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GENOME TOOLS FOR THE DEVELOPMENT OF RECOMBINANT LACTIC ACID BACTERIA AND THEIR SAFETY ASPECTS

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ABSTRACT

Lactic acid bacteria (LAB) are a phylogenetically diverse group with the ability to convert soluble carbohydrates into lactic acid. Many LAB have a long history of safe use in fermented foods and are recognized as food-grade microorganisms. LAB are also natural inhabitants of the human intestinal tract and have beneficial effects on health. Considering these properties, LAB have potential applications as biotherapeutic vehicles to delivery cytokines, antigens and other medicinal molecules. In this review, we summarize the development of, and advances in, genome manipulation techniques for engineering LAB and the expected future development of such genetic tools. These methods are crucial for us to maximize the value of LAB. We also discuss applications of the genome-editing tools in enhancing probiotic characteristics and therapeutic functionalities of LAB.Together with novel approaches such as CRISPR-Cas, the established systems for engineering now allow significant improvements to LAB strains.use of GM-LAB as cell factories in closed systems that prevent their environmental release is the least problematic aspect, and this is also discussed.

Introduction :

Fermentation is the oldest process of food preservation. As scientific studies have established that consumption of fermented food with live microorganism can promote human health beyond adding nutritional values, demand for fermented food or probiotic foods is increasing during the last two decades. Fermented foods occupy one-third of our diet, and more than 3000 different types of fermented foods and beverages are prepared and consumed by billions of people around the world. Probiotics are defined as "live microorganisms, which when administrated in adequate amounts, confer a health benefit to the host". Metchnikoff's probiotic theory of life prolongation was never directly tested, and researchers reported in 1924 that LAB present in yogurt,

specifically Lactobacillus bulgaricus, most likely do not reduce "putrifying" bacteria in the intestine because L. bulgaricus did not survive gastrointestinal (GI) transit.

Regardless, Metchnikoff's theory was born that bacteria could be health-promoting through modifying the composition of the bacterial population that inhabits our intestine. In 2017, this translates into the use of (tailored) probiotics to modify the gut microbiota to promote human health. Today we can engineer LAB in general, and recent advances have made it possible to engineer select probiotic strains in a high-throughput manner. Also, we have an increased appreciation and understanding of the role of the gut microbiota in health and disease. Metchnikoff's theory a step closer to reality. We will highlight the potential of clustered regularly interspaced short palindromic repeats (CRISPRs) and the CRISPR-associated enzyme (Cas) as a broadly applicable molecular tool to engineer probiotics. genetic engineering of LAB offers various tools to improve the strains and to enable greater viability and stability, and production and growth rates. The possibilities to effectively express therapeutic proteins and to use LAB as vaccines further strengthens their potential uses. However, no matter how effective a specific GM-LAB might be, a drawback that stands in the way of its marketing is that it has been genetically manipulated; this is mainly due to low consumer acceptance of GM microorganisms (particularly in the European Union [EU]) and regulatory restrictions as to their use. New methods such as recombineering and 'clustered regularly interspaced short palindromic repeats' (CRISPR)-Cas9 now allow highly controlled implementation of precise modifications [5]. While certain levels of acceptance of these methods has been achieved in the USA, they have not been differentiated from other methods of genetic modification in Europe. The obstacles that can be encountered when trying to bring genetically engineered LAB to the market are discussed here, as well as the steps that would be required for them to reach better acceptance.

Lactobacillus Strain Selection

The cheese bacterium Lactococcus lactis MG1363 was engineered to secrete murine interleukin-10 (IL-10). Oral administration of the recombinant lactic acid bacterium significantly reduced intestinal inflammation in two mouse models of disease. the presence of murine IL-10 in the colon of IL10−/− mice and (ii) that de novo synthesis of murine IL-10 by recombinant L. lactis during GI transit resulted in amelioration of intestinal inflammation. Subsequently, the L. lactis workhorse has been exploited to deliver a variety of recombinant proteins & DNA and has paved the way to harness other microbes as delivery vehicles. As these studies clearly demonstrate that L. lactis can be a successful delivery vehicle, what rationale supports the development of a therapeutic delivery platform using a microbe other than L. lactis ,One argument arises from the fact that L. lactis is a microbe that originates from a plant environment and has adapted to thrive in milk, not in humans . Because the optimal growth temperature of L. lactis is 30°C, some researchers may choose to select a strain that thrives at 37°C. A higher in vivo metabolic rate is predicted to yield increased therapeutic protein delivery, which may be desirable. Also, the efficiency of survival following GI transit can be a selection criterion.

