***Lactococcus lactis*: LAB model organism for bacteria-mediated therapeutic strategies**

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Abstract. *Lactococcus lactis* is a well-characterized, food-grade lactic acid bacterium (LAB) with generally recognized as safe (GRAS) status. Better understanding of this bacterium at a molecular level has led to the development of unprecedented genetic tools that enable the expression of heterologous proteins. Subsequently, the ability of *L. lactis* to express and deliver these proteins to eukaryotic hosts presents a promising approach to achieve potent treatments for various diseases. Here, we have reviewed the characteristics of *L. lactis* and the expression systems established for this LAB model organism. We also described the experimental applications of *L. lactis* in disease therapy, especially its role as a vector in vaccination strategies.

Keywords: bactofection, lactic acid bacteria, *Lactococcus lactis*, vaccine, vector

INTRODUCTION

1. Background

During the 90's, lactic acid bacteria (LAB) started to become popular starter cultures for the production of dairy and fermented food (Konings, 2000). This is particularly due to their ability to produce lactic acid as a major end product of carbohydrate fermentation (Kandler, 1983). Owing to their long history of use in food production, LAB have been proven as safe for human consumption and were certified as generally recognized as safe (GRAS) organisms by the US Food and Drugs Administration (FDA) (Kolida and Gibson, 2011). Currently, 13 genera have been classified under the LAB group, which include *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Paralactobacillus*, *Enterococcus*, *Carnobacterium*, *Lactosphaera*, *Leuconostoc*, *Oenococcus*,

Tetragenococcus, *Weisella*, and *Vagococcus* (Stiles and Holzapfel, 1997).

To date, efforts have been made to achieve a better understanding of LAB and to advance their application in health and nutrition. Improved knowledge of LAB has contributed to the development of heterologous protein expression systems, coupled with gene transfer mechanisms that have led to the employment of LAB as delivery agents for the treatment of diseases. Among them, *Lactococcus lactis* is the most-characterized LAB organism which has been extensively studied for biomedical applications. In this review, we discuss the properties of *L. lactis*, which made it an excellent LAB model. We also describe several *L. lactis*-based expression and delivery systems available, as well as a summary

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on the current *L. lactis* treatment strategies, particularly DNA vaccination.

2. Uses and importance of LAB

Major advancements in bacterial taxonomy tools and molecular biology tools in the past decades have led to revolutionary growth in studies involving the isolation and characterization of new LAB strains from various sources (Abushelaibi *et al.*, 2017; Guo *et al.*, 2010; Rubio *et al.*, 2014; Zoumpopoulou *et al.*, 2017). Morphologically, LAB are found in either rod or coccus shape and may exist as single cells or in chains (Settanni and Moschetti, 2010). Past studies have identified LAB as great starter cultures and additives for biopreservation of food, based on their ability to produce natural bacteriocins (Hirsch, 1951; Sashihara *et al.*, 2000; Sawa *et al.*, 2010, 2013) with antimicrobial effects against other strains, such as *Listeria monocytogenes* (Allende *et al.*, 2007; Amezcuita and Brashears, 2002; Perin and Nero, 2014), methicillin-resistant *Staphylococcus aureus* (Okuda *et al.*, 2013), and *Salmonella* (Kim *et al.*, 2015; Stevens *et al.*, 1991). Furthermore, the production of antibacterial proteins gives LAB a survival advantage over other strains and helps to maintain the equilibrium of the gastrointestinal tract (GIT) microflora, hence revealing their probiotic potentials. Initially described by Lilly and Stillwell (1965), probiotics is now used to refer to microorganisms that can confer health benefits to the host, which may include cancer prevention, decreased risks of inflammatory bowel syndromes and gastrointestinal infections, immune modulation, and relief of lactose intolerance symptoms, diarrhoea, and constipation (Salminen *et al.*, 1999; Schrezenmeir and de Vrese, 2001). The probiotic potential of LAB is also a result of their tolerance towards low pH and their ability to persist within the harsh, acidic environment of the GIT. Although acid tolerance is reported to be strain-dependent, several LAB, mostly belonging to the genus *Lactobacillus*, have been shown to display this characteristic (Goldin *et al.*, 1992; Jacobsen *et al.*, 1999; Oozeer *et al.*, 2006; Rubio *et al.*, 2014).

