**Lactic acid bacteria - promising vector vaccines: possibilities, limitations, doubts.**

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**Abstract**

Gram-positive and nonpathogenic lactic acid bacteria (LAB) areconsidered to be promising candidates for the development of new, safe systems of heterologous protein expression. Additionally, many experiments have shown that specific systemic and mucosal immune responses against selected pathogens can be elicited using modified LAB strains. For that reason they could be a good replacement of classical, often pathogenic, attenuated carriers. This solution offers many advantages in comparison to systemic inoculation, as well as, from immunological and practical points of view. The development of efficient mucosal vaccines is nowadays a priority in modern vaccinology, together with improvement of immunization efficiency, monitoring of antigen production *in vivo,* determination ofoptimal dose for vaccination,strain selectionand characterization.

**Key words:** lactic acid bacteria, live vector vaccines, oral administration, heterologous gene expression, gut microflora

**Introduction**

Vaccination is the most effective method of preventing and controlling of infectious diseases, that can often lead to eradication of the infectious factors from the environment. The World Health Organization (WHO) reports that licensed vaccines are currently available to prevent or contribute to the prevention and control of about twenty-five most common and dangerous infection diseases (WHO, 2012).

That is why vaccines play a profound role in the improvement of human and animal health. Induction of an effective immune response to the particular antigen is considered to be the main goal of vaccination. This approach should provide long lasting protection against particular infections. Recently, many researchers focused their interest on development of new, safe, mucosal vaccines which production is less time and cost consuming, less laborious and which are easy to apply. Additionally, they should prevent carriage of pathogens in the population with very limited influence on maternal antibodies in infants. Most often these vaccines are based directly on pathogenic antigens, in spite of that when given orally they induce low or non-existing immune response. The most possible reason of this low efficacy is connected with ineffective microbial adsorption, fast antigen degradation, and induction of mucosal tolerance. However, although development of efficient oral vaccines can cause many difficulties, advantages of this approach are still predominant. In recent works, two major trends can be observed (Husband 1993; Walker 1994; Lamm 1997; Wells *et al*. 1997; Holzapfel *et al.* 1998). One is based on synthetic systems like microspheres, liposomes, nanoparticles, or ISCOMS (immune stimulating complex). The other, on live viral or bacterial vectors that enables production of antigens *in vivo*. In the latter case, construction of stable recombinant strains that will synthesize sufficient amounts of antigen *in vivo* with no risk for the vaccinated host or the environment can be problematic. An innovative solution is the use of lactic acid bacteria (LAB), the choice is based on a number of very useful properties of these microbes. First of all, LAB are granted the so-called “GRAS status”, meaning that they are nonpathogenic and their application is safe for humans and animals. The absence of lipopolysaccharides (LPS) in their cell wall is a great advantage that eliminates the risk of endotoxic shock. Additionally, the food industry has a long and productive experience in the large scale production and safe storage of these microorganisms what can simplify preparation and storage of the potential vaccine (Holzapfel *et al.* 1998). LAB are mostly known for their widespread use as starter strains in food and feed fermentation technology, but also for the probiotic effect that some species or strains reveal in humans and animals (Marteau *et al*. 1993; Aguirre *et al.* 1993). Most LAB are quite acid resistant which enables effective survival after passage through the stomach - a vital point in oral vaccine administration. Some strains, mostly belonging to the *Lactobacillus* genus, effectively colonize cavities, where they play a crucial role in maintaining a balance in the natural microflora of the host. Adhesive properties are considered to be an important quality that ensures long lasting presence of particular LAB strains in the host and can extend the time of antigens presentation to the immune system. Specific lactic acid bacterial strains can also have an immunomodulatory effect on human and animal organisms, which has been confirmed for many antigens from infectious diseases, allergy promoting proteins, and therapeutic antibodies (Naukkarinen *et al*. 1986; Capron *et al.* 1995). The precise molecular mechanism of LAB-induced immunomodulation is not fully recognized, although it was confirmed that they can affect e.g. dendritic cells (DC) maturation and induce cytokine secretion through toll-like receptors (TLR), including TLR2. Some studies evidenced the role of LAB in peripheral T-cell hyporesponsiveness, and promotion of regulatory T-cell development by DC modulation (Rigaux *et al.* 2009). The natural adjuvanticity of LAB is an attractive aspect in oral vaccine development. LAB have also been shown to be efficient in producing heterologous antigens (e.g. *Helicobacter pylori* urease, LcrV antigen from *Yersinia pseudotuberculosis*, EP7 antigen of the human type 16 papilloma virus*,* HA of *avian influenza virus*) (Bermúdez-Humarán *et al.* 2002; Szatraj *et al.* 2014; Zhou *et al.* 2015). All mentioned aspects make LAB an attractive and promising host for oral vaccine production and delivery of many different compounds (Fig. 1).

