

Genetically Engineered Probiotics

¹Charu Gupta, Dhan Prakash and ²Sneh Gupta

¹Amity Institute for Herbal Research and Studies,
Amity University UP, Sector-125, Noida-201303, India

²Department of Zoology, R.G.P.G. College,
Chippi Tank, Meerut-250001, India

Abstract: Intestinal microbiota is a key component of both the metabolism and immunity. These can be helpful in healthcare, especially for the management of digestive diseases and food-borne illnesses. Through genetic engineering it is possible to fully express biologically active copies of such powerful molecules from food and commensal bacteria. Genetically engineered probiotics can be used to treat inflammatory bowel disorders such as Crohn's disease, ulcerative colitis, as well as other disorders that result from an overactive immune system. Genetically modified (GM) probiotics are also used as food additives to control the release of human growth factors by the modified bacteria to fight against injury and inflammation in the gut. For example the use of a plant sugar called xylan to stimulate the genetically modified human gut probiotic bacterium *Bacteroides ovatus* to produce specific proteins that can repair damaged cells and dampen down the immune system in the intestine that causes inflammation and disease. Studies have confirmed that administration of xylan with the genetically engineered probiotic bacteria resulted in a significant improvement of colitis, reduced weight loss, improved stool consistency, reduced rectal bleeding and accelerated healing of damaged colonic cells. GM probiotics possess potential in clinical applications like delivery of antigens for vaccines and thus are more readily accepted. This would provide a safer method of vaccination than the use of attenuated pathogens e.g. GM, *Lactococcus lactis*, produces IL-10 in the mouse intestine. The safety of such organisms that produce very powerful bioactive substances is of major concern as excess production of these substances in a healthy individual may be detrimental.

Key words: Bowel Disease • Colitis • Genetically Engineered • Inflammation • Probiotics

INTRODUCTION

Probiotic micro-organisms have been used since several years. Originating as food supplements, they are now most often administered orally and offer an attractive alternative for treatment of intestinal disorders. Lactic acid bacteria (LAB) 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 [1].

Probiotics have many potential therapeutic uses, but have not been universally accepted due to the lack of understanding of their action. LAB has been modified by traditional and genetic engineering methods to produce new varieties. Modern techniques of molecular biology have facilitated the identification of probiotic lactic acid bacterial strains, but only a few LAB have been modified by recombinant-DNA technology because of consumer resistance to their introduction to markets [2].

Lactococcus lactis is a food-grade, non-colonizing, non-invasive, Gram-positive bacterium, widely used in the preparation of food products and recognized as being

completely safe for human consumption. These LAB can be genetically modified to efficiently express any desired protein or peptide without disturbing their biological activity.

When delivered in the gastrointestinal tract, genetically modified *L. lactis* bacteria reside there for 8–48 h, during which time they continuously release therapeutic amounts of biologically active proteins. New prototypes of genetically modified *L. lactis* strains expressing single as well as multiple human α cell-specific Ag (s) are currently under investigation. Different safety agencies have already given their approval to the use of transgenic bacteria, so a phase I trial has been performed in Crohn's disease and the other is in progress for oral mucositis [3].

A better understanding of the mechanisms by which these micro-organisms act has now opened up possibilities for designing new probiotic strains. Through genetic engineering, it is possible not only to strengthen the effects of existing strains, but also to create completely new probiotics. These need not necessarily be composed only of bacterial products but can also include elements of regulatory systems or enzymes derived from a foreign-human-source. If designed carefully and with absolute attention to biological safety, the development of genetically modified probiotics like *Lactococcus lactis* has the potential to revolutionize alimentary health by secreting the actives of interest [4]. It is an elegant strategy for gut mucosal delivery.

Type-1 diabetes is an autoimmune disease against pancreatic beta cells and the scientists have demonstrated that oral treatment of diabetic mice with genetically modified *L. lactis* strain to secrete human pro-insulin and the immuno-modulatory cytokine IL-10 that is able to restore the tolerance of pancreatic beta cells in a safe, specific and long-lasting way [5].

