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Biotechnological Applications of Probiotics: A Multifarious Weapon to Disease and Metabolic Abnormality

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Abstract
Consumption of live microorganisms "Probiotics" for health benefits and well-being is increasing worldwide. Their use as a therapeutic approach to confer health benefits has fascinated humans for centuries; however, its conceptuality gradually evolved with methodological advancement, thereby improving our understanding of probiotics-host interaction. However, the emerging concern regarding safety aspects of live microbial is enhancing the interest in non-viable or microbial cell extracts, as they could reduce the risks of microbial translocation and infection. Due to technical limitations in the production and formulation of traditionally used probiotics, the scientific community has been focusing on discovering new microbes to be used as probiotics. In many scientific studies, probiotics have been shown as potential tools to treat metabolic disorders such as obesity, type-2 diabetes, non-alcoholic fatty liver disease, digestive disorders (e.g., acute and antibiotic-associated diarrhea), and allergic disorders (e.g., eczema) in infants. However, the mechanistic insight of strain-specific probiotic action is still unknown. In the present review, we analyzed the scientific state-of-the-art regarding the mechanisms of probiotic action, its physiological and immuno-modulation on the host, and new direction regarding the development of next-generation probiotics. We discuss the use of recently discovered genetic tools and their applications for engineering the probiotic bacteria for various applications including food, biomedical applications, and other health benefits. Finally, the review addresses the future development of biological techniques in combination with clinical and preclinical studies to explain the molecular mechanism of action, and discover an ideal multifunctional probiotic bacterium.

Keywords Probiotics · Hormones · Immunity · Metabolites · Metabolomics · Microbiota

Introduction

In 1908, Ellie Metchnikoff introduced probiotics as useful microbes to favorably improve the human gut microbiota, replacing harmful microbes, thereby boosting human health [1]. The term "probiotic" was first introduced by Lilly and Stillwell in 1965, as secreted microbial by-products that strengthen the growth of its neighbors [2]. Previous studies have illustrated probiotics as favorable therapeutics to treat gut microbiota imbalance [3, 4]. Probiotics are "live microorganisms" that confer beneficial health effects to the host when administered in adequate amounts [5]. Probiotics are inclusive of a broad range of microbes and their application, and they differentiate live microbes used as sources of useful compounds from those that are used for health benefits only [5]. Currently, the probiotics market is growing exponentially estimated to reach USD 61.1 billion in 2021, and is projected to reach USD 91.1 billion by 2026 (http://www.marketsandmarkets.com/PressReleases/probiotics.asp).

Probiotics are described as free of pathogens and antibiotic resistance and their activity, viability, and growth efficacy should be properly established [6, 7]. The most common species used in probiotics research and development belong to *Lactobacillus* spp. and *Bifidobacterium* spp. Moreover, other species are available in the market including the *Bacillus* spp., *Enterococci*, *Escherichia coli*, *Weissella* spp., and *Saccharomyces* [8]. Probiotics have been commercialized as lyophilized pills, but also as supplements to various food sources such as cheese, yogurt, and nutritional bars to

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enhance human health. Most of the probiotic strains belonging to the *Lactobacilli* and *Bifidobacterium* have been given the Generally Regarded as Safe (GRAS) status in the USA, and qualified for the safety status given by EFSA (European Food Safety Authority). The multifactorial selection criteria of probiotics used in various food sources are often divided into three categories: resistant to acidic gastric conditions, capable of attaching to the gastrointestinal mucosa, and immune system modulator [9, 10]. Diverse approaches have been undertaken by Asian countries mainly Indonesia, Malaysia, Philippines, Singapore, Thailand, and Vietnam to regulate probiotics marketing [11]. Among these countries, only Indonesia, Malaysia, Philippines, and Thailand have enacted specific regulations related to the legal definition of probiotics. Malaysia, Philippines, and Thailand have permitted to use of probiotics foods or health supplements; however, all six Southeast Asian countries have permitted for application of new microorganism to be used following different approaches and requirements [11].

