Review Article

Lactic Acid Bacteria as Vectors: A Novel Approach for Mucosal Vaccine Delivery

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Abstract

Lactic Acid Bacteria (LAB) has been used in food industry due to its classification as food grade microorganism. It has been used for food production as well as preservation on large scale. It is also considered as promising bacterial strain due to its probiotic activity that confirms human health. Moreover, it also shows resistance regarding its survival in Gastrointestinal Tract (GIT). Therefore, use of LAB as delivery platform for drugs as well as production of recombinant protein is a challenging approach for researchers now a day. As, it not only reduces the production cost of drug, but also act as live vector to synthesize and deliver target or therapeutic protein of interest. Moreover, it is possible to produce different proteins from same bacteria simultaneously. Thus altogether, this approach has not only provided an alternative option for intravenous administration of recombinant protein but also gives an alternative insight for delivery system of mucosal vaccine. This review aims to provide an overview in order to use specific species of LAB such as *Lactococci lactis* and *Lactobacillus* as vector for transfer of vaccine for mucosal as well as in recombinant form. Moreover, use of intron for desired genetic variation into target sites is explained to give directional insight for future studies.

Keywords: Lactic acid bacteria; Mucosal; Vector; Therapeutic; Introns

Introduction

For decades, Lactic Acid Bacteria (LAB) has been used for fermented foods [1]. The role of LAB is to use it as starter on large-scale for fermentation in order to get high quality and reproducible fermented food [2]. Basically, LAB are non-pathogenic Gram-positive bacteria classified into *lactococci* and *lactobacilli* and are termed as "GRAS" (generally recommended as safe) [3]. However, it has been found that certain strain of LAB i.e., *Lactobacillus* and *Bifidobacterium* has beneficial effect in improving health of people and animals. This beneficial effect is due to probiotic activities of LAB. Probiotic activity of LAB varies from species to species [4]. Some bacterial species maintain intestinal microflora after modulating bacterial flora in intestine, while few of them act as immune stimulator and prevent allergic reactions. Some species have been reported to provide protection against pathogen after releasing compound which inhibits the growth of pathogens by producing antimicrobial peptides *via* mucosal epithelial cells. Further, probiotic strains have also been reported to provide support against different diseases like diarrhea, inflammatory bowel disease, and autoimmune disorders [5,6].

Proper nutrition and effective vaccines both have been considered as important strategies for prevention of infectious diseases. It has been reported that LAB can act as an effective tool for both purposes, simultaneously. It has been used for producing functional food due to its probiotic ability that will not only strengthen the immune system, but also provides protection against infections. *Lactobacilli* have been reported to enhance antigen specific immune response due to its

adjuvant effect. Thus, LAB could be administered with target antigen in order to induce a more pronounced immune response [7]. On the other hand, advances in molecular biology has enabled us to produce recombinant strains of lactic acid bacteria that express antigen against pathogenic organism and strengthen the adaptive immunity after expression of certain cytokines [8] shown in Figure 1.
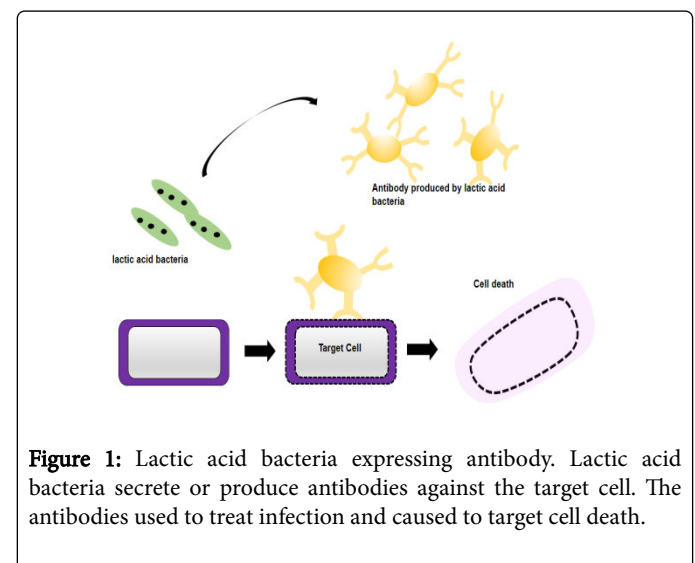

Figure 1: Lactic acid bacteria expressing antibody. Lactic acid bacteria secrete or produce antibodies against the target cell. The antibodies used to treat infection and caused to target cell death.

