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Review Article

Lactic Acid Bacteria as Mucosal Delivery Vaccine

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ABSTRACT

Mucosal surfaces of the body provide a congenial entry portal for all the known and emerging infective pathogenic microbes. Therefore, it is imminent that vaccination strategies need to be evolved for developing vaccines that are capable of hindering the entry of microbes through mucosal surfaces. The present day conventional vaccination strategies, though effective against some pathogens, seems ineffective due to certain drawbacks such as being ineffectual in generating immune response at mucosal surfaces and also of the difficulties experienced during administration of vaccine. Hence, novel strategies, such as development of oral/nasal mucosal vaccines vectored by probiotic microbes, can be thought of as an alternative as they are effective in inducing protective immunity at the site of infection, capable of eliciting systemic and mucosal immunity and moreover, easy to administer. This review outlines the efficacy of probiotic mucosal vaccines in modulating the immune system, particularly emphasizing on two major lactic acid bacilli as candidates for mucosal vaccine delivery vehicles in livestock and poultry.

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INTRODUCTION

For centuries, mankind had the tradition of using fermented food products (Sharpe, 1981) which were made with the aid of Lactic acid bacteria (LAB) which are categorized as *generally recognized as safe* (GRAS) by the United States Food and Drug Administration (USFDA). They are characteristically Gram positive, low GC content bacteria and are carbohydrate fermenters since they produce lactic acid as their major metabolic product. The different genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Vagococcus* and *Weisella* come under the group of lactic acid bacteria (Daniel et al., 2011). The strains of the genera, *Lactococcus* spp. and *Bifidobacterium* spp., have gained importance as probiotics by contributing to a normal host gut–microbial interaction as well as to promote the health of the host itself (Gareau et al., 2010). Generally, their ability to adhere to certain areas of the gastrointestinal tract has created interest among researchers to tap the potential of such microbes as vehicles for the delivery of biologically active compounds such as enzymes and vaccines (Pouwels et al., 1998). Several vaccination programmes prefer oral vaccine development to other vaccination modalities as they can elicit and modulate both mucosal and systemic immune responses, have lesser side effects and can be effected easily to a large population without the need of skilled personnel. Most of all, they prevent the carriage of pathogens in the population and does not interfere with inherent maternal antibodies in infants (Husband, 1993; Walker, 1994; Lamm, 1997; Wells and Pozzi, 1997; Bermúdez–Humarán, 2009). This review entails the advancements achieved so far by the use *Lactococcus* and *Lactobacillus* as mucosal delivery vaccine and vaccine adjuvant, with particular reference to the mucosal vaccines experimented for veterinary diseases. It also explains the challenges of *in vivo* application of such vaccines in livestock and poultry.

Suitability of Lactic Acid Bacteria for Mucosal Delivery Vaccines

The variation in heterologous protein secretory mechanisms by Gram–positive and Gram–negative bacteria shall be taken into account while choosing a suitable prokaryotic heterologous expression system for vaccine delivery. Gram–positive bacteria only have a single plasma membrane and a thick peptidoglycan–teichoic /teichuronic acids layer. Hence, the secretion of heterologous proteins in Gram positive bacteria is easier compared to Gram–negative bacteria, where an additional outer membrane has to be traversed before a protein is secreted into the external environment (Morello et al., 2008). The reason for choosing LAB for the development of mucosal delivery vaccines is their ability to prevent degradation of antigen in the gastrointestinal tract, being nonpathogenic and genetically modifiable, thus making them an excellent candidate as delivery vectors of proteins (cytokines, interleukins, etc.,) and antigens for developing novel therapeutic and disease control strategies (LeBlanc et al., 2013). The choice of the LAB strain used is first prioritized by its capability to effectively survive the gastric pH (Steidler et al., 2009) as well as bile (Sleator and Hill, 2006). Another factor to be considered is concerning its persistence in the gut surfaces. Unlike other LAB, *Lactococcus lactis* are incapable of colonizing the digestive tract of man or animals. They persist for less than 24 hours in the murine gut and in humans; the bacteria are voided from the gut in 3 days (Mercenier et al., 2000). Even then, this bacterium is preferred for vaccine development as it secretes less number of proteins as extracellular preoteases into the environment and is free of plasmids (Gasson, 1983). But, strains of Lactobacillus species,

