MINIREVIEW

Mucosal targeting of therapeutic molecules using genetically modified lactic acid bacteria: an update

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Abstract

Lactic acid bacteria (LAB) represent a heterogeneous group of microorganisms naturally present in many foods and those have proved to be effective mucosal delivery vectors. Moreover, some specific strains of LAB exert beneficial properties (known as probiotic effect) on both human and animal health. Although probiotic effects are strain-specific traits, it is theoretically possible, using genetic engineering techniques, to design strains that can exert a variety of beneficial properties. During the two past decades, a large variety of therapeutic molecules has been successfully expressed in LAB, and although this field has been largely reviewed in recent years, approximately 20 new publications appear each year. Thus, the aim of this minireview is not to extensively assess the entire literature but to update progress made within the last 2 years regarding the use of the model LAB *Lactococcus lactis* and certain species of lactobacilli as live recombinant vectors for the development of new safe mucosal vaccines.

Introduction

Lactic acid bacteria (LAB) have been safely consumed for centuries by humans in fermented foods, and they are thus 'generally recognized as safe' (GRAS) according to the U.S. Food and Drug Administration (FDA). Indeed, some specific strains of LAB, and in particular from *Lactobacillus* and *Bifidobacterium* genus, have been used as probiotics as they are thought to play an important role in maintaining a healthy microbiota as well as contributing to many health-promoting activities (Gareau *et al.*, 2010). In this context, it is not surprisingly that due to their intrinsic properties, they represent an attractive alternative for mucosal targeting of therapeutic molecules compared with other classical delivery systems (e.g. attenuated pathogens, liposomes, and microparticles). Indeed, mucosal immunization against infectious diseases is a public health priority mainly because mucosal surface represents the major portal of entry for many pathogens. Moreover, in large vaccination programs, mucosal vaccines (particularly via intranasal, oral, or genital) are more suited and convenient than systemic vaccines (i.e. generally injectables), because they are easier to administer and relatively inexpensive to manufacture. In addition, mucosal delivery of therapeutic proteins for chronic diseases and mucosal infections could enhance their potency and specificity and most importantly reduce potential side effect of the classical systemic routes of administration.

From this perspective, in the last 20 years, a large number of studies have reported the use of different LAB for the delivery of therapeutic molecules at mucosal level (Pouwels *et al.*, 1998, 2001; Seegers, 2002; Wells & Mercenier,

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2008; Bermudez-Humaran *et al.*, 2011) focusing mainly in the model LAB *Lactococcus lactis* (Bermudez-Humaran & Langella, 2004; Bermudez-Humaran, 2009) but also in the genus *Lactobacillus* (Pouwels *et al.*, 2001; Seegers, 2002). The aim of this minireview is therefore to summarize and discuss the research made within the last 2 years using these two bacteria genus and discuss major new developments and strategies. Ongoing challenges for the future use of LAB as mucosal delivery vectors will be also discussed.

Lactic acid bacteria as delivery vectors of beneficial molecules

Mucosal administration of therapeutic molecules offers several important advantages over systemic delivery such as *in situ* delivery of the protein of interest, reduction in secondary effects, easy administration, and the possibility to modulate both systemic and mucosal immune responses (Bermudez-Humaran, 2009; Bermudez-Humaran *et al.*, 2011). The rationale for the use of LAB as new delivery vectors was initially focused on the development of mucosal vaccines, which is derived from a large body of research that shows that this type of delivery system prevents the degradation of the antigen in the gut. Moreover, because LAB are nonpathogenic and genetically modifiable, they are excellent candidates as delivery vectors of therapeutic molecules and for the development of novel preventive and therapeutic strategies for humans diseases.

Today, two different strategies for the use of genetically modified LAB (GM-LAB) as live delivery vectors of health-promoting proteins exist: (1) LAB that produce and present proteins directly in mucosal sites and (2) the use of LAB as vehicles to deliver DNA directly into eukaryotic cells (Guimaraes *et al.,* 2006; Innocentin *et al.,* 2009) (Fig. 1). However, in this review, we will only focus on the first type of delivery (e.g. protein delivery by GM-LAB).