In another study, scientists deleted a gene from the bacterium *Lactobacillus acidophilus* which is commonly found in yoghurt. This gene is responsible for producing an enzyme that increases inflammation, a defining characteristic of Crohn's disease and ulcerative colitis. But the unaltered form of the bacterium also triggered production of a beneficial immune molecule, Interleukin-10 that helps to regulate the immune system. The goal of engineering the microbes was to deliver the beneficial effects without the harmful impact [6].

When the engineered microbe was fed to mice suffering with colitis and inflammation of the colon, the engineered bacteria prevented the weight loss and bloody diarrhea that typically accompanies this condition.

In addition, the treated mice had 90% less inflammation in their colon tissue than did their untreated counterparts [6].

Inflammatory bowel disease is one of the prime areas of interest for probiotic treatment, but there is little evidence to show that probiotics are effective in treatment or prevention of Crohn's disease. Some studies suggest that two different probiotic formulations can help prevent recurrence of ulcerative colitis. The mechanism of action of how probiotic bacteria influence the gastrointestinal system is still unknown but previous research suggests several possible mechanisms. Beneficial bacteria might temporarily alter the ratio of good to bad bacteria that inhabit the intestine, or they might specifically block activity of bad bacteria. Probiotics seem to influence the immune system by stimulating protective immune cells or blocking detrimental activities of immune cells [7].

Researchers looked in detail at the molecular effects of the engineered bacteria and found that the production of regulatory immune cells, rather than inflammatory immune cells was enhanced. The regulatory T cells, a type of immune cell, counteract the effects of harmful immune cells that attack the cells lining the gut [8].

According to another study, probiotic treatments do not have the same effects in humans as in animals and there is a big difference in showing protection in animal models (by treating the animals before symptoms occur) versus treating ongoing human disease [9].

Before beginning clinical tests, it is important to study the roles of more kinds of surface proteins in the engineered bacteria, to determine which are helpful and which are harmful. If the molecules, produced by the bacteria, can be identified that regulate the immune system, they may be used to develop drugs that have a similar effect. It is easier to modify and give controlled doses of chemical compounds than live bacteria, which can behave unpredictably once ingested [4].

Engineered probiotics as a treatment for colon cancer can also be explored. In preliminary studies in mice designed to mimic colon cancer, treatment with the modified bacteria reduced the number of polyps the animals developed by 90 percent. The scientists observed an average of only three small polyps in treated mice, compared to about 35 to 50 in untreated [10].

The bacteria's ability to reduce inflammation is not limited to the gut but the regulatory cells migrate throughout the body. It is hypothesized that these microbes may also be able to help treat other diseases linked to inflammation, such as rheumatoid arthritis and psoriasis [11].

Bioengineered probiotics expressing foreign gene products to achieve inhibitory effects against food-borne pathogens is highly desirable. Enteric infections account for high morbidity and mortality and are considered to be the fifth leading cause of death at all ages worldwide. Significant efforts have been directed toward the detection, control and prevention of food-borne diseases. Many antimicrobials including antibiotics have been used for their control and prevention. However, probiotics offer a potential alternative intervention strategy owing to their general health beneficial properties [12].

Genetically engineered probiotics are also useful in the treatment of parasitic infections. In a study, laboratory animals that were fed a modified version of a common genetically modified probiotic were completely cured of intestinal worms that belong to a family of parasites. It is believed that around 1.5 billion people are currently infected with the intestinal worms (Annual meeting of the American Society of Tropical Medicine and Hygiene (ASTMH). Soil transmitted helminthes (STHs) and other intestinal worms, though rarely fatal, are leading contributors to disease in school-age children in low-income countries and are amongst the most burdensome of the world's "neglected tropical diseases" or NTDs. STHs are found in soil that has been contaminated with human feces. Hookworms can linger in the intestines for years, where they feed on blood and tissue, robbing their hosts of iron and protein and interfering with absorption of critical nutrients. They frequently cause stunting and cognitive delays in infected children. They also can have long-term effects on educational achievement and productivity [4].

