Gene editing and genetic engineering approaches for advanced probiotics: A Review

Ruby Yadav, Vishal Kumar, Mehak Baweja, Pratyoosh Shukla*

Enzyme Technology and Protein Bioinformatics Laboratory, Department of Microbiology, Maharshi Dayanand University, Rohtak-124001, Haryana, INDIA

*Corresponding author: pratyoosh.shukla@gmail.com

Abstract

The applications of probiotics are significant and thus resulted in need of genome analysis of probiotic strains. Various omics methods and systems biology approaches enables us to understand and optimize the metabolic processes. These techniques have increased the researcher's attention towards gut microbiome and provided a new source for the revelation of uncharacterized biosynthetic pathways which enables novel metabolic engineering approaches. In recent years, the broad and quantitative analysis of modified strains relies on systems biology tools such as in silico design which are commonly used methods for improving strain performance. The genetic manipulation of probiotic microorganisms is crucial for defining their role in intestinal microbiota and exploring their beneficial properties. This review describes an overview of gene editing and system biology approaches, highlighting the advent of omics methods which allows the study of new routes for studying probiotic bacteria. We have also summarized gene editing tools like TALEN, ZFNs and CRISPR-Cas that edits or cleave the specific target DNA. Furthermore, in this review an overview of proposed design of advanced customized probiotic is also hypothesized to improvise the probiotics.

Keywords

Probiotics. Metabolic engineering. Gut Microbiome. CRISPR-Cas. Systems biology

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1. Introduction

Probiotics is currently a significant promising field for food manufacturers, mainly in dairy food industry with remarkable growth potential. It involves intake of live probiotic cultures to improve intestinal microflora for the overall health of consumers. The success of a probiotic product in the market will depend on the efficiency of the probiotic cultures used in the production. Probiotic microorganisms generally referred to as lactic acid bacteria (LAB) which are involved in the fermentation of dairy products, beverages, foods, and produces lactic acid as the end product of fermentation. Now a day's researchers are more focused on investigation of gut microbiota, which is a residence for the largest number of bacteria. Small intestine contains a dynamic and different bacterial composition which is driven by the introduction and conversion of small carbohydrates, whereas the colonic microflora is driven by degradation of the indigestible carbohydrates (Zoetendal et al., 2012).

It has been always challenging to sample and describe the gastrointestinal tract (GIT). But with the help of several projects such as human microbiome project, it has become easier to access GIT environment (Segata et al., 2012). Sustainable progress of system biology approaches to understand and optimize the metabolic processes involves various high throughput omics technologies such as genomics, metagenomics, metabolomic, transcriptomics, proteomics etc. The introduction of these techniques has increased the researcher's interest towards gut microbiota and provided a new resource for the exposure of uncharacterized biosynthetic pathways which enables novel metabolic engineering approaches (Lepage et al., 2013; Wilson et al., 2014). Metabolic engineering (ME) is a multidisciplinary field for improvement of strains

and high rate production of desired fermentation products and metabolites. Moreover, new transformation techniques have been developed which involves the silencing of specific gene/multiple genes within the same region of the chromosome. The genetic modification of microorganisms allows the introduction of desired genes that may have a positive impact on the food industry (Gupta et al., 2015). Another approach is selection of optimal genetic manipulation strategies for metabolic networks to achieve an optimized production (Kim et al., 2008). In recent times, metabolic engineering approaches have been upgraded to the systems level of integration of metabolic engineering strategies with systems biology. By this approach production rate can be optimized for genomic scale in a multiplexed way with less endeavor and time (Lee et al., 2011).

2. The human gut microbiota

2.1 Composition and metabolic activities of gut microbiota

Human gut microflora is complex, highly variable and host specific system. It consists of plentiful and different microbial communities. There is a beneficial symbiotic relation between host and its microflora. The host fulfills space and nutritional requirements of microflora and the latter contributes many beneficial effects to host health (Serban, 2011). The gut microflora plays a very important role in modulation of gut and immune system. The human gut flora has genes, approximately 100 times as many as in the human genome. According to Metagenomic studies, the gut microflora consists of 1000-1500 species. The predominant phyla are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (Tap et al., 2009). The large intestine of a healthy host consists of two major dominant phyla which are Bacteroidetes (gram negative

bacteria) and Firmicutes (gram positive bacteria). Many factors that affect the variation in quantity and quality of microflora along GIT are transit time, pH, mucus, digestive enzymes, bile acids, adhesion capacity, metabolic activity and environmental factors (McConnell et al., 2008). The beneficial role of gut flora in human health are protection against cancer, control over the differentiation and proliferation of epithelial cells of intestine, epithelial barrier development, complex carbohydrate fermentation, defense against pathogens, degradation of dietary carcinogens etc. (Serban, 2014).

At the time of birth, infant GIT is sterile. The initial colonization of the gut is determined by maternal flora and immediate environment around the child (Penders et al., 2006). Infant born via vaginal delivery got an exposure of microflora of the mother's vagina and intestine as passes through the birth canal of the mother. On the other hand, child born by cesarean delivery acquires early colonization of *Clostridia, Klebsiella* etc. and later or less colonization by Bacteroids, Bifidobacteria and *E. coli* (Gronlund et al., 1999). These children have a higher risk of asthma, atopy and allergic rhinitis due to lack of exposure to mothers microflora (Pistiner et al., 2008). The feeding type is also a major factor that influences the gut colonization. In breast fed infants Bifidobactera is dominant while in formula fed infants Bacteroides species are predominant (Harmsen et al., 2000). The use of antibiotics may also lead to alteration of gut microflora in early life (Celedon et al., 2004).

The major activity of human gut microflora is carbohydrate fermentation, which is a source of carbon and energy for the colonic bacteria. The short chain fatty acids (SCFA) producer species play a very important role in the degradation of complex carbohydrates. These species produces

SCFA and gases in collaboration with Bifidobacteria which meets the energy requirements of specialized bacteria, such as methanogens, sulfate reducing bacteria, etc. the regular intake of dietary fibers (prebiotics, plant based foods, etc.) increases the carbohydrate fermentation capacity which may improve the gut microflora (Tang et al., 2013).