Use of *L. lactis* as live delivery vector

Lactococcus lactis is a food-grade LAB widely used in the food industry and considered as the model LAB because many genetic tools have been developed for expression of the heterologous protein (Bermudez-Humaran & Langella, 2004). In addition, *L. lactis* is considered to be an efficient cell factory for protein production because it secretes relatively few proteins; the most commonly used laboratory strains (MG1363 and IL1403) are plasmid free (Gasson, 1983; Chopin *et al.*, 1984) and their genomes have been completely sequenced (Bolotin *et al.*, 2001; Wegmann *et al.*, 2007). Moreover, there is enough investigation today that supports the use of *L. lactis* as a mucosal delivery vector. Although the first report describing the use of recombinant *L. lactis* as a mucosal vaccine was performed in 1990 with nonviable bacteria producing a cell wall–anchored form of a *Streptococcus mutans* protective antigen (PAc) (Iwaki *et al.*, 1990); today, approximately 400 peer-reviewed publications have already been published describing recombinant *L. lactis* (with an average of ~20 new publications each year, Medline via PubMed database search) confirming the success of this LAB. In the following sections, we will provide a summary of the current research and advances made using recombinant *L. lactis* as live delivery vector of therapeutic proteins.

Use of recombinant *L. lactis* as mucosal vaccine

As mentioned above, the use of LAB and more particularly *L. lactis* as delivery vehicle was initially focused on the development of mucosal vaccines. Since then, many studies have reported engineered *L. lactis* strains as live mucosal vaccines for a large number of antigens derived from bacteria, viruses, and parasites [for review see (Bermudez-Humaran & Langella, 2004; Bermudez-Humaran, 2009)]. In this context, Cauchard *et al.* (2011) recently engineered a *L. lactis* strain secreting the virulence-associated protein A (VapA) from *Rhodococcus equi* (a Gram-positive bacterium that causes severe pneumonia in foals) and tested the immunogenic potential of the resulting strain in mice in combination with a recombinant strain of *L. lactis* secreting biologically active leptin (a pleiotropic hormone with significant immunomodulatory properties) (Bermudez-Humaran *et al.*, 2007). Mucosal administration (either intranasal or oral) of these recombinant strains led to a VapA-specific mucosal immune response and resulted in a significant reduction in *R. equi* viable counts in liver and spleen after a challenge with a virulent strain of *R. equi*. In 2011, Marelli *et al.* (2011) described the construction of recombinant *L. lactis* strains able to produce the rotavirus spike-protein subunit VP8 in cytoplasmic, secreted, and cell wall–anchored forms. Evaluation of the immune response evoked after mucosal immunization in mice shows that animals orally immunized with the *L. lactis* strain producing the cytoplasmic form of VP8 developed significant levels of intestinal IgA antibodies, while animals receiving *L. lactis* producing the cell wall–anchored VP8 form exhibited anti-VP8 antibodies at both local (i.e. intestinal) and systemic (i.e. serum) levels. Strikingly, specific VP8 antibodies evoked by *L. lactis* strains producing either the cytoplasmic (local antibodies) or the cell wall–anchored (serum antibodies) form of VP8 were able to block rotavirus infection by 50% and 100%, respectively. Later, Saez *et al.* (2012) developed a mucosal vaccine to control brucellosis based on recombinant *L. lactis* secreting *Brucella abortus* Cu-Zn superoxide dismutase (SOD). Mice immunized with this recombinant strain developed SOD-specific IgM antibodies together with