Several strains have been identified, including some lactobacilli, that better survive the conditions encountered during GI transit compared to L. lactis. Selection of strains that thrive at 37°C and can withstand the harsh in vivo conditions can thus be a key criterion. In addition, some research groups have exploited natural healthpromoting strains, such as Escherichia coli Nissle 1917. Although not within the focus of this chapter, E. coli Nissle 1917 is notable as the only probiotic E. coli in use for over a century and is now being developed as a therapeutic delivery platform. A clear advantage is that researchers can tap into the most comprehensive genetic toolbox available for bacteria. E. coli Nissle 1917 is one of the few Gram-negative probiotics, in contrast to the plethora of Gram-positive probiotics that are widely represented by lactobacilli. While lactobacilli are being exploited as delivery vehicles, strain selection will be key for successful design of tailored probiotics.

LACTOBACILLUS GENOME EDITING

One approach to enhance health-promoting properties is the ability to transform cells with exogenous DNA to genetically modify and engineer their genome. Depending on the type of application and the organism of choice, such engineering can be the first hurdle in the development of a LAB therapeutic. Several tools have been developed, but their application can be strain-dependent. Our discussion will not cover genetic engineering tools comprehensively, but we will briefly highlight some tools that have been developed and applied to lactobacilli. These include Cre-lox , bacteriophage integrases introns , and two-plasmid integration systems. To create DNA insertions or deletions following Campbell-like integration, the upp-encoded uracil phosphoribosyltransferase can be used in some strains to identify cells in which the second homologous recombination event occurred. Such tools have been very valuable to the LAB research community, but high-throughput engineering approaches to make subtle mutations in a LAB genome have been limited until more recently. The Britton laboratory developed SSDR in L. lactis, L. reuteri, and Lactobacillus plantarum. The procedure was optimized for use in L. reuteri and L. lactis, while proof of concept was demonstrated in L. plantarum. SSDR allows the user to create single-nucleotide changes in the chromosome without the need for antibiotic selection. Only an oligonucleotide is needed to generate the desired mutation(s) in the chromosome, and no cloning is required. SSDR can also be applied to make small deletions.

CRISPR–Cas-based systems : CRISPR–Cas system constitute adaptive immune systems in bacteria and archaea that can actively reject the invasion of foreign genetic elements such as phages and plasmids.