2.2 Probiotics and prebiotics for gut health

Various studies have shown the significant role of gut microflora on human health. The function of microflora includes barriers, trophic, immunological and metabolic functions resulting in improving the overall health of the host (Ivanov et al., 2012). So, an understanding of gut microflora and its activities is very important for the advancement in future health care research. The introduction of probiotics and prebiotics in diet may improve the metabolic activities of the gut. Probiotics are live microbial food supplements which, when administrated in sufficient amount confers various health benefits to the host (Goyal et al., 2013; Yadav et al., 2015). Prebiotic are non digestible carbohydrates, which pass through the small intestine in unmeatbolized form. On reaching the colon they undergo fermentation by colonic microflora and stimulate the growth of colonic bacteria. Examples of prebiotics are lactulose, inulin, fructooligosaccharides, wheat bran etc. Where synbiotics are the combination of probiotics and prebiotics which increases the efficiency of a probiotic.

As probiotic strains are able to colonize the gut and eradicate the harmful pathogens by competing for nutrient availability. So, they exhibit beneficial effects in the prevention or treatment of many diseases. Various studies have reported the role of probiotics in prevention of diseases such as colon cancer, cardiovascular diseases, inflammatory bowel disease (IBD), type

2 diabetes, pouchitis etc. Probiotic *Lactobacillus reuteri* strain is able to reduce the serum cholesterol level by reducing the absorption of fat from the intestine (Jones et al., 2012). Probiotics could be an important prophylactic strategy for various immune related diseases such as inflammatory bowel diseases (IBD), diabetes, and metabolic syndrome. Numerous studies reported role of *Lactobacillus* and Bifidobacteria in the prevention and treatment of intestinal inflammation. Probiotic *Lactobacillus fermentum* Lf1 could increase the activities of antioxidant enzyme system and reduces colitis.

3. Screening assay for selection of a probiotic strain

In vitro screening of probiotic bacteria is a low cost approach to assess the probiotic properties of many strains at the same time (Fig 1). Testing a strain for stress conditions similar to host body is utmost important for selection of that strain (Upadrasta et al., 2011).

It should be able to tolerate harsh GIT conditions such as low pH of the stomach, digestive enzymes, etc. For this purpose simple and most conventional test such as acid tolerance test can be performed**.** Similarly bile secreted by small intestine also affects the survival of probiotic bacteria by denaturation/damaging DNA, inducing misfolding of proteins and disruption of cell membrane (Lavermicocca et al., 2008). Therefore, bile salt (0.15-0.5% concentration) tolerance test is recommended for screening of a probiotic strain (Gorbach et al., 1992). Another important property of the selection is the safety aspect. The safety test, resistance to antibiotics includes determining the minimum inhibitory concentrations (MIC) by disk diffusion methods or microdilution broth test or by commercial kits (Argyri et al., 2013). Another safety test is hemolytic assay based on the zone of hydrolysis around tested strains on blood agar plates

(Pisano et al., 2014). Testing for production of enzymes (proteases, gelatinases and glycosidases), toxins (cytolysins) and biogenic amines is also an important safety aspect (Tan et al., 2013). Bacterial adhesion to mucus and intestinal epithelial cells is studied by cell surface hydrophobicity test using hydrocarbons such as xylene, hexadecane, toluene, etc. (Jena et al., 2013). Furthermore, various high throughput methods have also been reported which uses a commercially available immobilized mucin. Mucins are glycoproteins that strengthen the mucosal surface of intestine and act as a barrier against harsh environmental conditions (Laparra et al., 2009). Bacterial cell surface enzyme glyceraldehydes 3- phosphate dehydrogenase bind to mucin and evaluating the enzyme activity imparts a simple screening test for probiotic selection (Kinoshita et al., 2013). Assessment of autoaggregation activity which measures the adhesion capacity of a probiotic strain is also very important and desirable screening aspect (Botta et al., 2014). It is measured by determining the absorbance of bacterial cell suspensions left after a certain interval of time (Collado et al., 2008). Another important probiotic attribute is production of antimicrobial substances such as organic acids, bacteriocins etc. Numerous studies have reported that probiotics may possibly play an important role in maintaining gut microbiome homeostasis, prevention of gastrointestinal disorders or competitive exclusion of pathogenic microorganisms (Hickson, 2011). Furthermore, antagonistic effects of probiotic strains assessed by automated turbidometric assay for monitoring the growth of indicator bacteria is also a method of choice (Lahteinen et al., 2010). Moreover, bioluminescent indicator strains could also be used for the investigation of inhibitory compounds production (Lahtinen et al., 2007). Cross streak and radial streak assays are also very popular and efficient in assessing the antimicrobial activity of a probiotic strain (Coman et al., 2014). Probiotic microorganisms may competitively

exclude the pathogenic microorganisms. This property could be evaluated by co aggregation assay, which is based on measurement of absorbance (Collado et al., 2007). There are also many additional assay such as β-galactosidase assay. Screening of β-galactosidase enzyme is assessed by measuring the hydrolysis of o-nitrophenol- β-galactopyranoside. The production of vitamins (vitamin B, B12, K and folic acid) by probiotic strains meets the nutritional needs of human (Eck et al., 2013). The antioxidative potential of probiotic culture is assessed via linolenic acid test (Kullisaar et al., 2002).

3.1 Probiotic survival- importance and monitoring

For the selection of a probiotic strain it is very important that it should be viable during transit through GIT (Dave et al., 1998). Marteau et al., reported an approximate quantity of cells of *Lactobacillus acidophilus* (1.3-1.5% of oral inoculum) capable of surviving the GIT transit. It should also be able to tolerate acidic conditions of the stomach, gastric enzymes, toxic metabolites and intestinal bile salts (Kailasapathy et al., 2000). Survival and viability during processing, storage and shelf life of a food product is also very important. A probiotic product must contain 10^7 cfu/g viable bacteria (Ouwehand et al., 1998). This viability should not decline during food processing and transit through GIT (Martin 1996). Many factors such as pH, H_2O_2, storage temperature, dissolved O_2 etc. affect the viability of probiotic strain in a food product. Along with the viability a probiotic culture should also be able to colonize the GIT for carrying out beneficial effect. Numerous media are available for monitoring the survival of *Lactobacillus acidophilus* and Bifidobacteria probiotic cultures. Rogosa agar, modified Lactobacillus selective agar, bile medium, deMan Rogosa Sharpe medium etc. are the commonly available media for

monitoring *Lactobacillus acidophilus* survival (Shah, 2000). The studies also proved that some vitamins, minerals (manganese, magnesium) and simple sugars (glucose, fructose) can stimulate the growth and viability of Bifidobacteria and *Lactobacillus acidophilus* probiotic cultures (Kailasapathy et al., 1997). The use of prebiotics along with probiotic in a product also improves the effect of probiotic in that product.