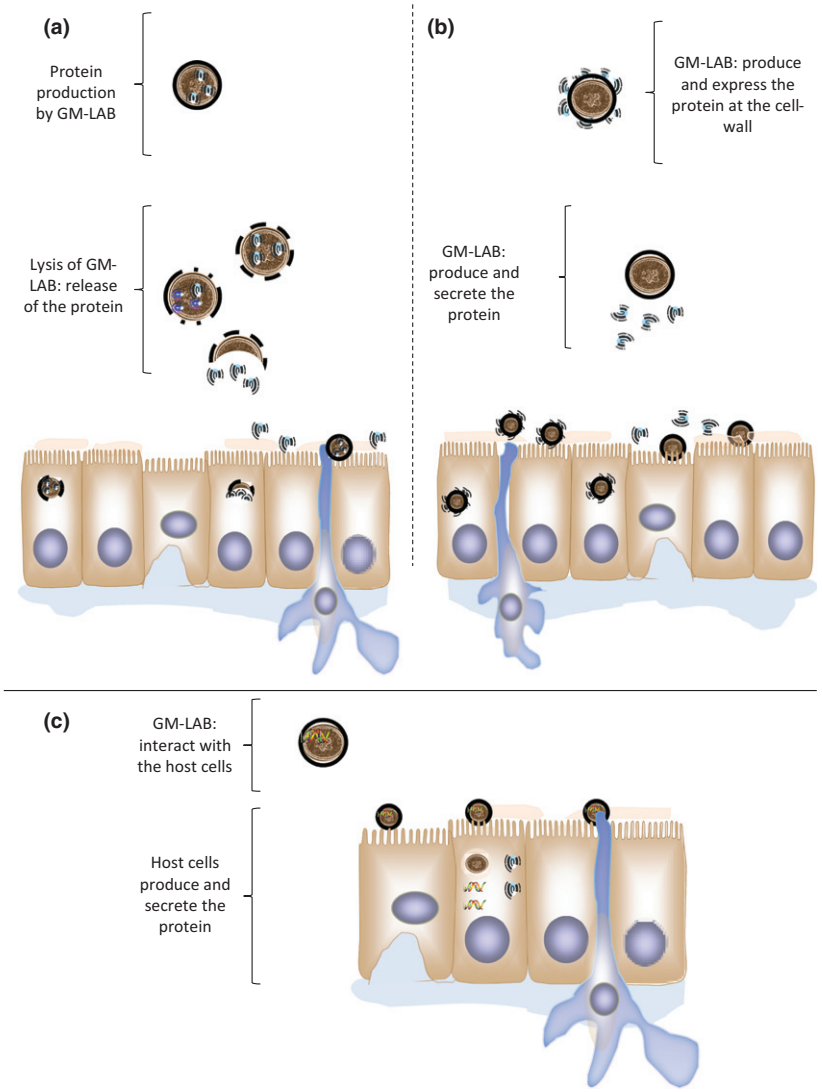

Fig. 1. Schematic representation of GM-LAB as delivery of protein antigens or DNA in the intestinal tract. (a–b) Protein antigen delivery. Protein producing GM-LAB reaches the intestinal tract and (i) after bacterial lysis, the protein is released into the intestinal lumen (a) or either (ii) secretes or (iii) or expresses an anchored form of the protein in the intestinal lumen (b). (c) DNA delivery. Bacteria harboring a plasmid containing an eukaryotic expression cassette designed to express the ORF of interest enter in target cells and escape from the phagolysosome, and after bacterial lysis, the plasmids are liberated on cytoplasm and then are transferred into the nucleus of the host cell where the expression of the ORF of interest occurs. The transduction and protein synthesis are by host cells machinery to release the protein of interest into the intestinal lumen.

SOD-specific sIgA in nasal and bronchoalveolar lavages (BAL). In addition, vaccinated animals were also protected against challenge with a virulent *B. abortus* strain. Furthermore, the immune response evoked in mice after immunization was improved when the *L. lactis* strain secreting SOD was co-administered with a *L. lactis* strain secreting biologically active IL-12 (Bermudez-Humaran *et al.*, 2003). Finally, in one of the most complete works performed in the last years using *L. lactis* as a live vaccine, Cousineau and coworkers demonstrate the interest in the use of *L. lactis* as a live vector against the Leishmaniasis, a parasitic disease affecting more than 12 million individuals worldwide (Kedzierski *et al.*, 2006). They first expressed a modified version of A2 antigen from *Leishmania donovani* in *L. lactis* in three different cellular locations: cytoplasmic, secreted, or cell wall–anchored forms and tested for their ability to generate A2-specific immune responses and as live vaccines against *L. donovani* infection in mice (Yam *et al.*, 2011). Subcutaneous immunization with *L. lactis* expressing the cell wall–anchored form of A2 induced the higher levels of antigen-specific serum antibodies, while mice immunized with *L. lactis* producing the cytoplasmic