Now a day, LAB is also considered as an important carrier for mucosal delivery system. There are several different reasons for selecting LAB as delivery vector. First, mucosal immunity is considered as highly important regarding to infectious diseases, as mucosal

surface is a main portal for entry of pathogens. Administration of therapeutic molecules through mucosal surface has several advantages over systemic routes i.e., feasible to administer without the use of syringe and needle, decreased side effects due to enhance potency & specificity, and ability to control both systemic and mucosal immune response [2,9]. Secondly, mucosal surface has been reported as potential route for delivery of vaccine due to its association with different lymphoid tissues such as nasopharynx, tonsils, salivary glands, respiratory tract and gastrointestinal tract [10]. Thus, follicle associated epithelium or Microfold (M) cells in lymphoid tissues help to overcome the invading pathogens by maintaining mucosal immunity. Additionally, M cells also help in transports the antigen across epithelium and initiate immune response at targets site [11]. However, there is one disadvantage for vaccination *via* mucosal route; a large amount of protein is required for administration due to its degradation at mucosal surface such as gastrointestinal tract. Only small amount has been found to survive and elicit the immune response [9].

This review focuses on utilization of LAB especially *lactococci* and *lactobacillus* as vaccine delivery system, its role in immunoprophylaxis, mucosal surface as route for vaccination as well as usage of intron system.

Delivery System for LAB

LAB is non-pathogenic and designated as Genetically Modified (GM-LAB) i.e., has ability to develop new material for treatment of various human diseases [12]. Initially, LAB was used as carrier for foreign antigen in 1990 to immunize against Streptococcus mutants after using PAc protein (antigen I/II) produced on cell surface. Intragastric immunization resulted in production of specific IgG and IgA antibodies. Thus, for the first time it was shown that LAB could be attractive alternative bacteria as vaccine vector [13]. LAB include microorganisms from different genus including *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Pedicoccus*, *Leuconostoc*, but l*actococcus* and *lactobacillus* have been considered as important vehicle as well as candidate for cloning and production of recombinant protein [14].

Other routes were also developed in order to minimize the chance of getting infection after an in contact with humans. For that purpose, intranasal and oral vaccine was also evaluated for *S. pneumoniae* and *Helicobacter pylori*, respectively [15,16]. As mucous membrane associated immunization is spread in whole body system where lymphocytes can easily move in body. So, oral immunization has been found to provide systemic immunity expressed by mucous membrane. LAB as carrier of antigen for *S. pneumoniae* were found to be effective *via* intranasal immunization. Moreover, *L. lactis* having *ppp A* gene from *S. pneumoniae* was employed for oral immunization of young and adult mice. Both routes enhanced specific antibodies in gut and stimulated systemic immune response [17,18].

Further studies were conducted to find out effect of carrier on the production of immune response level. Antimalarial vaccine was selected to check efficacy. Different strains of LAB producing Merozite Surface Antigen (MSA2) i.e. surface protein of *Plasmodium falciparum* were used. Different mouse lines with genetically variation were used. Combined oral and nasal immunization was employed. Significant difference was observed in the level and type of immune response. It clearly shows that immune response depends on type of animal used, genus of carrier as well as location of antigen [19]. Some studies showed that intranasal administration of *L. lactis* producing

intracellular antigen PspA was more effective as compared to purified recombinant protein [15].