4. Use of omics methods in probiotic research

Omics methods play an important role in human health and diseases. These techniques expand our knowledge in understanding the molecular mechanism of interaction between probiotic and the gut environment (Dimitrov, 2011). A few omics methods and their molecular structures are given in Table 1. The applications of probiotics in health area have gained a much attention and resulted in a need of genome analysis of probiotic strains.

Metagenomic studies are culture independent approaches which allow the study of multiple genomes in a given environment. Gut metagenomic sequencing enables us to identify a potential probiotic candidate for effective mechanism in the gut. The comparative metagenome analysis allows us to determine the diverse predominant phyla in the gut, determining antibiotic resistance of gut microflora and understanding the metabolism and physiology of probiotic bacteria (Manichanh et al., 2006; Diaz-Torres et al., 2006; Lee et al., 2010). LAB and *Lactobacilli* genome analysis have shown that each organism has a different characteristic feature. *L. plantarum* contains a gene which encodes a protein which is responsible for translation of a two component system that verifies their ability to interact with gut (Kleerebezem et al., 2010). The molecular studies of single gene/protein highlight the probiotic properties of a microorganism.

The introduction of omics methods allowed us to study a probiotic microorganism at the whole genome level (Gueimonde et al., 2012). Recently it has been reported that metagenomic approaches are quite relevant towards revealing the detailed physiological and genomic features of the complex gut ecosystem (Qin et al., 2010). These are tools for the complete understanding of in vivo probiotic behavior in contrast with simulated conditions and since these are cost effective they can be used in routine practice. Numerous metagenome analyses were performed for elucidating and discovering improved genes and their functions in specific digestive systems (Gill et al., 2006). The study of microbial diversity by metagenomic analysis provides us the knowledge of activity and composition of unknown ecosystem. Few contributions of metagenomic approaches to probiotic field are: development of novel methods for probiotic research, discovering new microorganisms, better understanding of mechanism of action of probiotic microorganisms with host, assessment of probiotic effects on gut micrbiome, identification of specific functions related to diseases etc. (Gueimonde et al., 2012). It is difficult to define the normal human microflora but with the advancement of metagenomic approaches the current understanding of activity of gut microflora can be further expanded. Thus, the combination of genomics, host response and microbiome studies allows us to select novel and effective probiotic strains for various health benefits to the consumers (Qin et al., 2010).

5. Genetically modified probiotic microorganisms

Genus *Lactobacillus* consists of a diverse group of microorganisms; among them are mostly the members of human gut microflora. Probiotic *L. acidophilus, L. gasseri, L. johnsonii, L. casei, L. plantarum, L. reuteri, L. salivarius, L. brevis* are the most important and common GIT bacteria.

The applications of LAB as mucosal delivery vehicle are in the current trend. It is because LAB can be engineered for the expression of a variety of heterologous and homologous proteins of virus, bacteria, and of mammalian origin (Suebwongsa et al., 2013; Liu et al., 2014; Qiu et al., 2013). In a recent study fourth plasmid (pRCEID7.6) of probiotic *L. casei* TISTR 1341 was sequenced. This plasmid replicon was further used for the generation of *E.coli/Lactobacillus* shuttle vector. A nucleocapsid protein (NP) encoding gene, obtained from influenza A virus was cloned into this new vector. Protein expression from this cloning would allow its significant use as mucosal vaccine (Suebwongsa et al., 2016). Genetically engineered probiotic microorganisms could be used as therapeutic, prophylactic and diagnostic agents. The use of synthetic probiotics as living therapeutic agents allows the controlled release of therapeutic at the correct infection location. These therapeutic probiotics secrete various antimicrobial agents as they detect the targeted pathogen. Thus, engineered probiotic could sense their environment and will expand their ability as therapeutic and diagnostic agents (Braff et al., 2016).

5.1 Metabolic engineering approaches for probiotic *Lactobacillus* and *Bifidobacteria*

Probiotics is an emerging field in food industry and it had attracted a lot of attention and shown a remarkable growth in this field. With the help of metabolic engineering approaches it has become feasible to understand probiotic mechanism of action and increasing possibility of designing desired probiotic strains. Metabolic engineering is a multidisciplinary field for the production of desired fermentation products and metabolites at a high rate and yield. Various applications of metabolic engineering in probiotics has been shown in fig 2. A few examples of using metabolic engineering approach in industrial probiotic strains are: transcriptomic studies of

Lactobacilli strains which reveal increased production of bioactive metabolites and improvement in yield of EPS biosynthesis in *Lactobacillus casei* LC2W through over expression of NADH oxidase. Higher activity of NADH oxidase enzyme leads to decreased NADH availability resulting in increased EPS yield (Li et al., 2015). The presence of residual galactose and lactose during the manufacturing of fermented food products may lead to numerous industrial as well as health concerns. Comparative genomic studies of *Lactobacillus casei* strain established that the occurrence of tagatose-6-phosphate pathway in this organism is a foremost contributor of galactose/lactose catabolism. Thus, it was found that due to presence of tagatose-6-phosphate pathway, *L. casei* could reduce the accumulation of galactose in fermented food products (Wu et al., 2017). Probiotic *Lactococcus lactis* produces tagatose which is a low-calorie sweetener and preferred sugar replacer as its characteristics are identical to sucrose (Lee et al., 2008). Tagatose production from lactose during growth of *L. lactis* is the only source of tagatose. Therefore, novel cost effective ME approaches for the production of tagatose was attained by using *Lactococcus lactis* strain. Amongst these approaches, reengineering of lactose plasmid which permits the uptake of lactose only by its PTS^{Lac} system and allows the metabolism of lactose-6-phosphate. In PTS^{Lac} system, cell takes up lactose as lactose-6-phosphate and metabolizes it into glucose and galactose-6-phosphate which further metabolized by the tagatose-6-phosphate pathway.Tagatose-6-phosphate accumulation within cell would force *L. lactis* to dephosphorylate it and resulting in *L. lactis* strain that could grow on lactose. An additional approach is lacC gene (gene for the enzyme tagatose-6-phosphate kinase) inactivation by using site specific insertion technique to redirect the cell for the secretion of tagatose during its growth on lactose medium. The strains bearing tagatose-6-phosphate pathway are generally used as

starter cultures in food and dairy industries due to their ability to remove galactose during manufacturing of food products (Yadav et al., 2017).