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which have probiotic properties, tend to persist longer in the digestive tract (Seegers, 2002). The positive effects of mucosal administration of LAB for vaccine delivery relies on the strain used as vaccine delivery vehicle, the therapeutic antigen produced by the vehicle and the disease chosen for vaccine development. The success in mucosal immunization using LAB depends on the inherent ability of the vehicle for heterologous antigen production, the immunogenicity of the selected antigen and the route of administration (Daniel et al., 2011). On comparing the effective route of administration for recombinant LAB having surface displayed human papilloma virus type 16 (HPV–16) E7 antigen and secretory interleukin–12 (IL–12), intranasal immunization is found to be better when compared to the oral route (Cortes–Perez et al., 2007). Generally, heterologous protein antigens are expressed by the recombinant LAB either within the cytoplasm or on the cell surface by surface display mechanisms or they are secreted to the intestinal lumen (LeBlanc et al., 2013). Innumerable lactic acid bacilli have been employed for live vaccine delivery, but it has been difficult to propose the most suitable location of the expressed antigen for eliciting immunogenicity because the strain differences may affect the amount of expressed antigen. Even a proportion of secreted antigen can remain cell associated depending on the efficacy of the fusion gene construct and the level of expression (Wells and Mercenier, 2008). Hence, research focus need to be diverted to develop strategies for modifying vehicles that support and efficiently express protein antigens by secretory and cell surface display mechanisms.

The subsequent literature gives a specific idea of the efficacy of various strains as a mucosal delivery vaccine, specifically pertaining to livestock and poultry diseases.

Application of Lactococcus Spp. for Mucosal Vaccine Delivery

The first experimental candidate was *Lactococcus lactis* based mucosal vaccine against dental caries which had the *Streptococcus mutans* surface protein (Pac) as the antigen of choice (Iwaki et al., 1990). Killed recombinant *L. lactis* cells having cytoplasmic localized expression of surface protein antigen (PAc), when administered orally resulted in salivary IgA and serum IgG responses against the antigen. Further, a recombinant *L. lactis* strain was developed that produced the highly immunogenic *Clostridium tetani* toxin, fragment C (TTFC – tetanus toxin fragment C) upto a level of 22% of the total soluble cell protein fraction via controlled expression of the TTFC gene (Wells et al., 1993). Here, mice were subcutaneously vaccinated with *L. lactis* expressing recombinant TTFC, for evaluating its immunogenicity and a subsequent lethal challenge in the experimental trial gave positive results. Later on, TTFC was used as a model antigen to study various parameters such as dosage, an ideal cellular location (i.e. cytoplasmic, secreted or surface displayed) of heterologous antigen in the LAB vehicle, route of administration (nasal, oral, intragastric or genital) and efficacy of co–expression strategies of the antigen along with cytokines or mucosal adjuvants. These methodologies have catered to define the most suitable vaccine delivery vehicle effectuating an optimal *in vivo* immune response against the designed vaccine. In the case of recombinant TTFC producing *L. lactis*, the nasal route was preferred and the best dose–response ratio was achieved when the antigen was surface displayed (Norton et al. 1996; Robinson et al. 1997). It is pertinent to discuss some of the recent findings on employment of LAB as vaccine vector (as antigen delivery vehicle or as DNA vaccine vector) for diseases of veterinary importance. During two decades of research many scientists have contributed to the knowledge on the efficacy and usage of various lactococcal strains as a mucosal delivery vaccine.