Immune response exhibited *via* LAB after using promoter/adjuvant was tested. *L. casei* with *pspA* gene of *S. pneumoniae* under control of lactose promoter provoked no immune response. Whereas, four different strains of bacteria (*L. lactis*, *L. casei*, *L. plantarum*, *L. helveticus*) were tested having *pspA* gene along with constitutive promoter. *L. lactis* exhibited low level of immune response, while other strains exhibited induce immune response with significant level of IgG and IgA. This difference in immune response is related to type of bacterial species as well as adjuvant potential [20].

DNA immunization enhances both humoral and cellular immunity. That's why, DNA vaccine is getting attention for researcher now a days. LAB is considered as potential candidate for DNA vaccine. Initial studies were done after incubation of *L. lactis* MG1363 strain having plasmid DNA along with CaCo-2 cell resulted in transfer as well as expression of plasmid DNA in eukaryotic cells [21]. Moreover, oral administration of *L. lactis* for cow's milk allergy showed presence of protein, complementary DNA (cDNA) as well as specific IgG and IgA antibodies in small intestine. There are two reasons that could cause high antibody level. It is due to transfer of plasmid DNA released by *L. lactis* in intestine and taken up by eukaryotic cells or *L. lactis* has been taken up by eukaryotic cells [22].

It has been thought that LAB cannot invade eukaryotic cells. Therefore, bacterial strains are specifically designed for interaction of eukaryotic cells. Plasmid DNA transfer has been studied extensively after using *L. lactis* along with reporter genes (i.e. cDNA). It expresses extracellular protein such as Fibronectin Binding Protein (FnBPA) or *L. Monocytogenes* Internalin (InIA). FnBPA was checked *via in vivo* and *in vitro* along with reporter genes. It was found that protein enhances the amount of DNA of reported genes in eukaryotic cells. But the amount of antigen produced is not increased. Moreover, mechanism of action has been found as different in both *in vitro* and *in vivo* experiments [23].

In vitro experiment was conducted after using *L. lactic* along with expression of InIA inetrnalin of *L. monocytogenes* and receptor i.e. E-cadherin. Experiment shows high level of invasiveness but structure cannot recognize receptor. Because InIa recognize human but not murine E-cadherin. Thus a modified strain of *L. lactis* was structured along with mutated InIa that can recognized murine E-cadherin. *In vivo* experiment was conducted and it shows increase level of invasiveness like *in vitro* experiments but amount of target protein is not increased. Thus, data suggests that LAB has high potential to act as DNA vaccine [24,25]

Vectors for *Lactococcus Lactis*

Lactococcus lactis is considered as model microorganism for LAB research due to its rapid use in treatment and prophylaxis. It is the first vector to be used for cloning of foreign genes [26]. Further, it is categorized as non-invasive and non-pathogenic bacterium along with GRAS (Generally Recognized as Safe). That's why; it is used as live vector for mucosal delivery of therapeutic protein. Because, it can resides protein due to its extraordinary safety profile. Moreover, it is considered as good candidate for production of heterologous protein, As it produces few protein in small quantity and only one protein in detectable quantity i.e., Usp45 [27,28]. First study exhibiting the potential of *L. lactis* as mucosal vector was done in 1990s. Mucosal vector was developed by killed recombinant *L. lactis* that produces

protective antigen (PAc) of *Streptococcus mutans* near cell wall [13]. In addition, most commonly used strain of *L. lactis* i.e. MG1363 is plasmid free and does not produce any extracellular protease and its genome has been sequenced. That's why; it has been used frequently in research [29].

Most commonly used expression system for heterologous protein is NICE, which uses nicin as promoter. Niacin is basically a bacteriocin, produced *via L. lactis* having adjacent eleven chromosomal genes encoding for biosynthesis as well as immunity [30].

Vectors for *Lactobacilli*

More than 180 species of *Lactobacillus* has been included in genus having different immunological, biological, ecological and molecular biochemistry aspects. The reason for variation is due to difference in ratio of Guanine (G) and Cytosine (C) content of DNA. Use of *Lactobacillus* as expression vector for cloning of gene is considered as challenging. Because there is huge variation in genetic diversity. Due to this variation, only a few plasmid replication systems are active for specific strains of *lactobacilli* [31].