ME of *L. lactis* has the potential to create cost effective production procedures as the current production process is a multistep enzymatic process which is very expensive. Some probiotic *L. lactis* strains produce nisin (bacteriocin), which is a safe and natural food preservative that increases the shelf life of food product by inhibiting the growth of spoilage causing microorganisms (Li et al., 2003). A novel finding which involves transcription induction of nisA gene in *L. lactis* LM0230 strain which is grown in M17G on exogenous galactose addition. This will help in expedition to maximize production of nisin by bacteria (Wang et al., 1999). Probiotic *Lactobacillus casei* LC2W is an EPS producing strain that contain EPS synthesizing gene, which makes it able to synthesize exopolysaccharide from medium containing skim milk (Ai et al., 2008). EPS biosynthesis regulation and improved production was investigated by using cofactor engineering.

Probiotics synthesize nutrients in the intestine and makes essential compounds available for microflora (Preidis et al., 2011). Genome sequence analysis of two strains (*L. reuteri* 6475 and 55730) showed that they carried out a synthesis of essential vitamins in human. The in silico studies revealed that both strains contain a complete metabolic pathway for vitamin B12 and folate biosynthesis. Further, mass spectroscopic studies had confirmed that both strains posses various folate derivatives for the host (Saulnier et al., 2011). It is considered that *L. reuteri* 6475 strain is able to synthesize amino acids (tyrosine, cysteine, histidine and arginine) along with other essential amino acids (Imura et al., 1998). These amino acids are important for infants and growing children. ‘Probiogenomics’ is a recently coined term for defining the whole genome

sequencing and studying health promoting ability, mechanism and diversity of probiotic strains (Ventura et al., 2009). The integration of transcriptome analysis enables us to investigate gene expression profiles onto metabolic models which are established by using genome sequencing data which could result into enhanced metabolic pathway predictions (Molenaar et al., 2005). A recent study of genome wide comparison was done on two *L. reuteri* strains (one commercial strain *L. reuteri* ATCC 55730 and other human breast milk derived). Metabolic models were created for both the strains to explain survival in host (OSullivan et al., 2012).

Bifidobacteria are beneficial large intestine dwellers which influence the gut flora in the overall health of the host. They have various beneficial effects on intestine by preventing the colonization of pathogenic bacteria. Among Bifidobacteria, *B. longum* is a very important probiotic bacteria and have more positive effects on the large intestine as compared to other species. *B. longum* DJO10A strain secretes effectual iron chelators which have the ability to withdraw iron from competitor bacteria (Lee et al., 2011). The complete genome sequence of this strain was deciphered and revealed that these bacteria shows change when they are taken from the intestine and grown in fermentation conditions. These changes do not offer any advantage in pure culture, but shows a tremendous effect in the intestine. It includes oligosaccharide utilization, resistance to arsenic and the loss of a genomic region needed to produce a broad spectrum lantibiotic (a type of anti microbial protein). A mutagenesis approach is proposed to try and understand the siderophore based iron uptake system in *B. longum*. A comparative genomic analysis of our intestinal strain of *B. longum* with a strain from the culture collection experimentally confirmed the identification of an antimicrobial peptide, belonging to a class of antimicrobial peptides called lantibiotics that has potentially the broadest antimicrobial

spectrum of any antimicrobial peptide described to date. This antimicrobial spectrum, extended to gram negative bacteria, including *E. Coli* and *Salmonella* perhaps the two greatest food safety problems in the food industry. It is expected that further research will further characterize this novel peptide and uncover its novel applications for the food industry.

5.2 Gene editing: An approach to develop customized probiotics

The genome engineering techniques have promoted the study of genome to a next level to offer the opportunity for its application in various fields like medical, agriculture, food and feed, etc. The gene editing techniques presents a potential technology for the improvement food products such as probiotics with better probiotic properties, providing resistance from biotic and abiotic stress. The major gene editing tools like Clustered regularly interspaced short palindromic repeats (CRISPR-Cas), transcription-activator like effector nucleases (TALEN) and Zinc finger nucleases (ZFNs have made it possible. These gene editing tools introduce double strand break (DSB) in the target gene, which are repaired by the error-prone non-homologous end joining (NHEJ) pathway or homology-directed repair (HDR) (Symington and Gautier 2011). Zinc finger nucleases(ZFNs) and Transcription activator-like effector nucleases (TALEN) are artificial restriction enzymes that edit or cleave the specific target DNA by using zinc finger DNA-binding domain or utilizing TAL effector DNA binding domains, respectively. The progress in gene editing tools and development of various methods for easy synthesis and assembly of TALENs, allows the efficient editing at multiple sites.

5.3 Gene editing and improvement of probiotic properties

Gene editing is quite extraordinary technique because of its capability to alter DNA by utilizing engineered nucleases called as molecular scissors. It finds application in diverse areas since the editing is done according to process fitment. The most efficient and simple technique of gene editing has been described as CRISPR-Cas (Kanchiswamy et al., 2016). There are approximately eleven CRISPR-Cas systems are reported that can be distinguished into three types Types I, II, and III which are further divided into 11 subtypes. Each type has its own specific Cas protein component which is named according to model organism.

Cas9 is a DNA endonuclease guided by RNA to target foreign DNA for inhibition. CRISPRs (clustered regularly interspaced short palindromic repeats) comprises an array of a 30-40 bp short, direct repeat sequence which is separated by spacer sequences that matches the foreign sequence. The mature CRISPR (cr)RNAs is formed by transcription and processing of CRISPR. The guide RNAs are derived from (CRISPRs). A ribonucleoprotein (crRNP) is formed from CRISPR (cr) RNAs and Cas proteins that induce the break in DNA/RNA of the invader (Hale et al., 2012). The CRISPR is marked by the specificity which is owned by guide RNA (gRNA), that permit specific binding to the desired DNA site. The guide RNA may self designed to target the specific DNA fragment. The gene of interest can be inserted or deleted from the system with the help of CRISPR/Cas9 by introducing double strand breaks into a target site (Auer et al., 2014). Suitable expression construct is required for successful accomplishment of CRISPR-Cas sgRNA sequence(s), the codon-optimized variant of Cas9, strong promoters suitable to derive transcription of sgRNA and Cas9.

The CRISPR-based tools are gaining widespread importance and rapidly being adopted by molecular biologist because of their high compatibility with bacterial system and archaea (Sontheimer et al., 2015).