form of A2 demonstrated the highest reduction in liver parasitemia after visceral *L. donovani* challenge. Later, the same group reported the construction of different lactococci strains expressing one of the best-studied *Leishmania major* antigens, the Leishmania homologue of activated C kinase (LACK), in the cytoplasmic, secreted, or cell wall–anchored forms, and a strain secreting biologically active mouse IL-12 (Hugentobler *et al.*, 2012a, b). Subcutaneous co-immunization with live *L. lactis* strains expressing the cell wall–anchored form of LACK and secreting IL-12 significantly delayed footpad swelling in *L. major*-infected mice. Furthermore, immunization with these two strains induced antigen-specific multifunctional TH1 $CD4^+$ and $CD8^+$ T cells and a systemic LACK-specific TH1 immune response. This same group then evaluated the effect against *L. major* infection in mice after oral immunization with recombinant *L. lactis* strains deficient in alanine racemase (alr-), an enzyme that participates in cell wall synthesis (Grangette *et al.*, 2004), expressing LACK antigen in the cytoplasmic, secreted, or cell wall–anchored forms alone or in combination with a *L. lactis* strain secreting mouse IL-12 (Hugentobler *et al.*, 2012a, b). They showed that oral immunization using live lactococci secreting both LACK and IL-12 was the only treatment that partially protected the mice against subsequent *L. major* challenge. Most importantly, protected animals displayed a delay in footpad swelling, which correlated with a significant reduction in parasite burden. These results demonstrate the potential of *L. lactis* as a live mucosal vaccine against *L. major* infection and provide the basis for the development of an inexpensive strategy to combat Leishmaniasis.

Engineered *L. lactis* strains to treat inflammatory and gastrointestinal diseases

The first description of a recombinant strain of *L. lactis* aimed to treat inflammatory bowel disease (IBD) was published over 13 years ago (Steidler *et al.*, 2000). In this pioneer study, the authors showed that oral administration of recombinant *L. lactis* secreting biologically active murine IL-10, a cytokine that has shown promise in clinical trials for treatment of IBD, prevented the onset of colitis in *IL-10*$^{-/-}$ mice and caused approximately 50% reduction in colitis in mice treated with dextran sulfate sodium (DSS). An important step forward toward the safe use of GM-LAB for human therapeutic purposes was the construction of a biological containment system for human IL-10 delivery by recombinant *L. lactis*. For this, Steidler *et al.* (2003) replaced the endogenous thymidylate synthase (*thyA*) gene in *L. lactis* by the human *IL-10* gene to generate a thymine auxotroph strain. The viability of this biologically contained strain was reduced by several orders of magnitude in the absence of thymidine or thymine in the media, and the containment was validated in pigs. Then, a small phase I clinical trial was conducted with this *thyA*-deficient *L. lactis* strain expressing human IL-10 in Crohn's disease (CD) patients showing not only that the containment strategy was safe and effective, but also that mucosal delivery of IL-10 by a GM-LAB is feasible in humans (Braat *et al.*, 2006). Subsequently, a phase II A trial was conducted and a press release was published in 2009. Although this assay confirmed the safety and the tolerability of the strain, the clinical results did not reveal a statistically significant difference in mucosal healing compared with the placebo group. Since then, several other studies confirmed the anti-inflammatory effects of the administration of GM *L. lactis* delivering IL-10 in different mouse models such as: decrease of a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced murine colitis by milks fermented by IL-10-producing *L. lactis* strains (del Carmen *et al.*, 2012), modulation of airway inflammation by *L. lactis* expressing IL-10 in a murine model of asthma (Marinho *et al.*, 2010), prevention of food-induced IgE sensitization by oral administration of an IL-10-secreting *L. lactis* in a mouse model of food allergy (Frossard *et al.*, 2007). All these results demonstrate the important role of IL-10 in inflammatory diseases in murine models. However, the absence of significant results in human trials led to suggest a less important role of IL-10 in humans and the potential of GM-LAB in delivering other new potential anti-inflammatory molecules (e.g. such as antioxidant enzymes or antiproteases) is currently under progress in many laboratories. From this perspective, it has been shown that GM *L. lactis* producing and releasing SOD, an antioxidant enzyme able to neutralize the reactive oxygen species (ROS), displayed anti-inflammatory effects in a TNBS colitis model in mice (Han *et al.*, 2006). More recently, Motta *et al.* (2012) engineered a *L. lactis* strain able to express and deliver human Elafin (a natural protease inhibitor that has pleiotropic anti-inflammatory properties *in vitro* and in *in vivo* models) to the site of inflammation in the colon to assess its potential therapeutic effect. Interestingly, in different mouse models of acute and chronic colitis, oral administration of Elafin-expressing *L. lactis* decreased elastolytic activity and inflammation and restored intestinal homeostasis. Furthermore, when cultures of human intestinal epithelial cells were treated with this strain, the inflamed epithelium was protected from increased intestinal permeability and from the release of cytokines and chemokines, both of which are characteristics of intestinal dysfunction associated with IBD. These results suggest that oral delivery of GM *L. lactis* secreting Elafin may be useful for treating IBD in humans (Motta *et al.*, 2012).