The development of oral vaccines targeting EspB (a type III secretory system protein [T3SS]) was designed by a research group wherein the T3SS protein was intracellularly expressed in *L. lactis* for immunizing BALB/c mice. Type III secretory systems (T3SS) are a group of proteins involved in pathogenesis and colonization of bacteria in the intestine of hosts such as humans and animals, and hence targeting such proteins as putative antigens for oral mucosal vaccine development was considered. In the study, although, post oral immunization of mice revealed absence of specific serum and faecal antibodies after ten days, an intraperitoneal inoculation of the purified recombinant EspB protein as a booster in mice resulted in a significant increase in serum IgG and faecal IgA levels. The results revealed that mucosal priming was favoured after lactococcal vaccination, but better optimized expression and delivery strategies for T3SS proteins need to be taken into account in order to improve the mucosal immune response (Ahmed et al., 2013). The comparative efficacy of recombinant *L. lactis* expressing FaeG (fimbrial adhesion) was explored when given orally and intramuscularly in piglets (Liu et al., 2013). The intramuscular immunization induced F4–specific systemic responses. It resulted in increase in the numbers of the IgG, IgM, and IgA antibody secreting cells in the jejunum and mesenteric lymph node, as well as the IgG and IgM antibody secreting cells in the spleen. A gene construct based on the conserved peptide stretch of the avian influenza M2 antigen ectodomain was used for a surface display based lactococcal vaccine utilizing *L. lactis* (LL). Experimental birds were vaccinated via the nasal route and also subcutaneously with keyhole–limpet–hemocyanin conjugated M2e (KLHM2e) (Reese et al., 2013). Upon challenge with high pathogenic avian influenza virus A subtype H5N2 the median survival times of both vaccinated groups were significantly longer (5.5 to 6 days) when compared to non–vaccinated birds (3.5 days). A recombinant mucosal vaccine against brucellosis was designed using *L. lactis* capable of secreting Cu–Zn superoxide dismutase (SOD) of *Brucella abortus* (Sáez et al. 2012). SOD–specific IgM antibodies together with SOD–specific sIgA in nasal and bronchoalveolar lavages (BAL) were noticed in inoculated mice and the vaccinated group were also protected from a challenge with a virulent *B. abortus* strain. The *L. lactis* strain secreting the virulence–associated protein A (VapA) from *Rhodococcus equi* was developed and experimented in conjunction with a *L. lactis* strain producing recombinant leptin, which was given orally and intranasally in mice (Cauchard et al., 2011). Mucosal administration 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*. An evaluation of the immune response of orally immunized mice with different recombinant *L. lactis* forms having the rotavirus spike–protein subunit VP8 being expressed in the cytoplasm, secreted or as a surface anchored antigen, showed the intracellularly expressed VP8 form to induce significant levels of intestinal IgA antibodies, while the cell wall–anchored VP8 form exhibited anti–VP8 antibodies at both mucosal and systemic levels (Marelli et al., 2011). Oral immunization of mice using recombinant *L. lactis* having intracellularly expressed and secreted forms of the potent superantigenic exotoxin, enterotoxin B of *Staphylococcus aureus* has shown to produce a protective immune response against the pathogen. Irrespective of the mode of expression of the enterotoxin B antigen, both of the recombinant strains were able to elicit cellular or systemic immune responses in mice. Moreover, the lactococcal vaccine

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strain having cytoplasmic antigen expression had a comparatively increased survival rate subsequent to *S. aureus* challenge in vaccinated mice (Asensi et al., 2013).

Application of Lactobacillus Spp. for Mucosal Vaccine Delivery

Though Lactobacilli are comparable to *L. lactis* as mucosal delivery vehicle, there are certain advantages in the use of lactobacilli as a live vector. They can persist longer in the digestive tract and some strains have intrinsic probiotic properties (Gareau et al., 2010; Kechaou et al., 2013). *Lactobaciilus plantarum* and *Lactobacillus casei* are the species commonly used to develop vaccine delivery vehicles.