The mechanism of editing often involves targeting using self designed guide sequence which is complementary to unique sequence at a site of interest, prompting break at a site, which is repaired at high rates using homologous recombination, thereby deletion or insertion of desired fragment. In terms of food applications, CRIPSR-Cas was applied in *L. reuteri*, one dairy starter culture, *S. thermophilus* (Selle et al., 2015; Oh et al., 2014). To design the customized probiotic the single-stranded DNA recombineering (SSDR) can be integrated with CRISPR-Cas to allow fine base changes in the chromosome. The approach can be followed using single step where recombineering oligonu-cleotide and the CRISPR-plasmid can be co-transformed in a probiotic probiotic bacteria expressing RecT and Cas9. The CRISPR-plasmid encoding CRISPR-array along with Cas9 and the tracrRNA cleave desired sequence until SSDR make the change (Oh et al., 2014; Van et al., 2014). Thus, such techniques provide the opportunity to enhance the scope of probiotics to maintain the health issues by making changes at a level of single nucleotide. The starter culture can also be implemented with such technology for the use at industrial level. A model of customized probiotic showing better immune response than usual probiotic has been shown in figure 3.

6. System Biology approaches to improve probiotics

Probiotics are living microorganisms which confer a number of health benefits to the host when enough amounts of microbes administered in the host body (Fijan, 2014). A number of factors

derived from probiotics which are capable of exerting various health benefits through a well described mechanism are also known as probiotic bioactives (Lew et al., 2013). Bioactives provides an alternative to the probiotics as it has a shelf life of longer period and safer than these probiotic bacteria or cells. Extracellular probiotic metabolites as well as cell wall materials have been reported to inhibit pathogenic growth and to treat immune deficiency-disorders, respectively, as described in previous sections (Lew et al., 2013). Recent advancement in life science technologies and systems biology approaches provides a prevailing tool to investigate interactions between probiotic-host biological systems and applications of probiotics. A combinatory approach of several computational methods can be applied to improve bioactives properties of probiotics to improve human health. These in silico approaches may be constructive to promote the thriving advancement of contemporary probiotics by straightening out the mechanisms which is operational between of probiotic-metabolite and host during interaction. Computations biology is in silico studies of proteins and genes, and their interactions within a cell system, or tissue system, or whole organism system. It is possible to understand complex metabolic pathways of biological system of probiotics with the help of computational techniques. In silico metabolic engineering in microorganisms has been employed in different areas such as industrial microbiology, medical microbiology and agricultural microbiology (Singh et al., 2011; Singh et al., 2015; Singh et al., 2016; Kumar et al., 2016). There are several in silico tools are available which have been widely applied to understand the metabolic pathways of cellular metabolic networks and to advance cellular properties through metabolic engineering. Metabolic pathway analysis (MPA), flux balance analysis (FBA) and metabolic flux analysis (MFA) are in the middle of the most accepted metabolic engineering tools for stoichiometric analysis of

metabolic networks. Flux is the rate at which material flows, flux is associated with the edges and carry a certain value. Understanding flux and managing it helps to regulate the metabolic network, thus to help to change biological process dynamics. The production of bioactive derived from probiotics can also increase through these tools FBA, MPA and MFA or properties of whole probiotics can be altered using same metabolic engineering tools. The renovation in quantitative structure activity relationships QSAR and 3D-QSAR3D atom-based models have also created a path to predict the molecular basis of interactions among activities of probiotic metabolite and their targets in the host organism. For instance, QSAR atom based models may be applicable to learn the connections among activity of fermentation products of probiotics such as antihypertensive activity and angiotensin converting enzyme- inhibitory peptides (Lin et al., 2008). A 3D-QSAR model for the probiotic-bioactive factors may be produced on the foundation of pharmacophore study together with an equation to explain the on the whole statistical importance of that model (Tan et al., 2014). A quick evolution in gene sequencing techniques has increased the number of probiotic strains with the whole genome sequencing, which further enhances the comprehension of the metabolic pathways with the help of genomic computational tools. In the past two years, there were a number of probiotic strains including several species of *Lactobacillus, Enterococcus, Bacillus Bifidobacterium* and *Escherichia* which genome have been fully sequenced which explained in details in Table 2**.** With the addition proteomics, transcriptomics and metabolomics data these genomes provides a clear picture for phenotype prediction which act as a bridge between biochemical information derived from genome and metabolic phenotypes, helps to generate quality metabolic genome-scale model (GEM) (Saha et al., 2014). There are certain genome-scale network models available for many microorganisms.

These models are very helpful tool to predict the production of various probiotics-bioactives and undesired secondary products, further with the help of these in silico tools it would be possible to enhance the production of the desired product and minimize the production of unwanted metabolites. GEMs may make available as a platform for invention of novel probiotics-metabolite in various other bacterial species and strains, including exopolysaccharides and vitamins, which wield a potential effect on human health (Saulnier et al., 2011).

We comprehend computational biology perspectives can be well applied to study probiotic interactions with human gut flora. These in silico studies are founded on definite tools which help to analyze huge amounts of genomic data, gene interactions, genome-scale models. Among various in silico methods OptKnock is a technique which searches for sets of gene knockouts that lead to the production of desired products (Burgard et al., 2003) and can be used for the same purpose in probiotics to deactivate the gene computationally and their effect studies through genome-scale model (GEM). On the other hand OptStrain that not only allows gene knockouts, but also incorporate coding genes for several novel enzymes from diverse microbial species to a specified microbial genome (Pharkya et al., 2004). In recent years, OptReg has been developed, which is a *in silico* tool helps to regulate metabolic enzymatic pathways either by positive or negative manipulations which is an additional benefit to OptKnock for the production of a desired metabolite (Pharkya et al., 2006). A snapshot of various in silico methods to study, probiotics has been described in fig 4.

Conclusion

The major challenge in the probiotic field is to improve the probiotic properties of the microorganisms by implementing various strain improvement approaches. The monitoring and analysis of food products, there is a rising need of rapid, accurate and definite techniques. Metabolic engineering approaches enhance the probiotic properties as well as production yield of these microorganisms, significantly. Consequently, the gene profiling and metabolic engineering approaches such as gene insertion, mutation, deletion or knock-out of the specific gene of these floras can be beneficial in modifying the organism for enhanced probiotic properties as well as production yield. We also conclude that these approaches enable us to identify a potential probiotic candidate for an effective mechanism in the human gut. CRISPR-cas based gene editing tool provide an opportunity to enhance the scope of probiotics to maintain the health issues by making changes at a level of single nucleotide. The starter culture can also be implemented with such technology for the use at industrial level.