Use of recombinant *L. lactis* to treat metabolic and auto-immune disorders

Other potential use of recombinant *L. lactis* strains is the treatment of metabolic disorders such as diabetes and obesity and auto-immune disorders such as atherosclerosis and encephalomyelitis. In this context, Amar *et al.* (2011) determined the effect after oral administration of a *L. lactis* strain producing biologically active human leptin (Bermudez-Humaran *et al.*, 2007) in high-fat diet (HFD) and *ob/ob* mice for 8 weeks. Body weight and fat mass gain were reduced over the time course of the experiment, and glucose intolerance was reduced, while fasted plasma insulin concentration remained mostly unchanged. This was associated with a slight reduction in adipose tissue inflammation as confirmed by the reduction in the concentration of some cytokine. Interestingly, in the leptin-deficient mice (i.e. *ob/ob* mice), the circulating leptin concentration remained undetectable suggesting only a possible local effect of the leptin-secreting strain. This observation confirms the interest in the use of recombinant LAB for the *in situ* production and delivery of therapeutic proteins. Then, Gao *et al.* (2012) reported the construction of a recombinant *L. lactis* strain able to produce and secrete biologically active mouse insulin-like growth factor-I (IGF-I). Indeed, the authors showed that recombinant IGF-I produced by GM *L. lactis* promotes NIH3T3 cell proliferation in a concentration-dependent manner. This strain could represent an attractive strategy for mucosal IGF-I delivery and thus to treat diabetes or even neurodegenerative diseases such as Alzheimer's disease (Saez, 2012). In 2011, Jing *et al.* (2011) investigated the effect of recombinant *L. lactis* producing either a cytoplasmic or secreted form of the *Mycobacterium tuberculosis* heat shock protein 65 (Hsp65) on a murine model of atherosclerosis. Oral administration of the two recombinant strains induced suppression of HSP65-specific proliferation. Strikingly, the inducible HSP65-specific tolerance exerted a protective effect on atherosclerotic lesion formation and endothelial damage in low-density lipoprotein receptor–deficient mice model. Lately, Rezende *et al.* (2012) showed that oral administration of a recombinant *L. lactis* strain secreting *M. leprae* Hsp65 prevented the development of experimental auto-immune encephalomyelitis (EAE) in mice.