Moreover, same trend has been found for *lactobacillus* promoters. Promoters have different activity level and is specific to selected strains [32,33]. Moreover, *lactobacilli* for expression of vector use different type of promoters like inducible promoter and PsIpA (a constitutive promoter encoding genes for slime layer of protein SIpA) [34]. Other promoters were induced *via* environmental conditions and few of them were induced by presence of carbohydrate e.g., PFOS (fructo-oligosacchride), Plac (lactose), and Ptre (trehalose). These promoters perform different functions and are generally suppressed by the presence of glucose. PFOS is found to enhance immunity. As fructo-oligosacchride is prebiotic, it stimulates the growth of beneficial bacteria in intestine [33]. Commonly used cloning vector for different strains of *lactobacillus* are pWV01, pSH71, pAMbeta-1 for *L. plantarum*, *L. acidophilus*, *L. gasseri*, respectively [31,35,36].

In 90th decade, genetically modified *Lactobacilli* produced heterologous protein for development of new generation of mucosal vaccines. In early 2000's, different species of *Lactobacillus* were successfully developed to use it as vehicle for delivery of protein *via* mucosal surface. This strategy was used for medical purpose. Further, it was found that it enhances the local immune response. Use of *Lactobacillus* as delivery vehicle was selected due to its specific characteristics e.g., persistence in digestive tract for long time and probiotic activity [37,38].

Moreover, *Lactobacillus* after genetic modification was used for developing a cloning system. Main feature of cloning vector for transfer of antigen is sequence of promoters showing inducible expression. A well-known system used for *lactobacilli* as inducible expression is Nisin Induced Controlled Expression (nice) [39,40]. In addition, these vectors are commonly used for heterologous protein expression and exhibit signal and secretion to allow protein expression [41,42]. Most expression systems are plasmid based due to ease of operation. On the other hand, integrated system provides a great advantage regarding genetic stability of strains but can be low in expression level.

Promoters are found to show different activity while using different strains of *lactobacilli* in an expression system. It shows difference in efficiency as well as plasmid copy number [43]. Furthermore, codon could be used for expression of heterologous protein from *E. coli*.

However, expression could be affected after using rare codon [44]. Moreover, Usage of codon for expression of heterologous protein in *lactobacilli* strains shows that highly expressed genes exhibit high usage of codon, while less expression shows less usage of codon, simultaneously [45,46].

Recombinant *L. Lactis* as Mucosal Vaccine

Lactobacilli have been used as delivery vector for the treatment of inflammation and Gastrointestinal (GIT) diseases [47-50]. Inflammatory Bowel Disease (IBD) consists of a group of disorders associated with inflammation of gastrointestinal tract [51-54]. Two most common forms of IBD are Crohn's disease and ulcerative colitis are considered to be associated with the influx of macrophages and neutrophils, resulting in continuous production of inflammatory mediator like cytokines and Reactive Oxygen Species (ROS) [55]. ROS include superoxide radical, hydrogen peroxide and hydroxyl radicals that cause cytotoxicity and mutation [56]. In order to detoxify ROS, cells have to develop a self-protection mechanism through antioxidant enzymes such as catalase and superoxide dismutase, which reduce oxygen and hydrogen peroxide [57]. Thus, therapeutic use of antioxidant enzymes in order to remove ROS is a promising method for prevention and treatment of an IBD. However, LAB such as *lactobacilli* has been found as an effective strain for prevention of IBD [58,59].

Genetically modified *Lb. casei* BL23 producing Superoxide Dismutase (SOD) and catalase (that degrade O_2- and H_2O_2, preventing the formation of HO) was induced in colitis rat model. Oral administration of *Lb. casei* producing SOD or catalase showed quick recovery of initial weight loss along with enhanced activity of enzyme in intestine as well as decreased level of inflammation in intestine, when compared with control rats group. It shows that genetically modified LAB producing antioxidant enzyme can be used for reduction and prevention of specific intestinal disorders such as IBD [60].