3. *Lactococcus lactis* as a model organism of LAB

3.1 Characteristics

The first bacterial pure culture of *Lactococcus lactis* (previously known as *Bacterium lactis*) was isolated from boiled milk, in 1873 by Joseph Lister (Stiles and Holzapfel, 1997). Subsequently, *L. lactis* became an important starter culture in the food industry, particularly for the production of cheese, milk, and buttermilk (Leroy and de Vuyst, 2004). *L. lactis* is described as a non-pathogenic, mesophilic, coccus bacterium of about 0.5 to 1 µm diameter (Teuber and Geis, 2006). This fermentative anaerobic bacterium is Gram-positive, and is therefore, less likely to induce immunogenic response when compared to Gram-negative bacteria such as *Escherichia coli* (Rueda *et al.*, 2014; Salminen *et al.*, 1998). Although *L. lactis* does not naturally colonize the digestive tract, the presence of its strains among the GIT microflora has been acknowledged in several studies (Grahn *et al.*, 1994; Klijn, Weerkamp, and de Vos, 1995; Schlundt *et al.*, 1994).

In many instances, it is worth noting that *L. lactis* is capable of adhering to intestinal cells. Using direct microscopic evaluation of Gram-stained samples, Lehto and Salminen (1997) reported that *L. lactis* subsp. *cremoris* ARH 74 is capable of adhering to human colorectal cancer cell line, Caco-2. The percentage of adhesion was determined as 16%, slightly higher than that of the positive control, *Lactobacillus* GG strain (Lehto and Salminen, 1997). Likewise, Kimoto *et al.* (1999) demonstrated that six out of nine *L. lactis* strains tested, are capable of adhering to Caco-2 cells. Among them, *L. lactis* subsp. *lactis* NIAI527 displayed the greatest level of adhesion (Kimoto *et al.*, 1999). Further investigations revealed that this strain could withstand low pH and bile, according to the number of colony forming units per millilitre (CFU/ml) obtained upon incubation for 30 minutes at pH 2.5 and under 0.5% oxgall, respectively (Kimoto *et al.*, 1999). Meanwhile, Ouwehand *et al.* (1999) have shown that *L. lactis* subsp. *cremoris* is able to adhere well to the mucus layer of human intestine. It was proposed by the authors that the study used mucus instead of intestinal cells in order to provide a better

representation of in vivo intestinal cells, which are typically covered by the mucus layer.

Like most LAB strains, *L. lactis* is also known to produce bacteriocins such as nisin (Biscola *et al.*, 2013; Kuipers *et al.*, 1993) and lactococcin (Alegría *et al.*, 2010; Holo, Nilssen, and Nes, 1991; Nissen-meyer, Holo, and Havarstein, 1992), effective against a considerable range of microorganisms, such as *Salmonella*, *Listeria*, and *Enterococcus*. Thus, based on these evidences, *L. lactis* is a very likely candidate for probiotic applications, comparable to *Lactobacillus*.

3.2 Genetic tools

As a result of extensive molecular studies of *L. lactis* and its subspecies, today, vast information regarding the genome of several *L. lactis* strains is available (Bolotin *et al.*, 2001; Bolotin *et al.*, 1999; Wegmann *et al.*, 2007). In addition, numerous reports on the genomic characterization of novel *L. lactis* strains isolated from a myriad of sources have also been published (Fernández *et al.*, 2011; Gao *et al.*, 2011; Siezen *et al.*, 2010). At present, improved understanding of *L. lactis* genome has provided a platform for the modelling of this bacterium's metabolic pathways (Flahaut *et al.*, 2013; Guedon *et al.*, 2005; Oliveira, Nielsen, and Förster, 2005). Consequently, this wealth of knowledge has led to the development of a wide range of genetic tools for *L. lactis*, which comprises of gene transfer and gene knockout mechanisms, construction of novel *lactococcal*-derived vectors, and establishment of regulated gene expression systems.