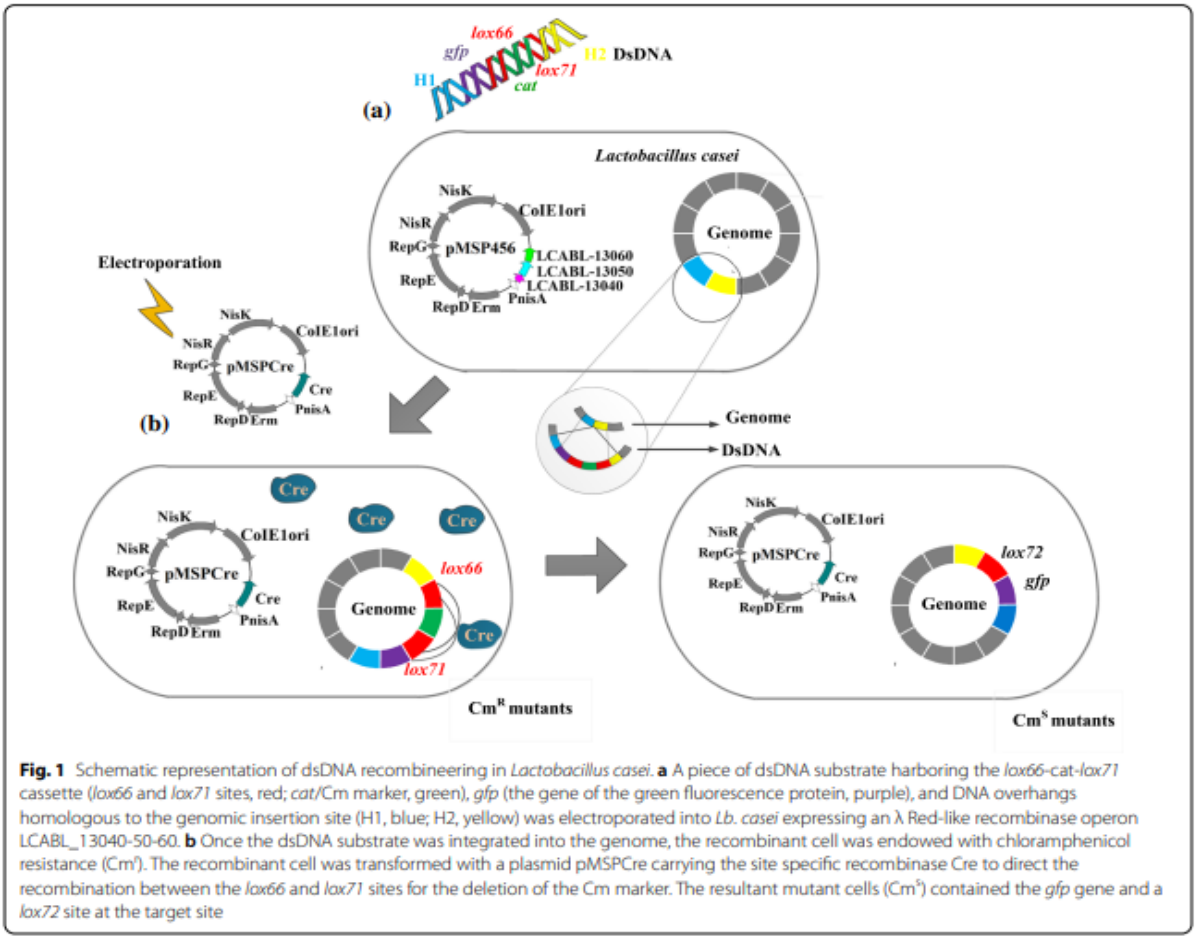

Fig. 1 Schematic representation of dsDNA recombineering in *Lactobacillus casei*. **a** A piece of dsDNA substrate harboring the *lox66*-cat-*lox71* cassette (*lox66* and *lox71* sites, red; *cat*/Cm marker, green), *gfp* (the gene of the green fluorescence protein, purple), and DNA overhangs homologous to the genomic insertion site (H1, blue; H2, yellow) was electroporated into *Lb. casei* expressing an λ Red-like recombinase operon LCABL_13040-50-60. **b** Once the dsDNA substrate was integrated into the genome, the recombinant cell was endowed with chloramphenicol resistance (Cm^r). The recombinant cell was transformed with a plasmid pMSPCre carrying the site specific recombinase Cre to direct the recombination between the *lox66* and *lox71* sites for the deletion of the Cm marker. The resultant mutant cells (Cm^s) contained the *gfp* gene and a *lox72* site at the target site

CRISPR–Cas systems has undergone explosive growth. In particular, the type II CRISPR–Cas9 system from S. pyogenes has been exploited as a facile and programmable platform for genome editing in a sequence-specifc manner in some eukaryotes and prokaryotes. Te system consists of Cas9 (an endonuclease), a trans-activating CRISPR RNA (tracrRNA) and a precursor crRNA array containing nuclease guide sequences (spacer) interspaced by identical direct repeats.