The *L. plantarum* based TTFC vaccine induced a higher TTFC specific antibody over *L. casei* in oral and intranasal immunization of C57BL/6 and Balb/c mice (Grangette et al., 2001). The recombinant *L. casei* expressing transmissible gastroenteritis coronavirus spike glycoprotein for intragastric administration stimulated antigen specific mucosal IgA production (Ho et al., 2005). Porcine Parvovirus VP2 protein based mucosal delivery vaccine using *L. casei* was able to increase serum antibodies (Xu and Li, 2007). The efficacy of two *L. casei* based porcine rotavirus oral vaccines expressing VP4 antigen and a VP4–LTB antigen fusion protein were capable of inducing serum IgG and mucosal IgA production in mice, but the IgA produced by *L. casei* (VP4–LTB) was higher when compared to the other strain. This highlights the utility of co–expression of proteins having adjuvant properties along with putative antigens (Qiao et al., 2009). A putative antigen of the enteropathogenic *E. coli* (EPEC), known as intimin β (a virulence factor), when used as a candidate gene for constitutive intracellular expression in *L. casei* CECT5275 for intranasal vaccination in mice produced antibodies that are capable of binding to the surface of enteropathogenic *E. coli* and inhibiting their adhesion to HEp–2 epithelial cells (Ferreira et al., 2008). Recombinant *L. casei* 525 expressing a fusion protein comprising poly–γ–glutamate synthetase A (PgsA; an anchoring matrix) and a fimbrial protein F41 (pilin) of enterotoxigenic *E. coli* (ETEC), was utilized as an oral mucosal vaccine in specific–pathogen–free BALB/c mice where significant mucosal IgA titres could be detected that prevailed for more than sixteen weeks with high levels of serum IgG responses specific for F41 fimbriae. A challenge of the vaccinated mice resulted in more than 80 per cent protection showing the utility of *L. casei* 525 as an efficient vaccine delivery vehicle against ETEC (Liu et al., 2009).

The cell wall motif known as the AcmA binding domain of *L. lactis* when utilized to co–express the VP1 protein of chicken anemia virus (CAV) via surface display on *L. acidophilus* for use as an oral LAB based vaccine in chicken, produced a moderate level of systemic anti–CAV neutralizing antibodies and a VP1–specific proliferative response in the splenocytes of immunized chickens (Moeini et al., 2011). A recombinant Lactobacillus strain co–expressing the Classical Swine Fever Virus (CSFV)–specific cytotoxic T lymphocyte (CTL) epitope 290 and the VP2 antigen of Porcine Parvo Virus (PPV) upon use as an oral vaccine in pigs stimulated mucosal and systemic CSFV–specific $CD8^+$ CTL responses along with the production of anti–PPV–VP2 serum IgG and mucosal IgA antibodies (Xu et al., 2011). The immunized group were protected from a CSFV challenge. Similarly a mucosal delivery vehicle was developed based on *L. casei* CICC 6105 using poly–γ–glutamate synthetase A (PgsA) as an anchoring matrix for the candidate antigens, K99 and K88 of enterotoxigenic– *E. coli* (Wen et al., 2012). Specific pathogen free (SPF) mice (C57BL/6) were orally immunized with the recombinant lactobacilli to evaluate the development of anti–ETEC K99 or K88 antibody responses, T–cell proliferation, and cytokine production by intracellular staining (ICS). The oral recombinant *L. casei* based vaccine, without using any adjuvant, was able to induce specific mucosal, humoral and cell mediated immune responses against the antigens. *Lactobacillus casei* IMG393 based oral mucosal vaccine against *Salmonella enterica* serovar Enteritidis (SE) was produced by generating strains expressing FliC (flagellar protein) alone and also expressing fusion proteins of FliC separately with cSipC (C–terminal region of a protein grouped under type III secretion systems protein) and OmpC (an outer membrane protein) respectively. Upon oral immunization in mice, the lactobacilli having co–expressed fusion proteins only had a comparable efficacy with that of lactobacilli vehicle expressing the FliC antigen alone (Kajikawa and Igimi, 2011).