The greatest progress was met through the development of nisin-controlled gene expression (NICE) system, in which the expression of any gene placed under the control of P_{nisA} promoter is induced by the addition of the substrate, nisin (de Ruyter *et al.*, 1996; de Ruyter, Kuipers, and de Vos, 1996). Upon further evaluation, the incorporation of the signal transduction genes, *nisR* and *nisK*, into the chromosome of *L. lactis* subsp. *cremoris* MG1363, has successfully generated the NZ9000 strain, a common host for the NICE system (Kuipers *et al.*, 1998). The overview of regulated protein expression by NICE system is illustrated in Figure 1.

Apart from regulated gene expression systems, several other genetic tools have been

developed in *L. lactis* to enhance bacterial uptake by host cells and to facilitate DNA delivery. For instance, Innocentin *et al.* (2009) reported that the development of *L. lactis* expressing either fibronectin binding protein A (FnBPA) of *S. aureus* or internalin A (InlA) of *L. monocytogenes* managed to improve the rate of *L. lactis* internalization into Caco-2 cells. Earlier, it was identified that the proteins FnBPA and InlA are involved in promoting invasion of *S. aureus* and *L. monocytogenes*, respectively (Dziewanowska *et al.*, 1999; Lecuit *et al.*, 1997). Similarly, Yagnik *et al.* (2017) designed a recombinant *L. lactis* expressing InlA to enhance the delivery of the plasmid pPERDBY, carrying a gene coding for enhanced green fluorescent protein (EGFP) to Caco-2 cells. Despite previous success in demonstrating that non-invasive *L. lactis* was capable of delivering the same plasmid into CHO-K1 and Caco-2 cells (Yagnik, Padh, and Desai, 2016), the incorporation of InlA expression has yielded three times greater expression of EGFP by Caco-2 cells (Yagnik *et al.*, 2017). Hence, it was proposed that this system could be applied for *L. lactis*-mediated treatment approaches, for enhanced therapeutic efficacy.

Figure 1. Schematic diagram of NICE system in *L. lactis*. Stimulation by extracellular nisin causes the histidine kinase, *nisK*, to undergo autophosphorylation and subsequently mediate phospho-transfer to the response regulator, *nisR*. This will then induce the transcription of genes under the inducible promoters, P_{nisA} and P_{nisF} .

In summary, the access to a complete characterization of *L. lactis* genome has provided substantial understanding of this bacterium's molecular biology and has inspired the development of state-of-the-art genetic expression systems, which in the future, could be replicated in other microorganisms, including other LAB strains. With a diverse array of genetic tools to choose from, *L. lactis* can be easily manipulated for biological and medical applications, ergo considered an excellent model of LAB.

3.3 Therapeutic strategies using *L. lactis*

The earliest report on tumor regression in cancer patients as a result of bacterial infection was described in 1813, involving *Clostridium perfringens* (Mowday *et al.*, 2016). Several decades later, William Coley's work on a vaccine derived from *Streptococcus pyogenes* and *Serratia marcescens* for the treatment of cancer (Richardson *et al.*, 1999) received enormous attention and has encouraged researchers to look into the role of bacteria for the treatment of diseases. As a result, several treatment strategies such as gene therapy and DNA vaccination have implemented the use of bacteria as delivery vectors. It has also been suggested that the employment of bacterial vectors could overcome the limitations of current DNA-based therapies. Through a process known as bactofection, bacteria could mediate DNA delivery into the host cells, thus enhancing the rate of gene transfer and enable the expression of heterologous proteins (Pálffy *et al.*, 2006). Furthermore, this process can be manipulated to achieve specific or selective targeted treatments, which could be useful against a variety of diseases. Unlike conventional treatments, immunization via bacterial vectors are relatively simple and cost-effective (Delany *et al.*, 2014).

Over the years, live bacteria, including *Salmonella* (Agorio *et al.*, 2007; Yang *et al.*, 2010), *Listeria* (Pilgrim *et al.*, 2003; van Pijkeren *et al.*, 2010), *Shigella* (Galmbacher *et al.*, 2010), and *Pasteurella* (Kamal *et al.*, 2017; Othman, Parton, and Coote, 2012) have been frequently tested as potential vector candidates. Unfortunately, the use of these bacteria, despite attenuated, is faced with criticisms from the lack of evidence to prove their safety. Therefore, the GRAS food-grade *L.*

lactis may be a potential alternative to achieve maximum treatment efficacy, while maintaining minimal health risks.