**LAB and gut-associated lymphoid tissue (GALT)**

The gastrointestinal tract is intensively exposed to pathogens and various biologically active factors, among them antigens and carcinogens. Lymphatic tissue associated with the gut plays a crucial role in the local and systemic immune response. Its role relies on mediating the migration and homing of the activated cells from the gut to other sites of the body. The mucosal immune system is responsible for about 60% of the immunoglobulin daily production. LAB are among bacteria which occur physiologically in the digestive tract of animals and humans. They ensure balance in the composition of the gut microflora and participate with other factors in regulation of many physiological processes such as allergies and inflammations (Gonzaga *et al.* 2009). Although the main goal of application of LAB in dairy food production is fermentation process of milk they are also used, because of their health-promoting effects, which include enhancement of non- and specific immune responses, as well as control of intestinal infections and anti-tumorigenic activity. Through their actions, connected with activation of inflammatory immune responses (IgA antibodies production), production of exopolysaccharides, and antibiotic-like substances, LAB assure the integrity of the gut mucous membrane. They also influence the distribution and the number of lymphoid cells in lymphatic tissues associated with the gut. However, the molecular mechanisms that lactic acid bacteria use to affect the immune system are unknown. Probably, LAB alone or their products are absorbed by M-cells and transported to lymphatic follicles where they are analyzed by the immune system (Mestecky 1987; Ernst *et al.* 1988; Lidbeck *et al.* 1993; Gibson *et al.* 1994). Several experiments showed that orally applied lactobacilli are assimilated by M-cells in mouse Peyer’s patches 6–12 hours after administration and can be found in mesenteric lymph nodes 48 hours after bacterial ingestion (Mozzi *et al.* 2010). It is possible that LAB can directly impact gut epithelium cells, which contain lymphocytes able to produce a wide scale of cytokines, and influence the local immunity (Fig. 2).

Interactions of LAB and products of their metabolism with immunocompetent cells, such as macrophages and T-cells, can stimulate production of cytokines. It is hypothesized that cytokines can be induced by LAB through two possible pathways: (i) cytokine secretion can be caused by antigen presentation to T-lymphocytes or (ii) after direct interaction between LAB and immunocompetent cells. These speculations are very possible in case of fact that lymphocytes and macrophages are equipped with specific receptors for LAB peptidoglycan (Fuller 1997). Two different scientific groups proved that the peptidoglycan of lactic acid bacteria can induce the secretion of IL-1 (stimulation of T- and B-cell proliferation), IL-6 (induction of B-cell differentiation) and TNF-α (anti-tumorigenic activity) by monocytes (Bhakdi *et al.* 1991; Dziarski 1991; Fernandes *et al.* 1995). TNF-α interferon induces expression of MHC class I and class II antigens, stimulates T-helper lymphocytes, activates macrophages which can enhance vaccine immunogenicity (Heumann *et al.* 1994). The most important factors that influence the immunomodulative character of LAB are connected with:

a) abilities of the immune system modulation characteristic for particular strains

b) dose of the bacteria applied to the host (Murray 1988; Perdigón *et al.* 1992; De Petrino *et al.* 1995)

c) bacterial status: live or dead bacteria (Paubert-Braquet *et al.* 1995)

 d) age and physiological state of the host

The fact that LAB are internalized in the gut via different pathways enabled them to induce different immune responses. The interaction of LAB with M cells activates mainly specific immune responses. The interaction with FAE cells (follicle-associated epithelial cells) induces a non-specific or inflammatory response. Interaction of LAB with epithelial cells can lead to enhancement of local immunity what can lead to antigen elimination (Portier *et al*. 1993; Gonnella *et al.* 1998). It occurred that *Lactobacillus casei, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* affect the systemic humoral immune response, which can be an attribute taken into consideration during selection of appropriate lactobacilli strains as an effective antigen carriers. Studies show that choice of specific LAB for oral administration is crucial for directed modulation of the systemic immune response according to the observed cytokine profiles. An significant increase of interleukins IL-10 and IL-4 levels was observed in mice fed with *Lact. delbrueckii* ssp*. bulgaricus* or *Lact. casei*. In turn, induction of IL-2 and IL-12 was observed only in case of *Lact. acidophilus*. The role of produced cytokines in the balance Th1=Th2 was determined. *Lact. casei, Lact. delbrueckii* ssp*. bulgaricus and Lact. acidophilus* increased the IgG1 response in favour to Th2. *Lact. acidophilus* induced the IgG2a response, with advantage for Th1. While, *Streptococcus thermophilus* was neutral for Th1=Th2 balance. The studies showed that different lactic acid bacteria stimulated different mucosal cytokine profiles. Thus, selection of probiotic strain with immunological properties must be well defined to influence cytokine expression that favour the claimed immune response (Martin *et al*. 1998; Hershberg *et al.* 2000).