Acknowledgements

RY and MB are grateful to M.D. University, Rohtak, India for University Research Scholarship (URS). VK is thankful to UGC New Delhi, India for awarding Junior Research Fellowship [F.17-63/2008 (SA-I)].

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Table 1 Omics methodologies for probiotics

Omics methodology	Definitations	Techniques	Biomarkers	References
Genomics	To study the genome of organism	DNA sequencing	Genes, Promoters	OSullivan et al., 2009; Zhou et al., 2010; Spor et al., 2011; Khoroshkin et al., 2016
Metagenomics	Metagenome study or study of genetic material obtained from any sample	Sequencing, Pyrosequencing	Screening of 16S rDNA libraries	Kleerebezem et al., 2010; Bottacini et al., 2010; Patro et al., 2016; Rebolla et al., 2016
Metabolomics	Study of set of metabolites present in cell/tissue of an organism or	HPLC, GC, MS, FTIR, CE/MS arrays	Metabolites-Antioxidants, lipids, vitamins sugars	Kekkonen et al., 2008; Martin et al., 2010; Rebollar et al., 2016

	study of chemical fingerprints left behind by cellular processes			
Proteomics	Structural and functional study of proteins or study of differential expression of proteins	X-ray crystallography, chromatography, LC-MS/MS, 2-DE analysis	Oligopeptides, proteins, peptides	De Cagno et al., 2011; Aires et al., 2010; Maria De Angelis, et al., 2016; Ruiz et al., 2016
Transcriptomics	Study of transcriptomes (set of transcripts) and their functions	q-PCR, RT-PCR, RNA sequencing	Transcripts of cDNA, RNAi, siRNA, mi RNA	Shima et al., 2008; Marco et al., 2010; Rebollar et al., 2016

Table 2 A description probiotic microorganisms with full genome sequencing of last Three years for application in systems biology

Sr. No.	**Probiotic Microorganism**	**Source**	**Genomic Information**	**Technique used for**	**Significance related to probiotic property**	**GenBank accession no.**	**Reference**
1.	*Enterococcus durans* KLDS6.0930	Fermented cream From China	A circular chromosome 2,867,090 and two circular plasmids.	PacBio RSII platform	Cell adhesion, Bile salt tolerance, and lipid metabolism molecular pathway	CP012366.1	Liu et al., 2016
2.	*Lactobacillus heilongjiangensis* DSM 28069	Traditional Chinese pickle	A circular chromosome 2,790,548 bp and No plasmids	PacBio SRMT and Illumina sequencing	Biosynthesis of riboflavin and folate	CP012559	Zheng et al., 2015

3.	*Lactobacillus helveticus* KLDS1.8701	Conventional sour milk from China	A circular chromosome of 2,096,031 bp and a plasmid	Illumina paired-end sequencing -16	Acid and bile tolerance, bacteriocin production and Exopolysaccharide production,	CP009907.1	Li et al 2015
4.	*Lactobacillus plantarum* LZ227	Raw cow milk	A 3,131,750-bp Circular chromosomeand 3 extra circular plasmids	Illumina paired-end sequencing and PacBioRSII sequencing	Biosynthesis of riboflavin and folate	CP015857-CP015862	Li et al 2016
5.	*Lactobacillus fermentum* F-6	Raw milk	A circular chromosome 2.06 Mb and No	454 sequencing and illumine paired-end	EPS production and citrate fermentation,	CP005958	Sun et al 2015

			plasmid	sequencing			
6.	*Bacillus coagulans* HM-08	Commercialized probiotic strain in China	A circular chromosome 3.62 Mb and no plasmid	Roche GS FLX system and illumine Miseq system	One *xyl* operon for xylose utilization	CP010525.1	Yao et al., 2016
7.	*Lactobacillus reuteri* ZLR003	Caecum mucosa of healthy weaned pigs	A circular chromosome 2,234,097 bp and No plasmid	Illumina Hiseq raw data and PacBio sequencing	-	CP014786	Zhang et al., 2016
8.	*Lactobacillus plantarum* LZ95	Newborn infant fecal	A 3,261,418-bp circular chromosome and two plasmids	PacBio RS II platform	Biosynthesis of riboflavin and bacteriocins	CP012122-CP012124	Li et al., 2016

9.	*Lactobacillus paraplantarum* L-ZS9	Fermented sausage	A circular chromosome 3,139,729 bp	Illumina Hiseq 2000 platform	Bacteriocins production and regulation.	CP013130	Liu et al., 2016
10.	*Bifidobacterium longum* CBT BG72	-	2.45-Mb chromosome and a plasmid	PacBio RS II	-	CP010453 and CP010454	Kwon et al., 2015
11.	*Lactobacillus helveticus* MB2-1	Sayram ropy fermented milk in China	circular chromosome of 2,084,058 bp with no plasmid	PacBio RS II platform	Gene cluster of 15.20 kb involved in EPS biosynthesis	CP011386	Li et al., 2015
12.	*Lactobacillus helveticus* CAU H18	Traditional fermented dairy product koumiss	Chromosome of 2,160,583 bp with no plasmid	PacBio sequencing Using single-molecule real-time (SMRT)	EPS biosynthesis and cell-surface aggregation-promoting factors	CPO12381	Yang et al., 2016

					(APFs) to enhance gastrointestinal tract (GIT) colonization.		
13.	*Lactobacillus plantarum* ZS2058	Sauerkraut	Chromosome of 319,7363-bp chromosome and three plasmids	Roche 454 pyrosequencing and Illumina paired-end sequencing technology	exopolysaccharides (EPS) producing gene and linoleic acid production	CP012343	Yang et al., 2015
14.	*Lactobacillus plantarum* P-8.	Conventional fermented cow milk in China	A 3,033,693-bp circular chromosome and six plasmids	Illumina paired-end sequencing and 454 sequencing	Bacteriocin production	CP005942.1	Zhang et al., 2015
15	*Escherichia*	Feces of a	Chromoso	Illumina	-	-	Reister

.	*coli* strain Nissle 1917 (EcN)	soldier who was the only one of his unit not suffering from dysentery	me with 5,441,200 bp and 2 plasmids	Paired-End libraries PacBio sequencing			et al., 2014
16	*Bifidobacterium animalis* subsp. *lactis* KLDS 2.0603	Feces of an adult human	Contains genomic chromosome of 19,469 bp	Roche 454 platform	EPS Biosynthesis, and transport of capsular polysaccharides	CP007522.1	Zhu et al., 2016