However, recombinant *Lb. casei* strains expressing IL-10 in combination with 5-amino salicylic acid (5-ASA) and Dextran Sulfate Sodium (DSS) were also induced in colitis rat model. It was found that recombinant *Lb. casei* have shown more effective prevention against inflammation [61].

Using the Intron System

Now a days, mutation has been generated in food microbiology with specific targets like cost reduction in food production units, maintenance of good quality as well as safety of food after ensuring food grade bacteria. Food grade bacteria used in food production are usually generated after variation in bacterial strains. However, non-food grade bacteria are generated without integration of heterologous DNA like antibiotic resistant markers or DNA sequence [62]. As, this mutation has been considered as stable during food production process as well as its passage in gastrointestinal tract, it shows that selection of an efficient tool for mutagenesis is very important [63].

Group II introns are versatile elements that can carry genomes after variation. Basically, introns are segments of inserting DNA along with coding sequence called exon. Introns are originated from messenger RNA (mRNA) *via* a process called splicing. Spliced part of mRNA is fused with exon to make an intron functional. However, success of group II introns depend on multi-functionality of splicing and mobility of reactions that forces DNA to work as an independent unit in order

to obtain an adaptable form having variant properties [64]. In this way, product with genetic variant has been developed along with desired trait. Such genomic variation has been used in various domain of life now a days [65].

Group II intron is RNA component that act as catalyst and found in different prokaryotic and eukaryotic cells [66]. Recently, it has been reported that group II intron is also found in variety of bacterial gene [67]. Moreover, group II intron can mobilized efficiently *via* a process called homing to the allele i.e., not a real intron [68] shown in (Figure 2). In addition, group II intron can be incorporated in double-stranded DNA at a specific target position [69]. Most of mobile intron have Intron-Encoded Protein (IEP) containing a reverse transcriptase that helps in splicing and homing activities such as DNA endonuclease and RNA maturase [70,71]. Mobile intron initiates activate after using structure of RNA in order to enhance the splicing catalytically whereas, IEP helps to conjugate intron RNA. As a result, exon and intron lariat-IEP Ribonucleo Protein (RNP) complex are formed. RNP complex are recognized as specific DNA target position and promote to integrate a single strand of target DNA *via* reverse splicing of intron RNA [72-75]. After that, IEP cut the other side of strands and use as a primer of target DNA. As a result, cDNA cloning of the resulting intron is integrated by recombinant of cell or repair mechanism [76-78].
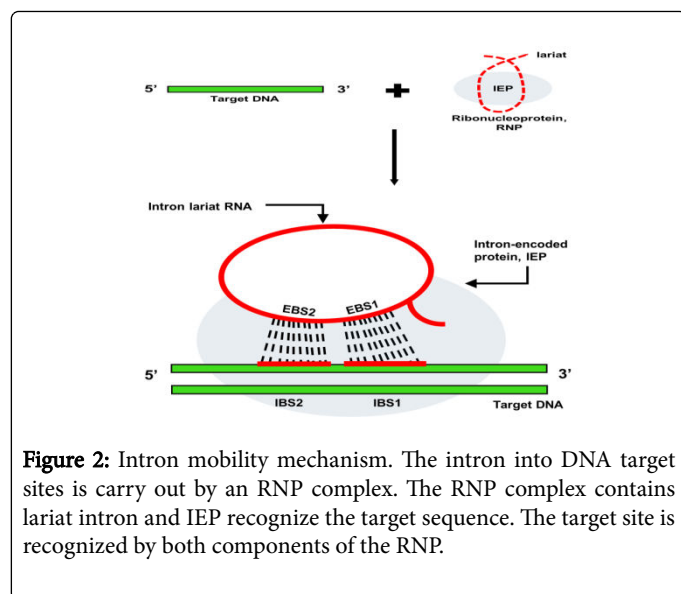

Figure 2: Intron mobility mechanism. The intron into DNA target sites is carry out by an RNP complex. The RNP complex contains lariat intron and IEP recognize the target sequence. The target site is recognized by both components of the RNP.