The non-specific immune response (the first line of defense) is connected with monocytes, macrophages, neutrophils and NK cells. Phagocytosis leads to intracellular reactions, and production of reactive oxygen and nitrogen radicals, also additional factors like TNF-α and IL-1 (Perdigón *et al.* 1988; Moineau *et al.* 1991; Balasubramanya *et al.* 1995; Tizard 2000). Results from different studies on animals show that the level of phagocytic cell functions, just like in case of systemic immune response, depends on the strain of applied bacteria. The secretion of lysosomal enzymes by macrophages in mice fed with fermented milk containing *Lact. casei* was more effective compared with the animals supplemented with *Lact. acidophilus* or *Strep. thermophilus*. Tortuero *et al*. (1995) reported an increase in interleukin-2 concentration in ileal tissues in piglets treated with *Strep. faecium* M-74 and *Lact. casei* spp. The histological preparation indicated an increased phagocytic activity of these cells. The differences in cell-wall composition were probably responsible for the different activity of tested lactobacilli strains (Tortuero *et al*. 1995). Moreover, strains which were able to survive in the gastrointestinal tract and adhere to the intestine mucous membrane induced a far more effective immune response (Perdigón *et al.* 1992). Stimulation of the non-specific immune system by fermented products is probably due to immune-active peptides produced during fermentation (Fiat *et al.* 1993). The induction of a mucosal immune response can be difficult to achieve due to the potential development of oral tolerance. Yet, Perdigón et al. presented that some of lactic acid bacteria strains are able to induce specific secretory immunity, while other enhance the gut inflammatory immune response. *Lact. casei* and *Lact. plantarum* were able to interact with Peyer’s patch cells which resulted in an increase in IgA, CD4+ cells, and specific antibodies directed against the stimulating strain. Some *Lactococcus lactis* and *Lact. delbrueckii* ssp*. bulgaricus* strains induced an increase in IgA+ cells entering the IgA cycle, but not CD4+ cells (Perdigón *et al.* 1999). In the study by Herías *et al.* (1999), gnotobiotic rats fed with *Lact. plantarum* in combination with *E. coli* showed lower amounts of *E. coli* in the small intestine and caecum one week after colonization, compared with a group colonized with *E. coli* alone. It appeared that colonized rats had a significantly higher total serum IgA levels and slighly higher IgM and IgA antibody levels against *E. coli* than those colonized with *E. coli* alone (Herías *et al.* 1999).

Health-promoting effects of LAB, enhancement of non specific and specific immune responses, as well as control of intestinal infections has been confirmed in a large number of different publications. Nader de Macias *et al.* (1992) reported increased resistance to *Shigella* infection mediated by high titers of anti *Shigella* antibodies in serum and in intestine secretions in mouse fed with fermented milk (Nader de Macias *et al.* 1992). Also, Paubert-Braquet *et al*. showed a correlation between an increased immune response and resistance to *Salmonella typhimurium* infection in mice fed with milk cultures (Paubert-Braquet *et al*. 1995). In another study, the application of *Lact. casei* and *Lact. bulgaricus* was reported to terminate corticoid-induced immunosuppression in mice with *Candida albicans* infection (De Petrino *et al.* 1995). Animals inoculated with lactobacilli showed a significant increase in specific and non-specific immune responses and reached higher levels of antibodies compared with non-immunosuppressed control animals. According to Alvarez *et al*. (1998), cultures of *Lact. casei* protect the host against *Salm. typhimurium* infection not only after the first application, but the effect is maintained after a simple revaccination (on day 15 or 30). A protective effect was observed when the number of IgA-secreting cells in the lamina propria and the level of secretory IgA in the gut fluid increased (Alvarez *et al*. 1998). This correlated with an increased number of polymorph nuclear cells that induced an inflammatory immune response and was noted to influence the mucous membrane integrity. It was discussed that the population of CD4+ and CD8+ T-lymphocytes as a whole could be increased, but the balance between them should be maintained. Higher level of cells in the CD8+ T-cell population could induce an inflammatory response through the cytotoxicity effect. On the other hand, the elevated number of CD4+ T-lymphocytes, peculiarly raised the Th1 population, could through the cytokine pathway, stimulate an enhanced expression of HLA class II. This event could be associated with a higher capture of antigens and overstimulation of the mucous.

