal 19-kDa fragment of MSP1 (Merozoite Surface Protein 1) from *P. yoelii* in *L. lactis* (LM2345 strain, lac-negative, plasmid-free) and demonstrated that oral administration reduced parasite burden in BALB/c and C57BL/6 mice [79]. In C57BL/6 mice, this vaccination regimen completely prevented infection and eliminated parasites [79]. Ramasamy *et al*. (2006) demonstrated that nasal and oral immunization of rabbits with recombinant *L. lactis* expressing MSA2 (Merozoite Surface Antigen 2) from *P. falciparum* elicited high IgG antibody levels [80]. They observed comparable results using two expression strategies: covalent anchoring to the cell wall peptidoglycan via the LPXTG motif and PrtP, or non-covalent attachment using a peptidoglycan-binding anchor domain [80]. Oral and nasal vaccination routes using the anchor domain elicited similar serum antibody titers [80]. Mice vaccinated with *L. lactis* expressing MSA2 in cytoplasmic or cell-wall-anchored forms exhibited humoral and cellular immune responses following nasal or oral immunization [81, 82].

Leishmania

Leishmania, a unicellular protozoan parasite transmitted to humans by blood-feeding sand flies, causes leishmaniasis. Leishmaniasis, a neglected tropical disease, remains uncontrolled due to the absence of an effective prophylactic vaccine. Several antigens from *Leishmania* or its sand fly vector have been investigated using the *L. lactis* system. To evaluate *L. lactis* as a vaccine platform against leishmaniasis, Yam *et al*. (2011) expressed a truncated A2 protein from *Leishmania donovani* in three forms: cytoplasmic, cell-wall-anchored (via M6 protein), and secreted [56]. Subcutaneous immunization with the cell-wall-anchored form elicited specific serum antibodies and reduced parasite load in infected BALB/c mice [56]. Subsequently, the LACK antigen from *Leishmania major* was expressed in cytoplasmic, secreted, and cell-wall-anchored forms, and its immunogenicity, alone or with IL-12 (secreted by *L. lactis*), was evaluated in BALB/c mice against cutaneous leishmaniasis [75]. Subcutaneous immunization with cell-wall-anchored LACK and secreted IL-12 protected BALB/c mice against *L. major* infection by inducing a Th1-polarized immune response [75]. The same group demonstrated that oral immunization with *L. lactis* (PH3960, alanine racemase-deficient) expressing LACK and IL-12 delayed footpad swelling and reduced parasite burden in BALB/c mice against *L. major*. However, LACK-specific antibodies were undetectable in the sera of immunized mice before or after parasite challenge [74]. Recent subcutaneous immunization with recombinant *L. lactis* expressing PpSP15 (from *Phlebotomus papatasi*) anchored to the cell wall via the PrtP signal peptide provided long-term protection against *L. major*, significantly reducing footpad swelling and parasite load in the lymph nodes of BALB/c mice. Cell-wall antigen expression likely enhanced cellular immune responses [55].

Trypanosoma

Trypanosoma, a genus of unicellular flagellated protozoa, includes species such as *Trypanosoma brucei*

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(causing African sleeping sickness) and *Trypanosoma cruzi* (causing Chagas disease). Researchers developed a formulation using two recombinant *L. lactis* strains: one expressing the TScf trans-sialidase enzyme from *T. cruzi* as a vaccine candidate, and another expressing c-di-AMP as a mucosal adjuvant, both on a single plasmid for cytoplasmic *in vivo* expression. Oral administration of this formulation elicited a specific immune response against TScf in *T. cruzi* [83].

Toxoplasma

Toxoplasmosis is caused by the protozoan parasite *Toxoplasma gondii*. Recombinant *L. lactis* enhances cellular and humoral immune responses against *T. gondii*. Oral immunization with recombinant *L. lactis* expressing ROP1 from *T. gondii* significantly increased humoral immune responses (IgG and IgA) in BALB/c mice but did not provide complete protection [84].