Use of *Lactobacillus* spp. as live delivery vector

The genus *Lactobacillus* contains more than 80 recognized species widely differing in biochemical, ecological, molecular, and immunological properties. This huge variation is reflected, for example, by the large differences in the DNA G+C content. Then, the control of transcription and translation may differ greatly from one species to the other, implying that knowledge generated for one organism may not simply be transferred to another. This means that genes that are efficiently expressed in one *Lactobacillus* species are not necessarily expressed in other species or are expressed with a different efficiency and/or with a different regulation of the gene expression (Pouwels & Leer, 1993; Pouwels *et al.*, 1998).

Use of recombinant lactobacilli as mucosal vaccine

Although there is much less research carried out with the genus *Lactobacillus* regarding their use as live mucosal vaccine (probably because its fastidious genetic manipulation and species-specific genetics tools, see above), in the last years the use of GM lactobacilli to produce heterologous proteins and to develop a new generation of mucosal vaccines has greatly increased. Indeed, today, more than 70 peer-reviewed publications have been published (Medline via PubMed database search) confirming the advantages of this genus to serve as live mucosal vaccine. Some of the advantages in the use of lactobacilli as a live vector certainly lie in the fact that they can persist longer in the digestive tract and some strains have intrinsic probiotic properties (Gareau *et al.*, 2010; Kechaou *et al.*, 2013). Markedly, comparable to *L. lactis*, several studies comparing and analyzing the expression of a variety of viral, bacterial, or eukaryotic origin proteins in several different strains of *Lactobacillus* spp. have been conducted (Seegers, 2002; Bermudez-Humaran *et al.*, 2011). The current research and advances made in the last years on the use of recombinant lactobacilli as live delivery vector will be discussed in the following paragraphs.

Fredriksen *et al.* reported the construction of two *Lactobacillus plantarum* strains secreting or anchoring at its cell wall an oncofetal antigen (OFA, a tumor immunogen expressed on all mammalian cancers). Mice orally immunized with these strains developed an OFA-specific immune response (Fredriksen *et al.*, 2010). In another interesting study, Adachi *et al.* (2010) showed that oral immunization in mice with a recombinant *Lactobacillus casei* strain expressing HPV-16 E7 antigen [an oncoprotein widely studied because of its implication in HPV-16-related cervical cancer (Bermudez-Humaran *et al.*, 2002, 2005)] was able to induce mucosal-specific E7 cytotoxic cellular immune responses. Then, Yoon *et al.* (2012) developed a HPV mucosal vaccine candidate using the pgsA protein to display a partial HPV-16 L2 peptide on the surface of *L. casei*. Oral immunization with this recombinant strain induced production of L2-specific serum IgG and vaginal IgG and IgA in mice. Strikingly,

the authors also showed that *L. casei* expressing the HPV-16 L2 peptide induced significant neutralizing activities against genital infection by HPV-16, HPV-18, HPV-45, and HPV-58 pseudovirions.

In 2010, in an effort to control AIDS epidemics, Vangelista *et al.* (2010) reported a promising strategy for the prevention of HIV-1 sexual transmission. This strategy consists of the use of a *Lactobacillus jensenii* strain (a human vaginal isolate) as live microbicide for the topical production of HIV-1 inhibitors (Vangelista *et al.*, 2010). For this, they constructed GM *L. jensenii* strains able to secrete the anti-HIV-1 chemokine RANTES, as well as C1C5 RANTES (a mutated analog that acts as a CCR5 antagonist and thus devoid of proinflammatory activity). Purified RANTES variants secreted by recombinant *L. jensenii* were shown to inhibit HIV-1 infection in $CD4^+$-T cells and macrophages, displaying strong activity against HIV-1 isolates of different genetic subtypes. This exciting work provides proofs for the use of GM lactobacilli producing RANTES molecules to block HIV-1 infection. Later, Kajikawa *et al.* (2012) constructed a recombinant strain of *Lactobacillus acidophilus* expressing the HIV-1 Gag at its cell surface by fusion with the signal peptide (SP) and anchor motif of a mucus-binding protein (Mub) from *L. acidophilus* with or without co-expression of the *Salmonella* flagellin (FliC) fused to Mub SP and anchor. Then, they showed that oral immunization in mice with recombinant *L. acidophilus* producing HIV-1 Gag resulted in an induction of interferon γ-producing cells at local mucosa, while immunization with *L. acidophilus* displaying both Gag and FliC resulted in an increase in Gag-specific IgA-secreting cells.