In conclusion, orally administered live LAB can modulate the systemic immune response that is highly related with the strain and the dose applied. All available in literature reports indicate LAB as a very interesting and attractive candidates for the development of new generation live vector vaccines.

**The choice of appropriate genus**

Based on the existing information about LAB, the choice of appropriate strain for the live vector vaccine development seems to be crucial to succeed. To make the choice more specific, so far three different genus of lactic acid bacteria have been quite well characterized not only based on the biological properties of strains but also according to the available molecular tools and experimental approaches as part of the LABVAC European research network:

a.) *Lactococcus lactis*

*Lactococcus lactis* strains does not colonize the digestive tracts. In mice, these microbes reside for about 24 hours and only passive transit is observed. In humans, lactococci persist in the gut for about 3 days (Klijn *et al.* 1995; Chamberlain *et al.* 1997; Havenith *et al.* 2002). Due to these facts attention has been focused on expressing various antigens inside *L. lactis* cells or in fusion with the cell wall. The first high-level inducible expression systems developed for *L. lactis* were based on the properties of the *E. coli* T7 bacteriophage RNA polymerase (pLET vectors). This system was used for a successful intracellular production of many heterologous antigens in *L. lactis* (e.g. diphtheria toxin fragment B, immunogen of *Shistosoma manson* 28 kDa, tetanus toxin fragment C (TTFC) (Wells *et al.* 1996; Robinson *et al.* 1997; Mercenier *et al.* 2000). Additional pLET vectors were modified to secrete the antigen (up to 3 mg l-1) as well as anchor it to the cell surface. Also a variety of TTFC fusion proteins, like TTFCHIV-gp120V3 loop fusion proteins were produced with quite good efficiency ranging about 2-20% of total soluble cell proteins. Analogous study has been performed with the glutathione S-transferase (P28) of the parasite *Schistosoma mansoni* (Capron *et al.* 1995). The P28 antigen has been efficiently expressed in *L. lactis,* separately and also as a fusion to TTFC. The immunogenicity of these antigens has been proven by *in vivo* experiments (Klijn *et al.* 1995; Chamberlain *et al.* 1997).Lactococcican also be treated as vehicles that can deliver cytokines. For example, the murine IL2- and IL6-encoding genes were expressed in *L. lactis*. The recombinant strains were shown to produce active interleukins in the culture supernatants at a level of 0.9 mg l-1(Steidler *et al.* 1995). In a second stage, *L. lactis* strains accumulating TTFC in the cytoplasm and secreting IL2 and IL6 were used for immunizations. A strong adjuvant effect was confirmed only for the live modified strains. The anti-TTFC serum IgG titers were 10- to 15-fold higher as compared to response generated by immunization with strains producing only TTFC, also they increased more rapidly. In contrast, the level of anti-lactococcal immune responses was not enhanced by co-expression of the cytokines (Walker 1994; Miettinen *et al.* 1996). Cell localization of the expressed heterologous protein, and the route of administration is also meaningful, and can differ with immune response level. Recombinant *L. lactis* producing three different types of TTFC: intracellular, membrane-anchored, extracellular, proteins were expressed. The strains were administered into mice subcutaneously, without any adjuvant. All three lactococcal strains were able to evoke protection against lethal toxin challenge (i.e. 5-20 x LD50 of tetanus toxin). The effective dose of *L. lactis* was mainly dependent on the amount of antigen produced. In case of the TTFC anchored to the cell membrane, protective antibodies were produced more intensively. In further experiments, mice were immunized by oral and intranasal routes. Nasal inoculation of mice with the strain expressing TTFC intracellularly using the lactococcal T7 system led to significant IgG serum antibody response, and 75% protection from lethal challenge with tetanus toxin (20 x LD50). The antibody titers were similar whether live or inactivated (mitomycin C- or formalin-treated) *L. lactis* were used. It was shown that mice can also be immunized orally with *L. lactis* strains producing TTFC at high levels (T7 expression system) or at a 10-fold lower level (pTREX1). TTFC-specific serum antibody responses of both the IgG1 and IgG2a isotypes were induced and significantly elevated levels of anti-TTFC IgA antibodies were detected in feces and gut secretions. Even though the antibody titers were lower than those following nasal immunization, the protective efficacy was similar (Wells *et al.* 1996; Robinson *et al.* 1997).