Giardia

Lee *et al*. (2006) first explored *L. lactis* as a vehicle for expressing antigens against the intestinal parasite *Giardia* [85]. Delivery of *L. lactis* expressing CWP2 (cyst wall protein 2) from *Giardia lamblia* as a vaccine candidate elicited CWP2-specific IgA antibodies and significantly reduced cyst formation post-challenge [85]. Another study compared *L. lactis* and *Streptococcus gordonii* as live antigen delivery vehicles for CWP2 from *G. lamblia* in BALB/c mice. Both systems reduced cyst formation, but *S. gordonii* was more effective, eliciting higher IFN-γ and intestinal IgA levels and further reducing cyst formation [86]. The authors attributed the superior performance of *S. gordonii* to its *in vivo* replication, which likely prolongs antigen exposure and enhances immune stimulation. In contrast, *L. lactis* does not efficiently colonize or replicate *in vivo*, potentially limiting antigen presentation duration.

Eimeria

Eimeria, an apicomplexan protozoan parasite with intracellular and extracellular life cycle stages, causes intestinal diseases. *L. lactis* expressing the 3-1E protein from *Eimeria tenella* alleviated disease symptoms in orally vaccinated specific pathogen-free (SPF) chickens [87]. Moreover, recombinant *L. lactis* expressing AMA1 (Apical membrane antigen 1) from *E. tenella* in cytoplasmic, secreted, and cell-wall-anchored forms was successfully used to vaccinate chickens against *E. tenella*. Chickens immunized with cell-wall-anchored EtAMA1 exhibited higher CD4+ T cell counts and IgG titers compared to other groups [88]. Another study evaluated the immunogenicity of *L. lactis* expressing both DCpep (dendritic cell-targeting peptide) and 3-1E antigens in chickens. Interestingly, oral administration of the cell-wall-anchored antigen significantly increased specific IgG and IgA antibodies, as well as CD4+ and CD8α+ cells, in peripheral blood [89]. The same group investigated the protective effects of oral immunizationwith *L. lactis* expressing cell-wall-anchored IMP1 (Immune Mapped Protein-1) against coccidiosis in chickens. Their results demonstrated an IL-2, IL-4, IL-10, and IFN-γ-dependent protective response in the spleen compared to the control group [90].

L. lactis as a delivery system for DNA vaccines

DNA vaccines are highly promising due to their safety, cost-effectiveness, ease of design and production, non-infectious nature, and ability to induce both cellular and humoral immune responses [91, 92]. However, limitations including inefficient mucosal delivery, antibiotic resistance genes in plasmids, enzymatic degradation, and low immunogenicity in humans hinder DNA vaccine applications. Non-pathogenic bacteria, such as *L. lactis*, can address some of these challenges. Bacterial DNA is protected from nucleases, unfavorable pH, and harsh cellular conditions [33]. Plasmid DNA delivered by *L. lactis* is released into the cytosol following intra-phagosomal degradation, translocates to the nucleus, and is expressed [33]. The expressed protein is presented via MHC class I or II, activating CD8+ T cells or CD4+ T helper cells, respectively. In 2004, researchers demonstrated that *L. lactis* could deliver therapeutic proteins to mucosal tissues as a live vector [93]. In 2006, *L. lactis* transformed with an *E. coli* shuttle plasmid containing a eukaryotic expression cassette was shown to deliver DNA vaccines to mammalian Cos-7 cells [94]. As depicted in Fig. 2B, researchers developed shuttle vectors for replication in both *L. lactis* and mammalian cells. These vectors include eukaryotic elements (*e.g*., eukaryotic promoter, Kozak sequence, and polyadenylation signal) to enable protein expression in cells such as epithelial cells [94]. Guimarães *et al*. (2006) successfully validated the pValac system, which includes the CMV promoter and BGH polyadenylation region [95]. After gene cloning, the pValac plasmid was transformed into *L. lactis*, and recombinant protein expression was detected in epithelial cells three days later [96, 97].