One of the earliest investigations on oral immunization using *L. lactis* was reported by Iwaki *et al.* (1990), using a recombinant *L. lactis* expressing a surface protein antigen (PAC) from *Streptococcus mutans*. Upon oral immunization with this recombinant strain, significant induction of PAC-specific immunoglobulin A (IgA) and IgG were detected in saliva samples of mice, implying that this recombinant strain may be a potent immunization strategy for dental caries (Iwaki *et al.*, 1990).

Since then, a considerable amount of researches have focused on the expression of tetanus toxin fragment C (TTFC) derived from *Clostridium tetani*, to elicit immune responses. In one study, Norton *et al.* (1997) showed that mice nasally immunized with recombinant *L. lactis* experienced an elevated production of IgG1 and IgG2b in response to TTFC. Also using a mouse model, Robinson *et al.* (1997) reported similar findings upon oral administration of *L. lactis* constitutively expressing TTFC. Meanwhile, Steidler *et al.* (1998) has successfully constructed a recombinant *L. lactis* co-expressing TTFC with cytokines to boost the immune response. Following intranasal immunization, co-expression of interleukin-2 (IL-2) or IL-6 with TTFC resulted in greater levels of TTFC-specific IgA and IgG production (Steidler *et al.*, 1998). Interestingly, further analysis on the different administration routes of TTFC-expressing *L. lactis* and the resulting cellular responses have revealed that intragastric and intranasal immunization contributed to comparable levels of IgG1 and IgG2a, but is predominantly composed of IgG1 when induced via intraperitoneal route (Robinson *et al.*, 2004). In other words, the route of administration of recombinant *L. lactis* may be an important determining factor to trigger the desired immune responses of the host.

In other investigations, immunizations with recombinant *L. lactis* were designed to combat infections from a variety of pathogens. In 2005, Miyoshi *et al.* (2014) designed a recombinant *L. lactis*, capable of secreting *Brucella abortus* heat-shock protein, GroEL, under the regulation of NICE system. Using the same expression system, Pontes *et al.* (2003) previously developed an *L.*

lactis strain producing L7/L12 ribosomal protein to provide immunity towards brucellosis. For vaccinations against *Helicobacter pylori* infections, the incorporation of a vital virulence factor during gastric colonization, urease subunit beta (ureB), have been widely reported (Chen *et al.*, 2006; Lee *et al.*, 2001; Zhang *et al.*, 2014). Although initially Lee *et al.* (2001) claimed that ureB failed to give immunoprotective levels of antibody production, findings from other studies have been quite favourable and *L. lactis* was concluded as a promising vector for anti-*H. pylori* immunization strategies. Apart from that, *L. lactis* has been genetically modified to express listeriolysin O (LLO) for intraperitoneal immunization acting against *L. monocytogenes* infections (Bahey-El-Din *et al.*, 2008, 2010). The expression of LLO by *L. lactis* has successfully induced the generation of IgG1 and IgG2a, along with an increase of CD8⁺ responses (Bahey-El-Din *et al.*, 2008). In an attempt to refine the *L. monocytogenes* vaccination strategy, *L. lactis* was transformed with a plasmid harbouring genes encoding for LLO and listerial antigen, P60 (Bahey-El-Din *et al.*, 2010). Even though no remarkable improvement was observed, it was suggested that the level of P60 were too low and may require additional optimization.