The only drugs available to treat hookworms in humans were originally developed to combat parasites that infect farm animals. They were only partially effective against the range of intestinal parasites that infect humans. There is also evidence of re-infections occurring rapidly after treatment and low levels of efficacy in some cases.

In an experiment, hamsters were deliberately infected with hookworms and then divided into two groups. One group received a bacteria *Bacillus subtilis*, which is often marketed as a "probiotic". The other group received the same probiotic, except the researchers modified it to express a protein derived from a closely related bacterium, *Bacillus thuringiensis* or Bt, which is known to be safe in humans but potentially lethal to intestinal worms. After five days of administration of bacteria, the intestine of the animals was examined for the presence of worms. There were no worms in the animals that received the

modified probiotic while those that did not received remained infected. This probiotic is a food-grade bacterial product that can be easily produced in large quantities in a simple fermenter and it can be manufactured in a form that has a long shelf-life and is also cost effective [2].

Bt is attractive because it is a well-understood, natural substance for controlling plant pests that is believed to be safe for animals and humans. It is frequently sprayed on organic crops and is mainly lethal to insects in their larva stage. Bt is also a bacteria used in genetically engineered corn and soybean to endow the crops with resistance to plant pests. It can be used as an effective intervention for intestinal worms as a treatment and not as a dietary supplement [12].

However, these results need to be replicated in other animals and also in humans but this is an important development to find a safe, affordable and effective way to confront a major global health problem.

Inter-relationship Between Human and Bacteria:

The human gastrointestinal tract microbiota consists of a large number of bacteria around 10-fold more than the total number of human cells that plays an important role in several physiological and metabolic processes and in the development of immune system. The collective adult human gastrointestinal tract microbiota is composed of upto 1000-1200 bacterial species [7, 13] with Gram-positive bacteria as the predominant species. Clostridium group is the most abundant in this complex ecosystem [13].

This micro-ecosystem is fundamental for the maintenance of homeostasis of a healthy individual [10]. This is a perfect example of mutualism between the host and bacteria where commensal bacteria provides essential nutrients to the host, metabolize indigestible compounds, defend against colonization of opportunistic pathogens and contribute to the development of intestinal architecture and stimulates the immune system [14]; whereas host provides nutrients and a stable environment to the bacteria [10].

Advantages of Probiotics for Human: The concept of probiotics is based on the observations made by Elie Metchnikoff (1907) in which the regular consumption of lactic acid bacteria in fermented dairy products such as yoghurt was associated with enhanced health and longevity in many people [15]. Most probiotics belong to group of LAB but some species and genera belong to other group also. Notable examples of probiotics are *Bifidobacterium sp.*, *Escherichia coli* Nissle 1917 and

east *Saccharomyces boulardii* [16]. The important criteria for a microorganism to be considered as probiotic are: i) it should have a clear beneficial effect on the host; ii) it should be non-pathogenic; iii) it should be able to survive transit through the GIT; iv) large number of viable bacteria must be able to survive prolonged periods upon storage [17].

Microbial Imbalances: Dysbiosis: Dysbiosis is microbial imbalance within the body. It usually occurs when the homeostasis (balanced microbial ecosystem) is broken due to overgrowth of opportunistic bacteria leading to a situation of illness. The apt correlation between dysbiosis and disease has been found with inflammatory bowel disease (IBD) where the proportion of Firmicutes, particularly *Faecalibacterium prausnitzii* was found to be low in patients that exhibited endoscopic recurrence 6 months after surgery [6, 18]. The composition of human microbiota is different in each individual, changes in phylogenetic distribution have been found in obese and diabetic individuals versus normal ones [19, 20]. The importance of human microbiota has been explained in hygiene hypothesis [21] which described that low exposure to infectious agents in early life explains the increased number of people suffering from allergies and asthma in developed countries. This hypothesis suggests that a well-balanced human microbiota is a factor that protects from such pathologies [22, 23]. There is some inter-relationship between microbial activities and health and disease. The production of short chain fatty acids such as butyrate has been proposed to protect against different illnesses [9, 24, 25].