Precursor crRNA is processed within repeat sequences to generate mature crRNA, which further forms a duplex with the tracerRNA. Te duplex interacts with Cas9, searches present DNA for a trinucleotide protospacer adjacent motif (PAM), and binds to proximal chromosomal complementary sequences (protospacer), inducing double-stranded breaks (DSBs) in the chromosome . lethal (unless repaired) DSBs stimulate the non-homologous end joining recombination (NHEJ) (existed in rare bacteria) or homologous recombination (HR) pathway to repair the DNA lesion, and thus desired mutations can be produced [34]. Moreover, CRISPR–Cas9 can be used as a counter selectable marker, as Cas9-induced DSBs in the wildtype allele allow rapid screening of expected mutants. In 2014, a RecT-assisted CRISPR–Cas9 approach was developed to perform codon

saturation mutagenesis and gene deletions in the chromosome of Lb. reuteri ATCC PTA 6475 . A similar approach was used in Lc. lactis NZ9000; seamless genomic DNA insertion or deletion (Fig. 2a, b) was efficiently accomplished within 72 h . Te CRISPR–Cas9 system was even used to modify the genome of the Lc. lactis virulent phage P2 and precise mutations were successfully achieved without the assistance of heterologous recombinases . Two plasmids carrying the recombineering template and CRISPR–Cas9 elements to achieve genome editing in Lb. plantarum WJL, but this failed in Lb. plantarum NIZO2877 and Lb. plantarum WCFS1, indicating that the genetic engineering feasibility of the method varied depending on the targeted gene(s) and strain. Variants of Cas9 have also been developed, such as Cas9 nickase (Cas9D10A), which generates chromosomal singlestrand breaks (nicks), circumventing the high lethality of DSBs induced by Cas9 . A CRISPR–Cas9D10A-based plasmid was constructed for genome engineering of Lb. casei LC2W, which allowed enhanced green fuorescent protein (eGFP) gene insertion and putative uracil phosphoribosyltransferase (UPRT) gene deletions with efficiencies of 35% and 65%, respectively . In addition to introducing point mutations, deletions and insertions in targeted genes, the CRISPR system can be used to regulate gene expression through CRISPR interference (CRISPRi) with catalytically inactive variants of Cas9 (dCas9), in which the endonucleolytic activity of Cas9 has been eliminated but the targeted binding function was still remains . CRISPRi systems served as robust tools for transcriptional regulation of the essential cell cycle genes in Lb. plant arum.

Gene integration into the chromosome

LAB have relatively simple metabolic pathways and can survive in the intestinal tract. Therefore, they are ideal candidates for delivery of cytokines, antigens, and other pharmaceutical molecules. Previously, expression of the target genes using plasmids was a common strategy for producing desired metabolites, but antibiotic must be added as selective pressure to maintain the presence of plasmids in LAB. Integration of target genes or gene clusters into the chromosome of LAB is preferable, to avoid the potential product safety risks and environmental pollution associated with antibiotic use. Using dsDNA or ssDNA recombineering strategy, genes of interest can be knocked-in to the target locus in the chromosome, but both the size and copy number of inserted genes are limited. To achieve integration of large DNA fragments or gene clusters at one or several chromosomal loci, the site-specific recombination systems can be adopted. The site-specific recombinase catalyzes the recombination between the recombinase recognition sites on a circular DNA and the chromosome. Moreover, after one round of integration, an additional recognition site is generated in the chromosome, so it is possible to achieve repetitive integration of target genes into the target sites. As proof of this concept, we constructed the recombinant Lb. casei BL23 strains in which the gfp gene or the fmbrial adhesin gene faeG from Escherichia coli was repetitively integrated into the chromosome using the Cre-loxP system. GFP and FaeG were stably expressed in the recombinant strains without supplementation of the culture with antibiotics, and the protein production was comparable to that of a plasmidengineered strain. In addition to the Cre-loxP system, other site-specifc recombinases from LAB prophages have been developed as genomic integration tools. Researchers constructed an integration vector with several new genetic traits using both the integrase and

attP sequences of phage ΦAT3. Te integration vector was capable of stably integrating the gfp gene into the chromosome of lactobacilli.