For vaccination against viruses, *L. lactis* has been tested to deliver viral antigens from H1N1 virus, human immunodeficiency virus (HIV), and human papilloma virus (HPV), among others. The most encouraging results were seen when *L. lactis* was designed to express E7 oncoprotein of HPV type 16 (HPV-16) for cervical cancer vaccines. The constitutive production of E7 protein was previously recognized in cervical carcinomas (McLaughlin-drubin and Münger, 2009), and recombinant *L. lactis* expressing this protein has been developed to deliver the antigen in mouse models. According to Bermúdez-Humarán *et al.* (2004), *L. lactis* expressing E7 protein under an induced system generated significantly greater levels of IL-2 and interferon gamma (IFN γ) when compared to a constitutive expression system. The different cellular localizations of E7 production also evoked different levels of immune response, with the highest response obtained when E7 protein expression was targeted to the cell wall. This HPV-16 vaccination strategy was later improvised

by utilizing *L. lactis* expressing both a cell wall-anchored E7 protein with IL-2 to provide a more potent and long-lasting immunity against HPV-associated cervical cancers (Bermúdez-Humarán *et al.*, 2005). Other antigens and proteins that have been expressed and delivered by *L. lactis* in animal and in vitro models are outlined in Table 1. This list includes the expression of carcinoembryogenic antigen (CEA) (Zhang *et al.* 2016a), anti-tumor necrosis factor (TNF) (Bernstein, Ho, and Lees, 2010), and various cytokines (Martín *et al.*, 2014; Shigemori *et al.*, 2017; Souza *et al.*, 2016) for the treatment of cancers and inflammatory bowel diseases.

4. Risks and concerns

It is commonly acknowledged that *L. lactis* is an attractive vaccine vehicle for DNA and protein delivery. However, its use have not been fully exploited due to the perception that it is less effective compared to pathogenic microorganisms, based on the non-invasive nature of this bacterium. As previously discussed, invasive recombinant *L. lactis* strains have been constructed through the expression of invasive proteins. Unfortunately, the incorporation of these proteins are feared to render the strains pathogenic. Furthermore, the use of genetically-modified strains may also present new challenges in terms of antibiotic resistance and gene transfer. In vitro, antibiotic resistant genes are often incorporated into genetic components such as plasmid DNA, to function as selectable markers. Although useful for an easy method of selection, these genes may give rise to resistant strains and could potentially transfer to other bacterial strains (Davison, 1999). Since the antibiotic resistance crisis is already a global threat that adds to the health and economic burden of the global population, it is feared that the use of genetically-modified organisms (GMO) will worsen the current deadly situation. Therefore, to avoid this potential hazard, alternative methods may be necessary to replace the use of antibiotic resistant genes for the purpose of vaccination. Should genetically-modified *L. lactis* be commercialized for disease treatments, the protocols for containment and handling of these strains will also need to be given extra attention.

Table 1. Examples of clinical and experimental studies using *L. lactis* as delivery vector for therapeutic purposes.

Target disease or pathogen	Antigen or protein	Experimental model	Sources
<i>Brucella abortus</i>	GroEL heat-shock protein	-	(Miyoshi <i>et al.</i> , 2014)
	L7/L12 ribosomal protein	BALB/c mice	(Pontes <i>et al.</i> , 2003)
<i>Clostridium tetani</i>	TtTFC	C57BL/6 mice	(Norton <i>et al.</i> , 1997; Robinson <i>et al.</i> , 2004; Robinson <i>et al.</i> , 1997; Steidler <i>et al.</i> , 1998)
<i>Helicobacter pylori</i>	UreB	C57BL/6 mice BALB/c mice	(Chen <i>et al.</i> , 2006; Lee <i>et al.</i> , 2001) (Zhang <i>et al.</i> , 2014)
	Cag12	C57BL/6 mice	(Kim <i>et al.</i> , 2006)
	HpaA and Omp22	BALB/c mice	(Zhang <i>et al.</i> , 2016b)
<i>Listeria monocytogenes</i>	LLO	BALB/c mice	(Bahey-El-Din <i>et al.</i> , 2008, 2010)
<i>Streptococcus pneumoniae</i>	PppA	Swiss Albino mice	(Medina <i>et al.</i> , 2008)
<i>Yersinia pseudotuberculosis</i>	LcrV	BALB/c mice	(Daniel <i>et al.</i> , 2009)
<i>Leishmania</i>	LACK	BALB/c mice	(Hugentobler <i>et al.</i> , 2012)
Influenza A viruses	Nucleoprotein	BALB/c mice	(Lei <i>et al.</i> , 2015)
H1N1	HA1 and nisP	BALB/c mice	(Joan <i>et al.</i> , 2016)
HIV	GAG-p24 antigen	BALB/c mice	(Chamcha <i>et al.</i> , 2015)
HPV-16	E7 protein	C57BL/6 mice	(Bermúdez-Humarán <i>et al.</i> , 2004, 2005)
Rotavirus	VP8 protein	BALB/c mice	(Rodríguez-Díaz <i>et al.</i> , 2011)
	VP6 protein	BALB/c mice	(Esteban <i>et al.</i> , 2013)
Hepatitis C	HCc core antigen	C57BL/6 mice	(Parlane <i>et al.</i> , 2011)
Hepatitis E	ORF2 capsid protein	BALB/c mice	(Gao <i>et al.</i> , 2015)
Cancer	Endostatin	Wistar rats	(Li and Li, 2005)
	Kisspeptin	HT-29 cell line	(Zhang <i>et al.</i> , 2016c)
	TRP2-tumor antigen	CHO-S cell line	(Kalyanasundram <i>et al.</i> , 2015)
	CEA	BALB/c mice	(Zhang <i>et al.</i> , 2016a)
Colitis	Heme-oxygenase 1	C57BL/6 mice	(Shigemori <i>et al.</i> , 2015)
	Anti-TNF	BALB/c mice	(Bernstein <i>et al.</i> , 2010)
Crohn's disease	IL-4	CHO cells and BALB/c mice	(Souza <i>et al.</i> , 2016)