Probiotics have previously been classified into mono- or multi-strain microbial products [12]. Microbial strains in multi-species probiotics belong to *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Eubacterium faecium*, *Lactobacillus acidophilus*, *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, and *Streptococcus thermophilus* [12]. Multi-strain/multi-species probiotic strains due to their symbiotic nature show a cumulative positive effect on health in terms of treating antibiotic-associated diarrhea, improving growth performance and mortality in broilers, and protecting against *Salmonella typhimurium* infection [12]. Similarly, Chapman et al. [13] showed that probiotic mixtures comprised of different strains produced a superior effect than that of single-strain probiotics, against pathogenic growth. Among the multi-species, *Bifidobacterium lactis* W51, *L. acidophilus* W22, *Lactiplantibacillus plantarum* W21, and *Lactococcus lactis* W19 have been proven to strengthen the gut barrier function and are also currently commercialized worldwide [14]. These probiotic mixtures were proven to be beneficial to patients with food intolerances by increasing the IL-10 levels and decreasing the cytokine Th2 [15]. Classical probiotics comprising the *Lactobacillus* and *Bifidobacterium* groups were shown as promising biological tools for the treatment of a wide variety of inflammatory and metabolic diseases [9].

The results of many clinical trials involving humans and mice studies have shown the efficacy of probiotics against multiple diseases including their ability to suppress hypertension [16], reduce irritable bowel symptoms [17], prevent inflammatory bowel disease [18], and post-operative complications [19]. Similarly, probiotics showed higher efficacy against allergic disorders [20], potent antimicrobial [21], and anti-colorectal cancer properties [22]. At the molecular level, probiotics initiate the gene activation to regulate the host cells' immune response including the regulation of brain behaviour through bidirectionalneuronal signaling [23].

The human gut microbiome in the form of probiotics can act as potential therapeutics against various issues like metabolic disorders, obesity, bacterial infection, and immunity [24]. Ingested microbes in the form of probiotics act as a source of useful compounds such as vitamins, bacteriocins, fatty acids, oligosaccharides, and various other immune-modulatory compounds that control the host immune system [25]. The human gut contains different microbes such as bacteria, fungi, and viruses; however, the consumption of probiotic bacteria alters the gut microbial composition in a strategic way to combat the pathogenic microbes in the intestinal niche [26]. However, in-depth studies in humans are urgently required to assess the probiotics-induced changes in gut microflora and these changes are associated with clinical benefits in the host. The recent discovery of high-throughput sequencing techniques coupled with other experimental approaches such as improved culturing methodologies, cost-effective genome and metagenome sequencing, and more powerful tools to unravel the bacterial genomes allows focusing on probiotics' relevant biological questions, facilitating patient-oriented therapeutics [27]. The development of new tools integrated with the analysis of the microbial community composition, transcriptome shotgun sequencing, and proteomics approaches allows the identification of potent probiotic strains [28]. Novel sampling systems provide information about the immune system, metabolism, and microbiome alteration, driving this field forward [29]. Ultimately, an integrated approach will support the establishment of mechanistic insight into effector molecules and will pave the way for researchers to developnovel, emerging concepts like postbiotics [30].

Accumulating evidence addresses the increasing importance of the identification/discovery of novel probiotic bacteria with robust functionalities that hold great potential to commercialize worldwide as functional products and therapeutics. In the present review, we summarize the use of bacterial strains as probiotics and provide an overview of microbial selection, screening trials, metabolic engineering, and molecular biology approach in combination with synthetic biology to address research developments in the rational design and engineering of probiotic bacteria.

Mechanistic and Molecular Insight of Probiotic Function

Previous studies have shown diverse probiotic effects through multifarious mechanisms like pathogenic protection, enhancement of immunomodulation, alteration of gut microbiota, improvement ofthe barrier function of gut

epithelium, and short-chain fatty acids (SCFAs) production (Fig. 1a) [31, 32]. The efficacy of probiotics to treat various diseases has been summarized in Table 1. Probiotics activity requires either direct contact or proximity to host cells for induction of inflammatory responses. The role of probiotics at an immune marker levelcomprises the modulation of mitogen-activated protein kinase (MAPK) signaling pathway, interleukin (IL)-6 & IL-8, tumor necrosis factor (TNF)-α, phosphoinositide 3-kinase (Akt), Interferon (IFN)-γ, and other contact-dependent mechanisms [72]. The specific effect of probiotics includes *Lactobacillus* (effector: lipoteichoic acid) mediated secretion of TNF-α, through toll-like receptor 2 (TLR-2), *B. longum* (effector: cell surface pilli) stimulated IL-10 secretion, modulation of proinflammatory cytokine and helper-T cell response by *B. longum* 36,524 (effector: exopolysaccharide), and *L. rhamnosus* (effector: cell surface appendages) mediated modulation of TNF-α, IL-6/10/12 in the intestinal mucous. Additional examples include *L. rhamnosus* (effector: pilli) induced generation of reactive oxygen species (ROS) and inhibition of NF-κB activation, *Ligilactobacillus salivarius* Ls33 (effector: peptidoglycan) mediated protection from colitis in IL-10 dependent manner, *L. acidophilus* L-92 (effector: surface layer protein slpA) mediated immune modulation, and *B. lactis* mediated induced IgA secretion [73, 74].