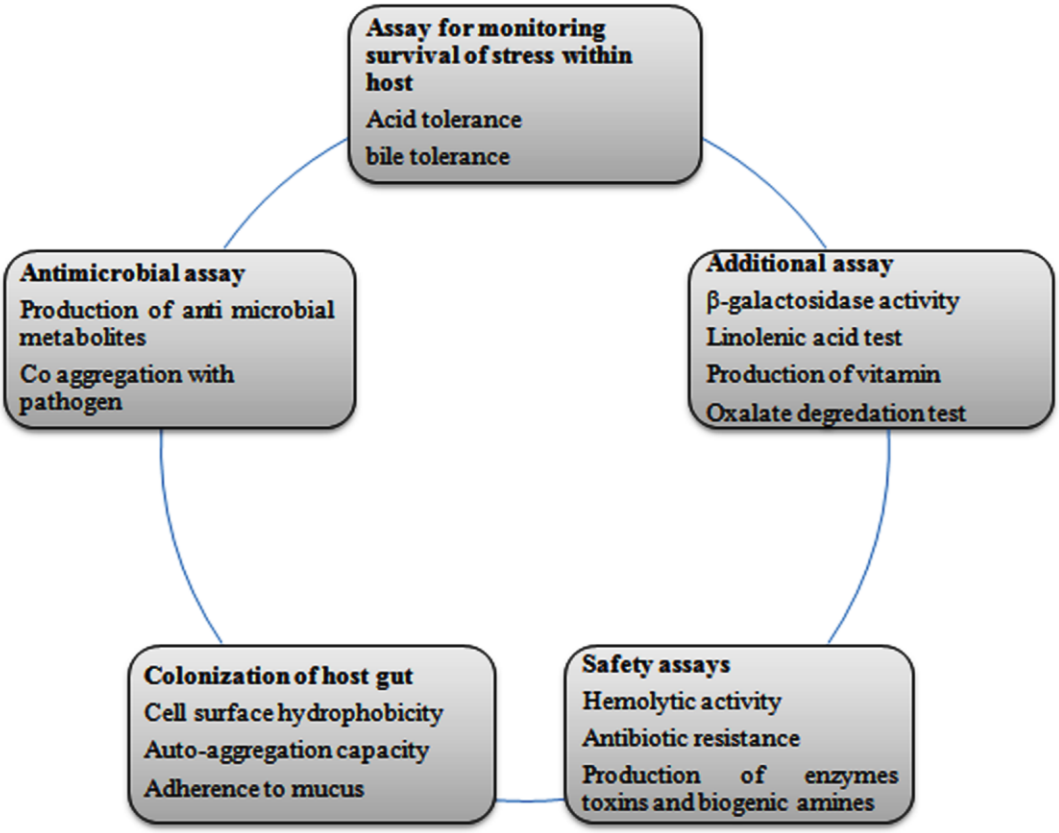

Fig.1 In vitro assays for screening of probiotic strain

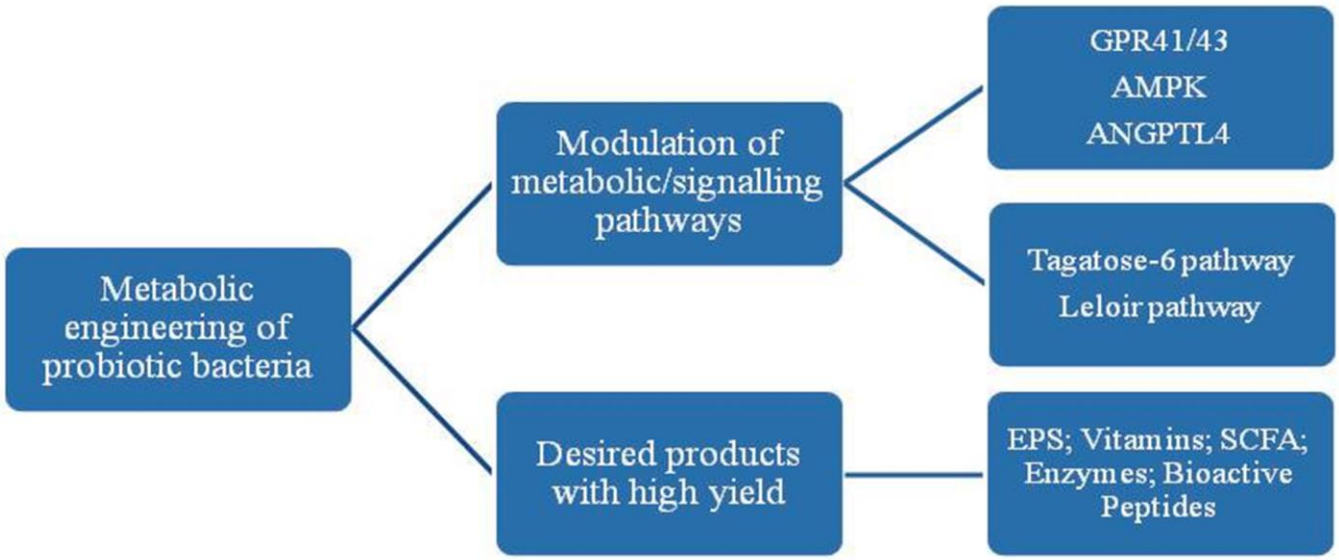

Fig.2 Applications of metabolic engineering in probiotics

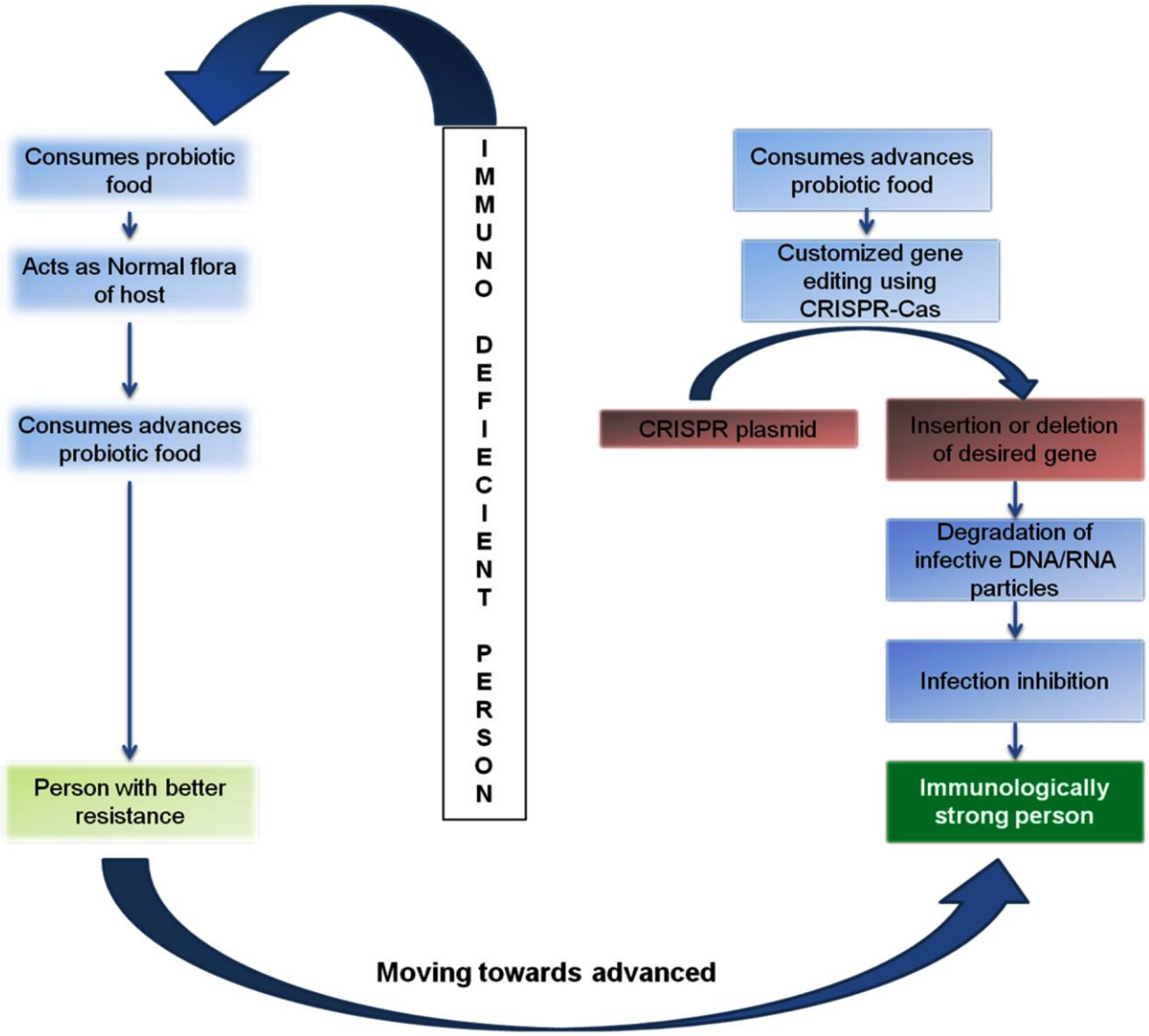