From the vector aspect, the use of Gram-positive, food-grade *L. lactis* already eliminates the risks surrounding issues with pathogenicity. In general, *L. lactis* is not known to cause any serious illness in humans. However, its effects on immunocompromised patients are not fully understood, and the risk of sepsis is a primary issue that should be thoroughly considered. In one study, Wagner *et al.* (1997) demonstrated that several LAB probiotic strains are mainly harmless

towards adult immunodeficient mice, showing no signs of mortality or morbidity after 4 to 12 weeks of colonization by probiotic strains. Upon further investigation, 36% and 21% of infant mice born to mothers colonized by *Lactobacillus casei* GG and *Lactobacillus reuteri*, respectively, were found to be dead in less than four weeks. Meanwhile, case reports on two premature infants suffering from sepsis due to *Lactobacillus* LGG were described by Kunz *et al.* (2004). In both cases, the infants have

been previously diagnosed with short bowel syndrome and have received intakes of *Lactobacillus* LGG probiotic supplements. In a rare event, an otherwise healthy 14-year old girl was diagnosed with mild gastroenteritis and was described as the first reported case of liver abscess as a result of *L. lactis cremoris* (Nakarai *et al.*, 2000). Thus, based on these findings, it can be concluded that although the use of probiotic strains are typically safe on healthy individuals, the risks of infections and side effects may be present in neonatal and immunocompromised patients. Throughout the process of vaccine development, clinical trials, and post-commercialisation, continuous monitoring and evaluation of *L. lactis*-based treatments need to be proceeded with caution to ensure that such incidents could be prevented.

CONCLUSION

Overall, the identification and characterization of *L. lactis* have led to significant advancements in its expression and delivery systems. By using *L. lactis* as a model organism, the established genetic tools could be extended to other LAB strains and help refine current vaccination and bacteria-mediated therapeutic strategies. The simplicity and ease of use of *L. lactis* also enable researchers to conduct necessary evaluations on a broad spectrum of diseases and their treatments, with much flexibility. But in order to fully exploit these potentials, several research questions are yet to be answered. Firstly, it would be of interest to determine the rate of DNA or gene transfer that could be achieved when using *L. lactis* as a vector. This information would be significant in order to verify the efficacy of this treatment approach. Once this is understood, various strategies could be designed to enhance the efficiency of gene transfer. Several possible aspects that could be investigated are the dosage, treatment administration route, and mechanisms for achieving targeted delivery. Other than that, it is also important to validate the safety of *L. lactis*-mediated treatments. Risks of infections and side effects in immunocompromised patients are some of the factors that should be scrutinized and studied over the course of treatment.

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