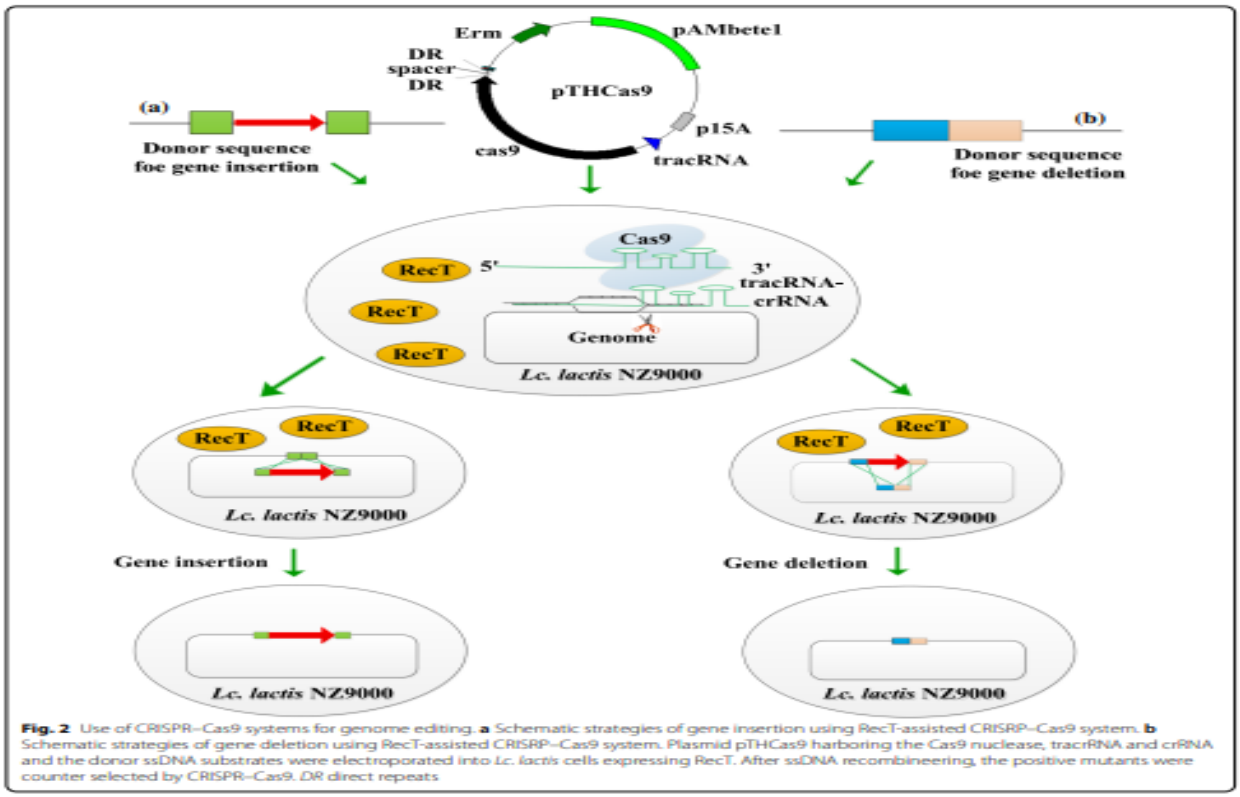

Fig. 2 Use of CRISPR–Cas9 systems for genome editing. **a** Schematic strategies of gene insertion using RecT-assisted CRISRP–Cas9 system. **b** Schematic strategies of gene deletion using RecT-assisted CRISRP–Cas9 system. Plasmid pTHCas9 harboring the Cas9 nuclease, tracrRNA and crRNA and the donor ssDNA substrates were electroporated into *Lc. lactis* cells expressing RecT. After ssDNA recombineering, the positive mutants were counter selected by CRISPR–Cas9. *DR* direct repeats

Applications of genome-editing tools in enhancing probiotic characteristics and therapeutic functionalities of LAB:

Potential tools that allow rapid, efficient genetic engineering of LAB. Tey enable production of tailored probiotics with specific traits, enhancing the probiotic characteristics and therapeutic functionalities of the bacteria.