Different vectors with constitutively active promoters of low to medium strength have been applied for heterologous gene expression in *L. lactis*. For example, the pTREX1 vector has been used to express TTFC and P28 proteins with efficiency of 1-3% of total cell proteins (48). These kind of vectors are suitable for the expression of membrane associated antigens which show some insolubility or toxicity to bacterial cells. In our previous work, Szatraj et al. (2014), adjustable *ptcB* promoter in *pIL253* vector for production of different kinds of viral haemagglutinin (HA) was used. The same vector was applied by Kasarello et al.(2015) during experiment connected with oral administration of *L. lactis* expressing synthetic genes of myelin antigens. Many researchers rely upon the use of commercially available nisin-inducible expression system, which is based on variety of different vectors, adapted for intra- or extracellular protein expression. Probably it is the most often used expression system in Gram-positivie bacteria nowadays (Kleerebezem *et al.* 1997). It is also worth to note that some particular *L. lactis* strains exhibit quite good persistence in the gastrointestinal tract of animals (Boguslawska *et al*. 2009) as well as an adhering capabilities (Radziwill-Bienkowska *et al.* 2014; Radziwill-Bienkowska *et al.* 2016). These finding make *L. lactis* more attractive as a vaccine delivery vector.

b.) *Streptococcus gordonii*

*Streptococcus gordonii* is a member of the normal human microflora. It colonises the oral and vaginal cavities, but persists only transiently in the digestive tract. Naturally competent genetic system of expression of heterologous proteins on the surface of *Strep. gordonii* strain CH1 is called “Challis” (Medaglini *et al.* 1995; Medaglini *et al.* 1997; Pouwels *et al.* 1998). It is based on chromosomal integration of recombinant DNA encoding the vaccine antigen fused with the M6 surface protein of *Strep. pyogenes*. In this system, it is possible that the antigen is simultaneously released to the medium and is present in the anchored form. In *Strep. gordonii,* recombinant proteins ranging in size from 15 to 441 amino acids have been produced effectively. E7 protein of human papilloma virus type 16, the V3 domain of HIV-1 gp120, an allergen from hornet venom, ovalbumin, the surface proteins (F and H) of the measles virus, the B subunit of the heat labile toxin (LTB) of *E. coli* can be distinguish in this group (Salminen *et al.* 1996). Nine chromosomal integration sites have been identified. What is interesting a recombinant strain expressing simultaneously at the cell surface two different antigens has been constructed. Conducted experiments revealed that use of this strain in single dose led to stable colonization in the oropharyngeal of animals, colonization was inoculum-independent (107-109 cfu), antigen expression was stable *in vitro* and antigen specific local (IgA) and systemic (IgG) antibodies were produced. The effectiveness of bacterial colonization was crucial for the observed immune response (Moineau *et al*. 1991; Tizard 2000). *Strep. gordonii* was the first recombinant commensal bacterium used as a live vector vaccine (Medaglini *et al.* 1995). According to its colonization capabilities, which allows prolonged exposure of the host to the antigen, this bacterium seems to be an attractive candidate for a vaccine delivery vector. Intragastric immunisation with strains producing LTB was the only one described so far. However, bacteria did not colonize the intestinal mucosa, but they did induce serum IgG and faecal IgA. Novel results indicate that phagocytosis of *Strep. gordonii* activates denritic cells (Moineau *et al*. 1991; Havenith *et al.* 2002). This is a great advantage as those cells represent efficient antigen-presenting cells, and are responsible for generating primary T cell responses. There are still some serious safety issues to solve before licensing such a system for human use. First, this carrier vector was formerly classified as *Strep. sanguis*; but the M6 protein originating from *Strep. pyogenes* for a long time has been considered as a virulence determinant. Moreover, the system leads to chromosomal integration of a gene fusion together with an associated drug resistance marker what constitutes an additional disadvantage (Mercenier *et al.* 2000).