Gram *et al*. (2007) compared *L. lactis* and *E. coli* expression systems encoding HIV-1 gp120 for immune activation following three intramuscular injections in mice. The *L. lactis*-based DNA vaccine (pLL120, ~7.8 kb) elicited higher antibody titers but lower cellular responses compared to *E. coli*, which was compensated by additional CpG motifs [98]. De Azevedo *et al*. (2015) used recombinant *L. lactis* harboring *Staphylococcus aureus* FnBPA (Fibronectin Binding Protein A) or *Listeria monocytogenes* mInlA (mutated Internalin A) DNA (pValac, ~3.7 kb) to directly transfect mouse bone marrow-derived dendritic cells [99]. Pereira *et al*. evaluated oral immunization with *L. lactis*-FnBPA+ (MG1363 strain) carrying pValac:ESAT-6, which elicited cytokines and antibodies against tuberculosis [100]. Another study demonstrated that intranasal DNA immunization of C57BL/6 mice with *L. lactis*-FnBPA+

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expressing Ag85A (pValac:Ag85A) from *Mycobacterium tuberculosis* induced a significant Th1 response, with elevated IFN-γ, TNF-α, and IL-6 levels, as well as IgG and anti-Ag85A IgA [101]. The same group demonstrated that oral DNA immunization with *L. lactis* expressing fused ESAT-6 and Ag85A antigens elicited significant cellular and humoral immune responses [102]. Table 4 summarizes studies on DNA vaccines using *L. lactis* as a carrier.

Plasmid stability is critical for the efficacy of *L. lactis*-based vaccines. Plasmid loss can reduce antigen expression and vaccine efficacy [33]. Strategies to address this include chromosomal integration, plasmid stabilization systems, and optimized culture conditions. Stable gene expression is essential for consistent vaccine performance and successful clinical translation.

L. lactis as an adjuvant

A key advantage of live-vectored vaccines, such as those using *L. lactis*, is the vector's inherent adjuvanticity [103]. Non-replicating vaccines, such as protein or inactivated vaccines, often require adjuvants to enhance immunogenicity. Certain *L. lactis* strains, such as NZ9000, exhibit innate adjuvant properties when administered orally or nasally, enhancing protective immune responses against diseases such as cancer [104-106]. Indeed, *L. lactis* is considered superior to other bacteria as a delivery vehicle due to its potential adjuvant efficacy [65, 95]. Studies have demonstrated that the adjuvanticity of *L. lactis* enhances vaccine efficacy, particularly for pneumococcal antigens delivered nasally or orally [65]. Moreover, the adjuvant effect of *L. lactis* has been confirmed in immunized mice [98].

L. lactis vaccine delivery pathways

Inducing a robust immune response involves challenges such as selecting the immunization route, antigen, dosage, and administration method. Antigen degradation, immune tolerance, and gut microbiota are additional critical factors. Oral and mucosal vaccines face harsh conditions, such as stomach acid and digestive enzymes, which can degrade antigens before they reach immune cells. Repeated antigen exposure, particularly in mucosal tissues, can induce immune tolerance rather than activation. An imbalanced gut microbiome can either enhance or suppress vaccine efficacy [33]. Addressing these challenges requires optimized formulations, protective delivery systems, and adjuvants to enhance antigen stability and immune activation. *L. lactis* is a promising vaccine delivery platform, capable of utilizing various administration routes and stimulating diverse immune cells. However, further research is required to fully evaluate the efficacy of *L. lactis* across applications and compare its immunogenicity with other vaccine platforms. *L. lactis* has been administered as a vaccine platform via cutaneous [33, 70], subcutaneous [55, 56, 75], intra-muscular [98], intra-dermal [83, 98, 107], oral [74], and intranasal [80] routes. *L. lactis* facilitates thetransfer of DNA plasmids to immune cells, such as dendritic cells, via phagocytosis [99]. Mucosal administration of recombinant lactic acid bacteria, particularly via the oral route (Table 3), offers several advantages over systemic inoculation [33, 74]. Several studies have reported subcutaneous administration as an effective route for immunization against parasites such as *Leishmania* [55, 56, 75].