Table 1 Current tools available for lactic acid bacteria genome editing

Tools	Examples of partial applications	Characteristics	References
Plasmids-based allelic exchange	*Lc. lactis, S. thermophilus, E. faecalis*	Homologous recombination-dependent; marker free; time-consuming	[19, 20]
DsDNA recombineering	*Lb. plantarum, Lb. casei*	Recombinase-mediated; high efficiencies for both deletion and insertion; marker-dependent	[29, 30]
SsDNA recombineering	*Lb. reuteri, Lc. lactis, Lb. plantarum, Lb. gasseri*	Mutation efficiency 0.4–19%; applicable to genomic mutagenesis; marker free	[31]
CRISPR–Cas-assisted recombineering	*Lb. reuteri, Lc. Lactis*	High efficiency (up to 100%) for small deletions (< 1.0 kb in *Lb. reuteri*, < 100 bp in *Lc. lactis*); marker free	[35, 36]
CRISPR–Cas9D10A	*Lb. casei*	Used for both gene deletion and insertion (25–65%); simplified editing procedure; marker free	[39]
CRISPRi	*Lb. plantarum, Lc. lactis*	Used to repress multiple target genes simultaneously; reversible effects; precise targeting; marker free	[40–43]

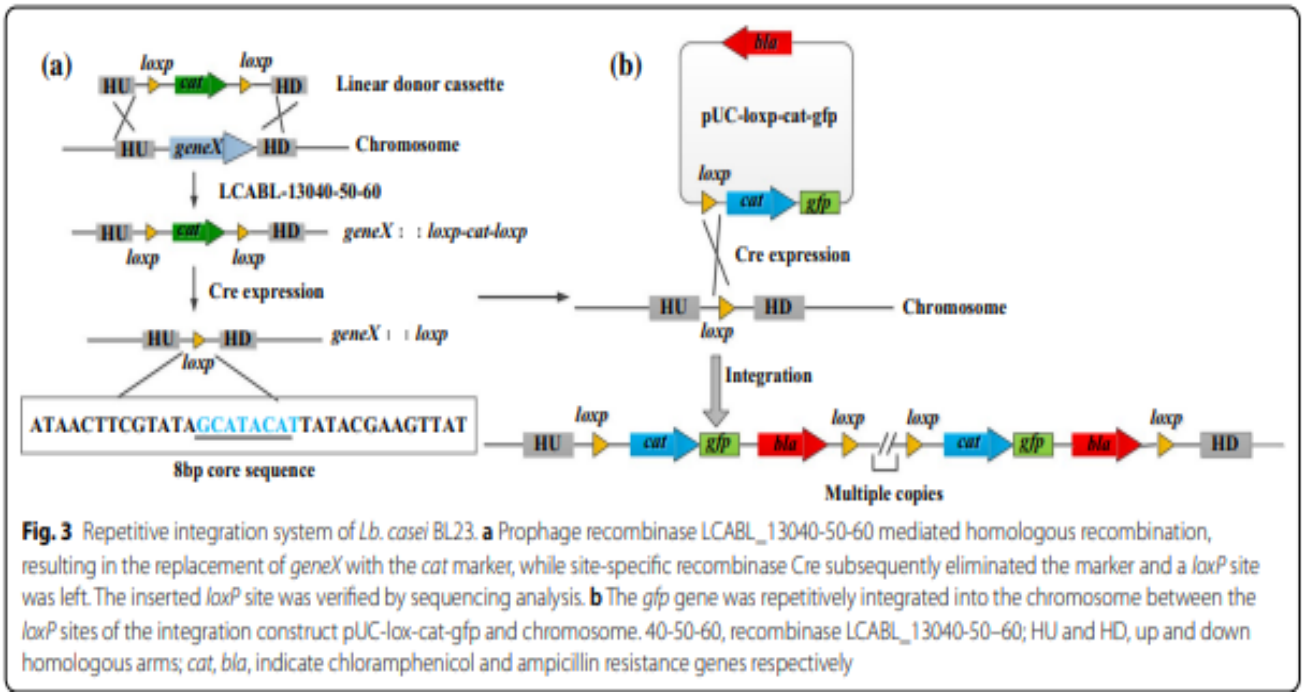

Fig. 3 Repetitive integration system of *Lb. casei* BL23. **a** Prophage recombinase LCABL_13040-50-60 mediated homologous recombination, resulting in the replacement of *geneX* with the *cat* marker, while site-specific recombinase Cre subsequently eliminated the marker and a *loxP* site was left. The inserted *loxP* site was verified by sequencing analysis. **b** The *gfp* gene was repetitively integrated into the chromosome between the *loxP* sites of the integration construct pUC-lox-cat-gfp and chromosome. 40-50-60, recombinase LCABL_13040-50–60; HU and HD, up and down homologous arms; *cat*, *bla*, indicate chloramphenicol and ampicillin resistance genes respectively

Enhanced probiotic strains and starter cultures: Bacteria must endure a variety of harsh conditions either in industrial environments or in the gastrointestinal tract, including oxidative and osmotic stress, acid and bile, pathogens, and the host immune response. These stresses pose a challenge to survival and effective colonization. Exopolysaccharides (EPS) is important for stress resistance. Researchers have demonstrated that spontaneousmutations of the hypothetical membrane-anchored protein Balat_1410 and the putative tyrosine kinase EpsC altered EPS properties in Bifdobacterium animalis subsp. lactis and Lb. johnsonii, respectively, resulting in cells that were resistant to gastrointestinal stress. S. thermophilus cannot grow on galactose and ferments only the glucose portion of lactose the residual galactose is excreted into the medium, which would