c.) *Lactobacilli*

*Lactobacilli* are known as safe bacteria possessing a number of properties that render them highly suitable for delivering many different compounds to the mucosa. The immunomodulating capacity of lactobacilli together with the possibility of targeting antigens to specific sites of the bacterium seems to be an attractive opportunity. Two types of *Lactobacillus* strains: “commensal” and “dietary”, may be taken into account as a potential vaccine vehicles. Commensal strains are expected to combine health-promoting properties with the ability to adhere in the area of the oral cavity, stomach, intestine, vagina and urethra. Dietary strains are mainly used in milk industry as a starter for production of fermented milk, meat or vegetable products. It should be taken for consideration that different strains vary in their immunomodulatory characteristic, what can have a great impact on their natural vaccine potential. Especially, their capacity to adhere to the relevant epithelial surfaces is an important property. Adhesion can be mediated through direct adherence to the antigen sampling cells (M cells, epithelial cells, mucus), or through aggregation with resident bacteria. The second way leads to an intense competition with the endogenous bacterial community, what can effect with prolonged antigen exposure. Both *in vitro* and *in vivo* models have been used to select or screen for adherent *Lactobacillus* strains (Pouwels *et al.* 1998). Nevertheless, converging data indicate that colonisation is host- and tissue- or even site-specific. It is unlikely that one, particular strain will constitute an ideal vector to deliver antigens to different hosts or to different mucosal cavities within the same host (Medaglini *et al*. 1997). Four non-recombinant chromosomally marked (Rifr Smr) lactic acid bacterial strains: *L. lactis* MG1363, *Lact. fermentum* KLD*, Lact. plantarum* NCIMB8826 and *Lact. salivarius* UCC433118 were given orally to human volunteers as a fermented milk product. The two latter strains were found far superior in their ability to survive the passage through the stomach. They were able to reach quite high feasible counts in the ileum, what is important in perspective to Peyer’s patches localization (Mercenier *et al.* 2000). It seems that the isolation of the final candidate strains from the targeted host is most appropriate but there are studies with mice model describing a variety of strains with human or murine origin that can be effectively use to colonise at least two body cavities of mouse animal model. For example, *Strep. gordonii* was shown to persist for several weeks in the oral cavity and the vagina of mice, while *Lact. paracasei* LbTGS1.4 (vaginal murine isolate) and *Lact. plantarum* NCIMB 8826 (human saliva isolate) survive in the gut or the vagina for over a week (Salminen *et al.* 1996). The importance of selecting the most favorable *Lactobacillus* strain is also linked to the strong strain-specificity of the currently used gene cloning and expression tools (Havenaar *et al.* 1992; Mercenier *et al.* 1996). Optimal translation, transcription and targeting sequences can differ significantly with the species and might even be strain dependent. For the strains studied within the LABVAC network, all efforts led to heterologous gene expression levels close to those obtained in lactococci(Wells *et al*. 1995). Different antigens were produced in the cytoplasm (up to a few percent of total protein content), in the culture medium (up to 13 mg/L) or at the cell surface (Gonzaga *et al.* 2009). Recent improvements in gene expression included the development of plasmid expression vectors of increased stability and chromosomal integration systems which target specific or random loci. Those systems are mostly based on a different non-replicative or unstable plasmids or, alternatively, on recombinant conjugative transposons (Wells *et al*. 1995). On the one hand, the latter offer the possibility to rapidly test the expression of the specific antigen in a variety of recipient strains. However, this approach may lead to integrants which carry an antibiotic resistance marker, with inactivated gene(s) essential for persistence or immunomodulation. A system, based on a nonreplicative plasmid, with non-disruptive integration achieved in the tRNA-Ser locus has been described by Dupont et al. (1995). Another integration vector, especially suited for *L. plantarum*, that can lead to insertion either at the tRNA-Ser or at the L-LDH locus was constructed (Mercenier *et al.* 2000). Inactivation of the L-LDH does not impair the growth of *Lact. plantarum in vitro* or *in vivo*. The recombinant plasmid carries the antigen-encoding DNA in a translational fusion with the L-LDH gene. Second homologous recombination leads to resections containing only the heterologous gene and no antibiotic marker. Both systems were used successfully to produce antigens in lactobacilli. Recombinant transposons allowed Rush et al.(1997) to express the *E. coli* LTB at the cell surface of different lactobacilli. Integration of the M6:gp41E (gp41E is a HIV-1 derived epitope) or the TTFC encoding gene was achieved in the chromosome of *Lact. plantarum* NCIMB 8826. In both cases, integration led to higher production levels comparing to insertion at the tRNA-Ser locus. The integrants produced comparable antigen amounts than the recombinant strains carrying multicopy plasmids. Expression of TTFC in different cellular compartments of a number of different *Lactobacillus* strains was also performed (Pouwels *et al.* 1993). It should be noted that improvement ofexpression levels often relied on modification of the translation initiation region or translational fusion with well-expressed endogenous genes. Few regulated promoters are available for lactobacilli. The nisin-inducible expression system originally designed for *L. lactis* was also implemented in *Lact. plantarum* NCIMB 8826(Kleerebezem *et al*. 1997). This required integration of the sensor and regulatory genes, *nisK* and *nisR*, into the chromosome of the host and to optimize the induction conditions. The nisin system turned out to be very efficient and allowed for high expression level of gp50, TTFC and GFP (Green Fluorescent Protein from *Aequorea victoria*). Since the antigen production level can be controlled by conditions of induction, it is possible to investigate the effect of antigen quantity on the level and duration of the specific immune response. The GFP+ strains that do not require addition of exogenous substrate or co-factor to emit fluorescence represent an ideal tool to perform *in vivo* studies. Their usefulness has been confirmed *in vitro* (phagocytosis by macrophages) and *in vivo* (intragastric and intranasal administration to mice) (Geoffroy *et al.* 2000). It is also intended to use the GFP marker to follow the survival of strains in the environment. Although the function of lactobacilli as adjuvants or carriers was established early on, the immunogenicity of recombinant strains by the intraperitoneal route was demonstrated relatively recently. Latest experiments carried within the LABVAC network confirmed, that after nasal administration all lactobacilli producing TTFC at the level of a few percent of total cellular protein content, including the *Lact. plantarum* NCIMB 8826 integrant, induced production of serum IgG and local IgA responses (Mercenier *et al.* 2000). A controlled comparison of TTFC-producing LAB strains by the oral route has been undertaken as well. Ongoing work includes the selection of strains appropriate for human use, identification of adhesion factors and also use of S-layers for antigen presentation (Mercenier *et al.* 1996).