Fig. 3 Design of advanced customized probiotic hypothesized using CRISPR Cas technology

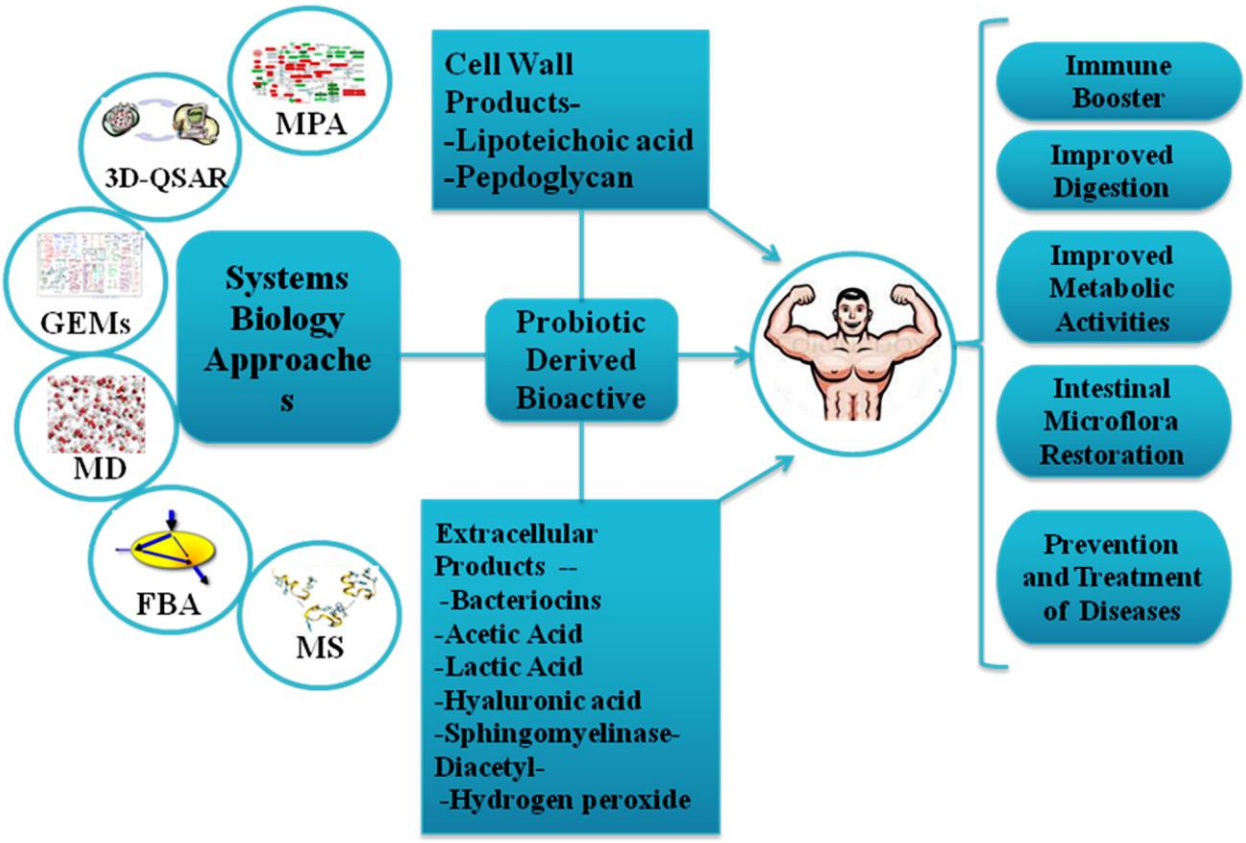

Fig. 4 A snapshot of *in silico* approaches to improve probiotic properties