Ll.ltrB-lactococcal intron is first bacterial group II intron with splicing and genetic mobility [79,80]. Homing occurs in Ll.ltrB through a complex pathway that reaches the homing part *via* complex of RNA and IEP intron that are located in the target position of DNA [75,81]. Like other group II intron, Ll.ltrB includes Exon Binding Site (EBS) sequence that forms a combination of Intron Binding Sites (IBS) for homing. In addition, the role of IEP protein in Ll.ltrB is positioning of RNA in annealing and target of double-stranded DNA material that permits RNA-DNA interaction [81].

Moreover, specificity of intron is determined *via* base pairing. Thus, determinants of specificity and EBS of intron can be recalibrated in order to permit the intron homing in new DNA position. Ll.ltrB intron can be retargeted for insertion of plasmid and chromosomal target. It is efficiently done in *E. coli* and other enteric bacteria [82,83]. In addition, biochemical and genetic data is identified as thumb rule to recognize the target position and it enables to design all the intron with targeted gene [81].

Use of Lactococcal Group II Intron

Ll. ltrB-*lactoccoci* has potential to use it as an agent for targeted genetic traits. Thus, development of genetic system after using *lactococci* has given a new direction to structure and function of group II intron [65]. Moreover, food grade plasmid cannot hold antibiotic resistance gene or DNA sequence of *E. coli*. Therefore, intimin gene that appears on the cell surface of *E. coli* O157 i.e. Green Fluorescent Protein (GFP) was inserted directly into chromosomal DNA of *L. lactis*.

Group II intron can easily move the genetic elements that can be invasive into a specific gene [68]. Ll.ltrB, lactococcal group II intron was used to achieve the multi copy delivery of heterologous genes after using the genome of *L. lactis* IL1403-ucd. However, there was no need to use selectable markers for transfer of genes [84].

Ll.ltrB was retargeted in order to invade three transposase gene (*tra* gene). *Tra* gene was present in IL1403-ucd, but each copy of 9, 10, 14 was found in tra904, tra981 and tra983 respectively [85]. Intron invasion of Tra904, tra981 and tra983 allele group showed high frequency and individual segregate property. Therefore, it has to copy from 1 to 9 in tra alleles [82,86,87]. Whereas, in order to obtain carrier of large copy number of heterologous gene, GFP markers replicate in Ll.ltrB after targeting tra904 and resulting intron (Ll.ltrB: GFP) was induced to invade *L. lactis* tra904 allele. Thus, segregates with Ll.ltrB: GFP were obtained with 3, 4, 5, 6, 7, 8 copy from tra904. In general, increasing the number of chromosomes of Ll.ltrB: GFP leads to high expression of GFP in the strains. The highest levels of GFP expression was found in specific 6 copy that generates GFP at similar level to obtain the multiple cloning plasmid. In addition, the highest level of GFP expression was stable more than 120 generations. It shows that stable integration of multiple replication of heterologous genes can be obtained *via* group II intron carrying a bacterial genome. Also, *L. lactic* can undergoes DNA transformation and reveals new horizons for future research [84].

Conclusion

Based on our study, together with data obtained from others, we can emphasize the interests in using LAB strains to develop novel therapeutic protein mucosal delivery vectors, which should be tested in human clinical trials. Therefore a bio-contaminant strategy to prevent the dissemination in the environment of this genetically modified LAB should be developed before they can be used in humans. There is a need to optimize some more aspects of LAB as vaccine delivery system. *Lactococci* and *Lactobacilli* both can be used targeted delivery of mucosal vaccines against many diseases, but there is need to improve at various levels i.e., nature of molecule delivered at the targeted site, expression systems for increasing the quantity of delivered molecule, nature of *lactobaccili spp.* as *Lactobacilli casei* gives more advantages in comparison to *Lactobacilli lactis*. Efforts should be continued due to the future of prophylactic and therapeutic strategies based on recombinant *Lactococci* and *Lactobaccili* requires a clear demonstration of their efficacy in human clinical trials, which would lead to a better acceptance.

Acknowledgment

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