**Doubts**

The use of genetically modified organisms always raises concerns about their uncontrolled persistence and spreading in the environment. Also transfer of antibiotic selection markers or other genetic sequences between microbes cannot be ignored. Especially, when many efficient methods that improve adaptation skills, such as conjugation, transformation, retromobilization or transduction have been identified. Toomey et al. examined the involvement of LAB, (*Enterococcus* *faecalis* and *L. lactis*) in spreading of resistance determinants between lactic acid bacteria and pathogenic strains, such as *E. coli*, *Listeria* spp.*, Staphylococcus* *aureus and Salmonella* spp*.* No resistance transfer was noted in case of first two strains. Unfortunately, erythromycin resistance was transferred to *Listeria* spp from donor strains*.* Additionally, a high frequency erythromycin and tetracycline-resistance transfer was observed between LAB species. The prevention systems can be based on: active and passive approach. The first one is based on the conditional genetic control through an activation of a “killing” gene. Expression of this gene depends on an environmentally responsive element or the total repression of the gene (Toomey *et al.* 2010). Active containment systems present a large amount of foreign DNA, what excludes their use in humans (Pouwels *et al.* 1998). Many of these systems are plasmid borne and their function is maintained only in case of plasmid integration in the bacterial chromosome what can limit lateral dissemination (Dziarski 1991; Fernandes *et al.* 1995). Mutations can inactivate a “killing” gene or lead to constitutive expression of the essential gene. Combining of more than one system in a recombinant strain can be a solution to avoid these kind of problems. Passive containment systems are based mainly on complementation of an auxotrophy or other gene defect by supplementation with the intact gene or the necessary metabolite. Passive systems are often bacteriostatic rather than bactericidal (Murray 1988; Bhakdi *et al.* 1991; Heumann *et al.* 1994). Among them, two systems with potential for human application can be distinguished. The first one is based on alanine racemase mutants that require D-Ala, and can be generated in a large number of LAB strains. The second one is based on a thymidine synthase (*thy*A) mutant of *L. lactis* and combines the elements of a passive and active systems together. Absence of the essential component induces bactericidal effect in the former and bacteriostatic in the latter. The gene can be exploited by replacing an essential gene involved in the survival of the LAB with an antigen of interest. Integration of the antigen gene into the chromosome enables to avoid use of antibiotic selection markers and a plasmid-based system, thus providing more stability. In a potential situation when the GM strain would be released to the nature the transgene would be eliminated from the genome and the GM strain would revert to a wild-type. Steidler et al. replaced *thy*A with a synthetic human IL-10 transgene in *L. lactis* what resulted in creation of the Thy12 strain. The mentioned strain, after validation studies in pigs, has been approved for experimental therapy in humans with IBD in the Netherlands. It would be greatly interesting to determine in the future the specificities of double mutants that could offer additional benefits over a single *thyA* mutation, such as increased antigen expression in the presence of two transgene copies, redundancy with respect to biological containment, and the applicability of the mutant using different kind of transgenes (Steidler 2003).