The use of genetically modified *Lactobacillus salivarius* expressing Salmonella OmpA (via surface display) for use as an oral mucosal vaccine in chicken when explored, revealed that oral infection with transformed *L. salivarius* elicited significant humoral responses (Rahbarizadeh et al., 2011). A *L. plantarum* based oral vaccine was devised by expressing two distinct versions of the extracellular domain of Invasin, a multidomain virulence protein of *Yersinia pseudotuberculosis*, capable of stimulating the innate immune response by initiating pro–inflammatory reactions and cellular internalization in host cells. Four different N–terminal anchoring motifs were considered for cell wall targetting of the extracellular domain of invasin onto *L. plantarum*, i.e., two lipoprotein anchoring domains, a transmembrane signal peptide anchoring domain and one LysM–containing protein motif as cell wall anchor. Though all of the modified bacterial strains were capable of potentiating the NF–κB pathway in monocyte cell cultures, a distinctive response was obtained in constructs which had the lipoanchor fused–complete invasin extracellular domain. Hence, vaccine antigens co–expressed with anchored extracellular domains of invasin can be capable of potentiating antigenic immunogenicity in the host and thus represents a promising modality in the development of LAB based mucosal delivery vaccines (Fredriksen et al., 2012).

Safety Concerns

A debatable concern involved in the use of lactic acid bacteria based mucosal vaccines is the potential hazard of introducing genetically modified organisms to the environment. Such engineered bacteria which express antigens and antibiotic markers using replicating plasmids, can have the potential for horizontal transfer of plasmid to other bacteria, thereby posing the potential threat of introducing pathogenic antigens to the non–pathogenic bacteria and antibiotic resistance markers to the environmental microflora. In such instances, gene modifications for the development of auxotrophic mutants that are incapable of multiplying in the environment can be thought of as an alternative. Steidler et al (2003) worked on engineering LAB strains possible for biological containment. They replaced the *thy*A gene (coding for thymidylate synthase) with the expression cassette for human IL–10 in *L. lactis*, thereby developing an auxotrophic strain dependent on thymidine, which is present in low amounts in nature. A vaccine delivery vehicle was designed in *L. lactis* which had the LLO (Listeriolysin O of *Listeria monocytogenes*) gene chromosomally integrated. This is considered as an alternative to employing expression vectors so as to reduce the use of antibiotic markers and also, the likelihood of horizontal gene transfer to other bacterial species in the natural environment is greatly minimized (Bahey–El–Din et al., 2010). As an alternative to the

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usage of genetically modified LAB, Lin et al (2012) developed a new vaccination strategy involving the exogenous anchorage of avian reovirus (ARV) sigma C onto the cell wall of lactic acid bacteria. The heterologous antigen with autolysin AcmA as fusion protein was expressed in *E. coli* and exogenously anchored on the surface of *Enterococcus faecium*. This vaccination method could enhance both mucosal and systemic immunity in murine models. Hence, LAB vaccine development should focus either for the safe containment methods of genetically modified lactic acid bacteria or to devise cell wall adhering recombinant antigen fusion proteins along with LAB strains for mucosal vaccine delivery systems.

CONCLUSION

Mucosal vaccines are considered advantageous over injected vaccines as they are efficient in eliciting systemic and mucosal immune responses in the host, easy to administer and require only minimal trained personnel. Lactic acid bacteria, which are claimed to be nonpathogenic and easy for genetic modification, are excellent mucosal delivery vectors for heterologous antigens and therapeutic proteins. By developing chromosomally modified bacterial strains, with minimal usage or involvement of recombinant plasmids, the hurdles based on its safety concerns can be countered. Other methods such as developing heterologous antigens capable being exogenously adhered onto lactic acid bacterial cell wall can be considered. This would obviously favour for clearance in conducting clinical trials which can eventually be made use for preventive and therapeutic intervention for infectious and non–infectious pathologies of veterinary importance.

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