Table 4. Examples of using recombinant *L. lactis* as a DNA vaccines carrier

Delivery Vehicle/Plasmid	Disease	Deliver DNA to	Antigen	Animal model/Cell type	Immunization route	Observed result	References
L. lactis (MG1363/pLIG)	Listeria monocytogenes	Mammalian epithelial cells	Bovine β-lactoglobulin	Cos-7 cells	Transfection with LipofectAmine	Antigen expression and secretion observed 24 and 48 hours post-incubation	[94]
L. lactis (pLL120)	HIV	Mice	gp120 (HIV-1BX08)	BALB/c	Intramuscular	Induced specific humoral and cellular responses against HIV	[98]
L. lactis (NZ9000, MG1363/pValac)	-	Mouse bone marrow-derived dendritic cells (BMDCs)	Cow milk allergen β-lactoglobulin (BLG)	BMDCs	Incubation with BMDCs, inducing maturation	Delivered DNA vaccines to dendritic cells or across epithelial monolayer, enhancing mucosal vaccine potential	[99]
L. lactis (MG1363/pValac)	Tuberculosis	Mice	Fibronectin-binding protein A (FnBPA)	BALB/c	Oral	Increased pro-inflammatory cytokines (IL-17, IFN-γ, IL-6, TNF-α)	[100]
L. lactis (MG1363/pValac)	Tuberculosis	Mice	Fibronectin-binding protein A (FnBPA)	C57BL/6	Intranasal	Transferred DNA to BMDCs, inducing significant Th1 response	[101]
L. lactis (MG1363/pValac)	Tuberculosis	CHO cells, mice	Fused ESAT-6 and Ag85A	BALB/c	Oral	Increased IFN-γ, TNF-α, IL-17, and humoral immune responses	[102]

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Concluding remarks

L. lactis provides a safe and effective platform for developing vaccines against parasitic diseases, particularly due to its ability to deliver antigens to mucosal surfaces. Next-generation sequencing (NGS) has facilitated the discovery of new parasite antigens, and *L. lactis* is well-suited to express and deliver these candidates. The microaerophilic nature of *L. lactis* may limit protein yields, but this can be mitigated through fermentation and genetic optimization. Future research should prioritize improving antigen stability, investigating mucosal adjuvants, and developing multivalent vaccines to maximize the potential of *L. lactis* against complex parasitic infections. These vaccine candidates should be evaluated in animal models using diverse approaches, including purified recombinant proteins produced *in vitro* or expressed *in vivo*.

To this end, bacterial expression systems offer advantages over other platforms (*e.g*., yeast, insect, or mammalian cells) due to their rapid growth, high protein yields, cost-effectiveness, and established genetic manipulation techniques. These attributes make bacterial systems ideal for the scalable production of antigens for parasitic disease vaccines. Additionally, certain bacteria can function as live vectors, directly delivering antigens to the host immune system. Indeed, *L. lactis* is a promising bacterial expression system for parasitic disease research due to its safety, ability to induce mucosal and systemic immunity, and versatility in delivery routes, such as oral and intranasal administration. Although *L. lactis* is generally safe, its use in vaccines requires caution, particularly for immunocompromised individuals. Potential risks, such as bacterial translocation or unintended immune responses, must be considered. However, several challenges must be addressed for the *L. lactis* vaccine platform. First, the genetic construct and antigen localization significantly affect immunogenicity; for instance, surface-displayed antigens enhance immune recognition, whereas cytoplasmic expression may limit accessibility. Second, the immunization route must be carefully chosen based on the target parasite and desired immune response; oral or intranasal delivery is effective for mucosal pathogens like *Giardia*, while systemic infections like *Plasmodium* may require parenteral administration for robust systemic immunity. Finally, the microaerophilic nature of *L. lactis* reduces protein yields compared to aerobic bacterial strains, necessitating further optimization.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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