**Research networking**

The selection of model colonizing versus non-colonizing vaccine species or strains is a crucial step in designing vaccines. On one side, continuous *in vivo* synthesis of the antigen at the desired mucosal surface should stimulate the immune system. However, when using immunostimulatory LAB, high level of synthesis should not be expected. On the other hand, non-colonizing LAB can act as live microparticles pre-loaded with the antigen. This approach will assume high-level expression of protective antigens. Still it is not possible to predict which system is better according to optimal antigen presentation, a parameter which is known to affect its immunogenicity. Strains that are able to efficiently colonize humans need to be selected most of all on the basis of safety, physiological and metabolic criteria. Vaccine strains should be genetically stable to allow the expression of protective epitopes in different cellular locations. From the immunological point of view, analysis of the immune response, the nature and the intensity according to the mode of antigen presentation, the immunization route and the nature of the bacterial vector should be well characterized as it is essential for the final immune-protective effect.

Two research networks, focused on a common model system, have been organized (contracts BIO2-CT94-3055 and BIO4- CT96-0542). Three potential LAB vaccine vehicles were being chosen: *L. lactis* (a prototype of a non-colonizing strain), lactobacilli (as colonizing bacteria) and *Strep. gordonii* (an oral commensal bacterium with a stable antigen presentation system). The European project is concentrated on examining antigens of bacterial and viral origins and comparison of the three bacterial systems. The first system is based on the tetanus toxin fragment C (TTFC). It is a 47 kDa non-toxic polypeptide with the ganglioside-binding domain. It is a useful model because of its well-established immunogenicity and the availability of a lethal mouse challenge model (Fairweather *et al.* 1987). The approach is an effective means of evaluating the capability of a mucosal delivery system to elicit a systemic humoral immune response. The second candidate is the gp50 protein of the porcine pseudorabies virus (Aujeszky’s disease virus, ADV). This pathogen infects animals through the respiratory tract. The best way of its inactivation is induction of mucosal immune responses. This approach was designed to evaluate the potential of LAB as live vaccines to target viral mucosa-associated diseases. Additionally, challenging mice or pigs by the ADV will enable to evaluate bioactivity of the induced antibody responses (Mercenier *et al*. 2000).

**Conclusions**

LAB expression system can be considered as a promising one not only for efficient antigens delivery, but also for production of many biologically active compounds. Unfortunately, it is difficult for now to compare and make sensible conclusions based on the conducted studies, what is caused by diversification of LAB strains according to their physiology and genetics. Still there are too many impenetrable areas that are crucial for creation of an effective mucosal vaccine. It is necessary to broaden knowledge about the LAB strains applied locally and levels of antigen production inside of the host. The development of effective mucosal vaccines is also inextricably linked to understanding of the immune response mechanisms and the cellular and molecular pathways involved in its control. More comprehensive knowledge about the type of cells induced and the type of the induced immune response, the roles of cytokines, or phagocytic functions would be helpful. Currently, hopes are turn to new systems of immunogenic antigens targeted to specific areas, cells or even receptors. The development of recombinant lactic acid bacteria vaccines is in its early stages. Fortunately, specific immunological experiments, new recombinant strains and vectors continue to be constructed and described in detail what can lead in a near future to standardization of LAB vector vaccines production.

**Conflict of interest**

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication.

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**Figure legends**

Fig. 1. Actual LAB application.

Fig. 2. Food grade vaccines based on Lactic Acid Bacteria.

After oral administration of food grade vaccine the mucosal and systemic responses are stimulated after antigen presentation by M cels (M) and dendritic cells (DC) in the intestine. In consequence lymphocytes induce adaptive reactions that involves IgA, IgG production and recruitment of cytotoxic cells.