Kandasamy *et al.* (2011) used the strain *L. rhamnosus* GG, an isolate successfully used to induce tumor regression in an orthotopic model of bladder cancer (Seow *et al.*, 2010), to secrete either the prostate-specific antigen (PSA) or a form of PSA fused to IL-15, a cytokine known for its effects in both the activation and migration of neutrophils and T cells and dendritic cells activation and proliferation. They thus showed that recombinant *L. rhamnosus* GG activated neutrophils and induced DC maturation, T-cell proliferation, and PSA-specific cytotoxic T lymphocytes. In addition, IL-15 enhanced direct DC activation of CTL (Kandasamy *et al.*, 2011). Later, Moeini *et al.* (2011) used the AcmA binding domains of *L. lactis* to display the VP1 protein of chicken anemia virus (CAV) on *L. acidophilus*. Oral immunization of chickens with this recombinant strain resulted in a moderate level of systemic anti-CAV neutralizing antibodies. A VP1-specific proliferative response was also observed in splenocytes of immunized chickens.

Xu *et al.* (2011) showed that a GM strain of *L. casei* co-expressing a cytotoxic T lymphocyte (CTL) epitope of the classical swine fever virus (CSFV) and the VP2 antigen of porcine parvovirus (PPV) (two highly contagious pathogens, resulting in enormous economic losses in pig industries worldwide) can efficiently stimulate mucosal and systemic CSFV-specific $CD8^+$-CTL responses and protect pigs against CSFV challenge.

Finally, other recent studies demonstrated lactobacilli prototype vaccines against pancreatic necrosis virus (IPNV, a pathogen that infects wild and cultured salmonids) (Min *et al.*, 2012; Zhao *et al.*, 2012), a highly pathogenic avian influenza (HPAI) (Wang *et al.*, 2012), and porcine epidemic diarrhea virus (PEDV) (Liu *et al.*, 2012).

Use of recombinant lactobacilli to treat IBD

Similar to *L. lactis*, the *Lactobacillus* genus has also been used as delivery vector to treat inflammatory and gastrointestinal diseases and more particularly (IBD). From this perspective, LeBlanc *et al.* (2011) reported the effect of a GM *L. casei* strain producing either catalase (CAT) or SOD (two antioxidant enzymes) in a TNBS colitis model. Oral administration of mice with the CAT- or SOD-producing *L. casei* strain showed a faster recovery of initial weight loss, increased enzymatic activities in the gut, and lesser extent of intestinal inflammation compared with control animals. These results suggest that GM-LAB producing antioxidant enzymes could be used to prevent or decrease the severity of certain intestinal pathologies such as IBD. In another very recently study, Qiu *et al.* (2013) studied the effect of a recombinant strain of *L. casei* expressing IL-10 combined with 5-aminosalicylic acid (5-ASA) in a DSS-induced colitis mice. The results showed that recombinant *L. casei* expressing IL-10 combined with 5-ASA was more effective in preventing the inflammatory signs than the wild-type strain of *L. casei* with 5-ASA.

Conclusion

This study reviewed how GM-LAB, and more particularly *L. lactis* and lactobacilli, can be used as novel therapies for a wide variety of diseases. Examples of the most recent applications in different diseases that allow LAB to express numerous beneficial compounds were provided. The consumption of engineered strains by humans is still highly controversial due to the public perception that genetic manipulation is not 'natural'. Scientists must perform well-designed studies where the results are divulged to the general populations to inform consumers of the obvious beneficial effects these novel techniques can confer with the minimum of risk to their health and to the environment. Throughout the course of history, most novel treatments have met resistance from potential

benefactors; it is thus important to show that the potential benefits are superior to the risks of novel treatments to be completely accepted by the population as a whole.

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Authors' contribution

J.G.L. and C.A. contributed equally to this work.

Competing interests

The authors declare that they have no competing interests.

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