Probiotics for Dysbiosis: The administration of beneficial microorganisms to restore the normal ecosystem is a strategy to improve the health status of the patient and/or to prevent a normal healthy individual from acquiring dysbiosis in the future. There is scientific evidence on the use of probiotics as therapeutics against traveler's diarrhea, irritable bowel syndrome (IBS), IBD, lactose intolerance, peptic ulcers, allergy and autoimmune disorders among others [24, 9]. The colonization of GIT with Bifidobacteria properly shapes gut microbiota, induces oral tolerance and decreases the frequency of allergic disorders [25]. Perinatal administration of a probiotic strain of *Lactobacillus rhamnosus* GG (LGG) reduces the development of atopic eczema in children due to the anti-inflammatory properties of this probiotic

[25-27]. Moreover, LGG is also found effective in reducing atopic dermatitis as it enhances the production of the anti-inflammatory cytokine IL-10 [28]. Oral administration of *L. casei* Shirota strain inhibits specific IgE production [29].

There are various reports that have confirmed that selected strains of probiotics that have been genetically modified has various therapeutic effects in IBD, e.g. *L. casei* BL23 strain has shown anti-inflammatory effects in an animal model, other probiotic strain like VSL#3, LGG, BIFICO, *E. coli* Nissle) have shown positive effects in human trials suffering with pouchitis, UC and CD [11]. Likewise *Lactobacillus acidophilus* has a modulatory effect in intestinal pain [30].

From the above studies, it is pertinent to say that equilibrium of the microbiota in the gastro-intestinal tract is very important as its disturbance can contribute to several intestinal diseases such as IBD. Gut microbiota performs various functions like metabolize nutrients, produce vitamins and degrade toxic products such as carcinogens, food additives, bile salts and cholesterol etc [12]. Probiotic bacteria are also helpful in intestinal disorders like IBS (irritable bowel syndrome), that is characterized by chronic abdominal pain, discomfort, bloating and alteration of bowel habits.

Genetically Modified Microorganisms: New Prospects
Delivery of Therapeutic Molecules: Administration of probiotics is a natural way to treat dysbiosis within the GIT. Commensal bacteria can be genetically modified to function as potential live delivery vectors for target compounds such as food grade LAB at the mucosal level [31, 8]. Recombinant bacteria are used for producing the heterologous molecule of interest *in vivo* that can be used as vaccines. The main advantage of using the genetically engineered bacteria or viruses is that they can be used as carriers to produce heterologous antigens that increase the immunogenicity of weakly immunogenic antigens. The live bacterial vectors have an additional advantage over viruses that they have a genome that is able to harbor many heterologous genes, in contrast to viruses where the capacity to encapsulate foreign DNA is limited. This is an effective strategy to deliver therapeutic molecules to the mucosal tissues in order to avoid degradation and promote uptake of the antigen *in situ* and stimulate adaptive immune responses rather than the tolerogenic immune responses that are observed in feeding studies with soluble antigens [32].

There are two types of bacterial vectors used to deliver compounds at the mucosal level: attenuated pathogens and non-pathogenic bacteria. Gram-positive commensal or food-grade bacteria constitute good alternatives to pathogenic bacteria [33]. The reason behind using food-grade LAB is that they are used in the fermentation and preservation of food and is categorized under GRAS (Generally Recognized as Safe). The other advantage of LAB as delivery vectors for vaccine purposes is due to their potential to elicit both antigen-specific immune responses at mucosal surface and effective systemic immune responses. Studies have shown that LAB vaccines elicit antigen-specific IgA response in faeces, saliva or broncho-alveolar and intestinal lavage fluids and antigen-specific IgA-secreting cells in the lungs and mesenteric lymph nodes [34].