have adverse efects on galactosemia patients. Spontaneous mutation in the galKTEM promoter of S. thermophilus produced a mutant strain with galactose-consuming ability. Using the emerging genome editing technologies, such as the CRISPR–Cas systems, the introduction of single nucleotide mutations would undoubtedly faster than spontaneous mutation through consecutive cultures. Other galactose transformation pathways could also be introduced into S. thermophilus using genomic integration strategies.

Engineered LAB for delivery of biotherapeutics: LAB is appealing as vaccine carriers as they are able to induce both mucosal and systemic immune responses, and are free from the risks of conventional attenuated live pathogens,, the application of engineered Lc. lactis to secrete interleukin-10 (IL-10) for the treatment of infammatory bowel disease (IBD) in colitis-induced mice. In Lc. lactis, the essential thyA gene (encoding thymidylate synthase) was replaced by the IL-10-encoding gene; when deprived of thymidine or thymine, the viability of the strain decreased by several orders of magnitude, essentially preventing its accumulation in the environment. Lc. lactis without thyA has been evaluated in human clinical trials, and even though the trial did not satisfy expectations regarding efficacy, the bio-containment strategy was highly successful. Since the use of IL-10 for IBD treatment, many other cytokines have been produced in Lc. lactis, including IL-12 and IL-6. Apart from delivering cytokines, LAB have also been developed as cell factories for production and delivery of allergens. For example, Lc. lactis CHW9 was used to produce peanut allergen Ara 2 Lc. lactis NZ9800 was used to deliver the major birch allergen Bet-v1 and Lb. plantarum NCL21 was used to produce a major Japanese cedar pollen allergen, Cry j1, that can suppress allergen-specific immunoglobulin E response and nasal symptoms in a murine model of cedar pollinosis. Recombinant LAB are regarded as a potential alternatives to current therapies for type I diabetes; for example, recombinant Lc. lactis NZ9000 expressed fusion protein HSP65-6P277 to improve glucose tolerance in a mouse model. In the field of anticancer therapeutics, recombinant Lc. lactis NZ9000 secreting tumor metastasisinhibiting peptide Kisspeptin was used to inhibit HT-29 cell proliferation and migration through the induction of apoptosis pathways and by down regulating matrix metallopeptidase-9 expression. Other cancer antigens expressed using Lc. lactis include an E7 antigen against human papilloma virus type-16 and a glycosylated tyrosinase related protein-2 tumor antigen against melanoma. In addition to protein and peptide-based therapeutics, metabolites with medicinal applications are produced by LAB, such as γ-amino butyric acid and hyaluronic acid. The former is a non-proteinaceous amino acid with hypotensive, anticancer, ant anxiety, and diuretic properties, and the latter is a carbohydrate polymer used in wound healing and to treat dermatitis.