Lactococcus lactis is a model LAB species that is used for the heterologous expression of therapeutic proteins such as antigens, cytokines and enzymes.

Recombinant LAB and Commensals in IBD:

The conventional therapies for IBD are anti-inflammatory drugs combined with immunosuppressive agents [35]. Recombinant *L. lactis* strain is used for producing and delivering IL-10, an anti-inflammatory cytokine, *in situ* in different mouse models [36]. It has been shown that daily mucosal administration of recombinant *L. lactis* secreting IL-10 led to a 50% decrease of the colitis that was induced by the administration of dextran sodium sulphate (DSS) in mice. This beneficial effect was dependent on the *in situ* secretion of IL-10 by the live lactococci. The delivery of immunosuppressive cytokines by other bacteria has also been studied e.g. probiotic potential of IL-10 expressing *E. coli* Nissle 1917. The use of LAB to produce Trefoil Factors (TFF) at the mucosal level to treat IBD has been also studied. TFF are a class of non-mitogenic peptides that helps in the protection and repair of the intestinal epithelium [37]. These peptides have strong protective effects for repairing mucosa after damage. They are usually administered by an oral route where they are absorbed at the intestinal level. Intra-gastric administration of recombinant *L. lactis* secreting TFF leads to the expression of the active peptides in the colon and prevents and repairs damage due to acute colitis [38].

Recombinant *L. lactis* was also used to treat colitis that secretes Lcr V antigen, anti-inflammatory protein produced by *Yersinia pseudotuberculosis* to escape the immune response of the host.

Recently LAB secreting elafin have been tested in chronic and acute colitis models and it was found that inflamed epithelium was protected from increased intestinal permeability and from the release of cytokines and chemokines by LAB secreting elafin [39]. In addition to these recombinant probiotic strains, mutants in specific genes encoding potential probiotic functions (mucosal adhesion factors, resistance to acid, specific cell wall components etc.) have been engineered to compare their biological effects with that of their wild-type counterparts.

Grangette *et al.* [40] has used a mutant of *Lactobacillus plantarum* with impaired capacity to incorporate D-alanine in teichoic acids [40]. This mutant was found to be more protective in a mouse model of colitis than the wild type strain. Moreover, the lipoteichoic acid deficient *L. acidophilus* enhances IL-10 production by dendritic cells and macrophages and down-regulates IL-12 and TNF- α under *in vitro* conditions [41].

As discussed in the above paragraphs that *Lactococcus lactis* is the most widely used LAB in the production of heterologous proteins and is considered as the live delivery vector model [42]. But the main drawback is that this bacterium has a short survival time (~24 hrs) in the human GIT thus leading to a reduced time of action.

As an alternative, *Bacteroides ovatus* was selected over *L. lactis* by Hamady *et al.* [43] to deliver the proteins. This bacterium has the ability to colonize the colon and can utilize xylan. They were able to develop a xylan-regulated delivery of human keratynocyte growth factor-2 to the inflamed colon [44] and the human TGF- β 1 to treat colitis in mice [45]. These promising results confirm the potential of the use of recombinant commensal for *in vivo* delivery.

Future Prospects: Public interest in probiotics is on the upswing. Scientists are bringing this traditional therapy into the 21st century by genetically engineering the microbes to enhance their effect on the immune system. They hope that the new bugs will ultimately help in the treatment of inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, as well as other disorders that result from an overactive immune system.

Current probiotic research encourages the search and characterization of gut bacteria as a model for finding new natural and/or engineered probiotic strains that can be used to restore the normal balance of the human gut ecosystem. The complete knowledge of molecular mechanisms and active compounds in commensal and probiotic strains is still lacking. Taking into account

that the human GIT is composed of 10^{13} - 10^{14} microorganisms, it is important to focus on the exact role of bacterial active compounds in homeostasis and immune response.

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