Table 2 Therapeutics produced from various recombinant lactic acid bacteria

Therapeutic products	Disorder/disease	Strains
Interleukin-10 (IL-10)	Inflammatory bowel disease (IBD)	*Lc. lactis N/S*
Interleukin-12 (IL-12)	Asthma	*Lc. lactis* NZ9000
Interleukin-6 (IL-6)	Adjuvant	*Lc. lactis* IL1403
Peanut allergen Ara2	Hypersensitivity type I	*Lc. lactis* CHW9
Birch allergen Betv1	Hypersensitivity type I	*Lc.lactis* NZ9800
Japanese cedar pollen allergen Cry j1	Hypersensitivity type I	*Lb. plantarum* NCL21
HSP65-6P277	Diabetes mellitus type I	*Lc. lactis* NZ9000
Kisspeptin	Colorectal cancer	*Lc. lactis* NZ9000
HPV-16-E7	HPV-16 induced cancers	*Lc. lactis* NZ9000
Glycosylated tyrosinase related protein-2	Skin cancer	*Lc. lactis* MG1363
γ-Amino butyric acid	Hypertension, anxiety	*Lb. pentosus* SS6
Hyaluronic acid	Wound healing, dermatitis	*Lb. acidophilus* PTCC1643

N/S not specified

Activate

The Consequence of Genome Modification: Genome modification of LAB can result in either inactivation of a given cell function or introduction of a new function. To achieve the inactivation of a function, a gene can be either deleted or mutated so as to diminish its function. Lactobacillus has three active bile salt hydrolase (bsh) genes and can persist in the mouse gut; Lactobacillus helveticus, on the other hand, cannot persist because it has a frame-shift in the bsh gene resulting in its inactivation. L. lactis NZ3000 carries an in-frame deletion of the chromosomal lacF gene, and therefore it will only grow when lactose is provided. Developments in the field have brought new techniques that now allow precise removal or replacement of genetic elements, and that have already been successfully applied to LAB , often with the aim of achieving biocontainment and avoiding the spread of unwanted genetic elements into the environment. When introducing a new function, a gene can be activated by mutation of the promotor (e.g., to allow or increase protein expression) or by alteration of a gene by point mutation (e.g., to increase the catalytic activity of the enzyme). A new function can also be introduced through the stable genome integration of a new gene. Genome integration results in low numbers of gene copies, but the integrant is not dependent on environmental or developmental factors. Another option is to introduce a new gene with a plasmid . in this case, multiple copies of recombinant genes can be introduced by increasing the plasmid copy number.

Conclusions and future perspectives: Overall, the examples we have provided illustrate how food-grade bacteria in general, and probiotic lactobacilli in particular, can be used to promote human health. As long as the scientific community continues to expand the genetic toolboxes for LAB, we envision that LAB will provide great potential to modulate the microbiota. In particular, the application of CRISPR-based technologies has the potential to eradicate target microbes in a strain-specific manner. Advancing this field must include considerations of the ancillary forces and dimensions that drive and enable technological advances, such as

intellectual property, regulatory processes, and consumer acceptance. The ongoing CRISPR intellectual property battles are somewhat hindering the adoption of this technology for food, agricultural, and clinical applications, given the lack of clarity around freedom to operate. Furthermore, regulatory approvals are still pending in some cases, and regulatory processes are unclear and/or yet to be defined in others. Lastly, consumer acceptance of genetically modified organisms remains a challenge that extends beyond the scientific dialogue, and much progress remains to be achieved. Nonetheless, the momentum of the ongoing microbiology renaissance remains strong, fueled by CRISPR technological advances and our increasing awareness of the microbiome. Recombineering and CRISPR-Cas9 techniques, in particular, will greatly facilitate targeted and trace-less genome modification. It is questionable whether a strain obtained via random mutagenesis (i.e., a method acceptable for regulatory bodies) is safer than a strain obtained via targeted and knowledge-based methods [113,178]. Therefore, all genome editing tools should not be treated in the same manner, but rather evaluated separately.

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