Most mice studies have demonstrated that probiotics inhibit pathogenic colonization either by attachment to epithelium cells through competition for mucosal binding sites, by producing antimicrobial compounds, or by blocking the ability of pathogens to adhere (Fig. 1b). Collectively, most of these responses were shown in different probiotic strains in mice models. Also, the response is strain-dependent (Table 2). Many lactic acid bacteria (LAB) produce compound such as bacteriocins which show antimicrobial activity [83]. Bacteriocins have been shown to disrupt the quorum sensing (QS) signaling pathway. Production of bacteriocin Abp118 by *L. salivarius* UCC118 inhibits the infection of *L. monocytogenes* in mice [84]. *L. acidophilus* La-5 has been shown to inhibit the virulence factor expression of pathogenic *E. coli* O157:H7 in an in-vitro mice model [85]. *L. acidophilus* A4 antagonized the adhesion of *E. coli* to the intestinal epithelium by upregulating the secretion of IL-8, TNF-α, and mucin-2 [86]. Among other lactic acid bacteria, *L. acidophilus* GP1B improved the survival of mice subjects, following infection with *Clostridium difficile*. Additionally, *Limosilactobacillus reuteri* RC-14 repressed the virulence of *Staphylococcus aureus* through toxic shock syndrome toxin-1 [87]. Among other functionalities, probiotic bacteria have been shown to maintain gut barrier function through the up-regulation of tight junction proteins like claudin-1 and occluding [88]. The hydroxy cis-12-octadecenoic acid (HYA) produced by *L. plantarum* has been demonstrated to modulate the tight junction proteins by inducing the secretion of INF-γ and TNF-α, via mitogen-activated protein kinase (MAP-K) and extracellular signal-regulated kinase (ER-K) pathway [89]. Additionally, many probiotics can secrete metabolites, improving colonic epithelial resistance, enhancing the upregulation of mucous secretion (MUC1, MUC2, MUC3) in colonic epithelial cells, and modulating the microbiome population.

In mice, probiotic metabolite HYA ameliorated the pathogen-induced epithelial barrier disruption by preventing the degradation of E-cadherin and beta-catenin in a GPR 40-dependent pathway [90]. *L. rhamnosus* secreted proteins p40 and p75, which play a key role in maintaining intestinal epithelial stability by inhibiting cytokine-mediated apoptosis. In an in-vitro cell-based assay, *Lactobacillus* strains have been shown to secrete MUC-3 in HT29 cells and MUC-2 in Caco-2 cells; however, these effects require direct mucosal adherence. Various *Bifidobacterium* and *Lactobacillus* resist bile salts by producing the bile salt hydrolase (BSH), thus minimizing weight gain and also reducing the cholesterol, and triglyceride levels in mice [91]. Sarkar et al. [92] reported that probiotics can also regulate the host's antidepressant and anxiolytic effects via regulating the signaling to the central nervous system. In *L. rhamnosus* JB-1 administrated mice, corticosterone response was attenuated through the expression of mRNA for γ-GABA-A and GABA-B brain receptors [93]. Mice fed with *L. reuteri* (strain ATCC PTA 6475) restored the oxytocin level, and improved their social behaviour when fed with the high-fat diet, which resulted in gut dysbiosis [94].

Up to now, a few in-vivo proteomics studies involving large-scale characterization of microbiota-mediated changes have been displayed especially in gastrointestinal tract (GIT) models [95]. Proteomics studies involving probiotics have provided insight into molecular mechanisms and metabolic pathways, required to overcome the challenges of acidic pH in GIT (pH 2.0) and bile salts in the small intestine. Most proteomic studies highlighted the over-expression of proteins regulating the carbohydrate metabolism, over-expression of Clp family proteins (GroES, GroEL, DnaK, DnaJ), and proton translocating ATPase to maintain cytoplasmic homeostasis. Additionally, the expression level of proteins involved in transcription, translation, DNA repair, nucleotide, and amino acid biosynthesis were highly affected [96, 97]. Furthermore, proteomic evidence showed enhanced exopolysaccharides (EPS) production, supporting the hypothesis that EPS may confer protection to bacterium against the adverse environment in the GIT. On the other hand, in *L. rhamnosus* the under-expression of EPS biosynthesis genes resulted in a less protective EPS layer, which was found to assist in gut adhesion [98]. Moreover, proteomic evidence supports that *Lactobacillus* exposure to bile salts triggers the expression

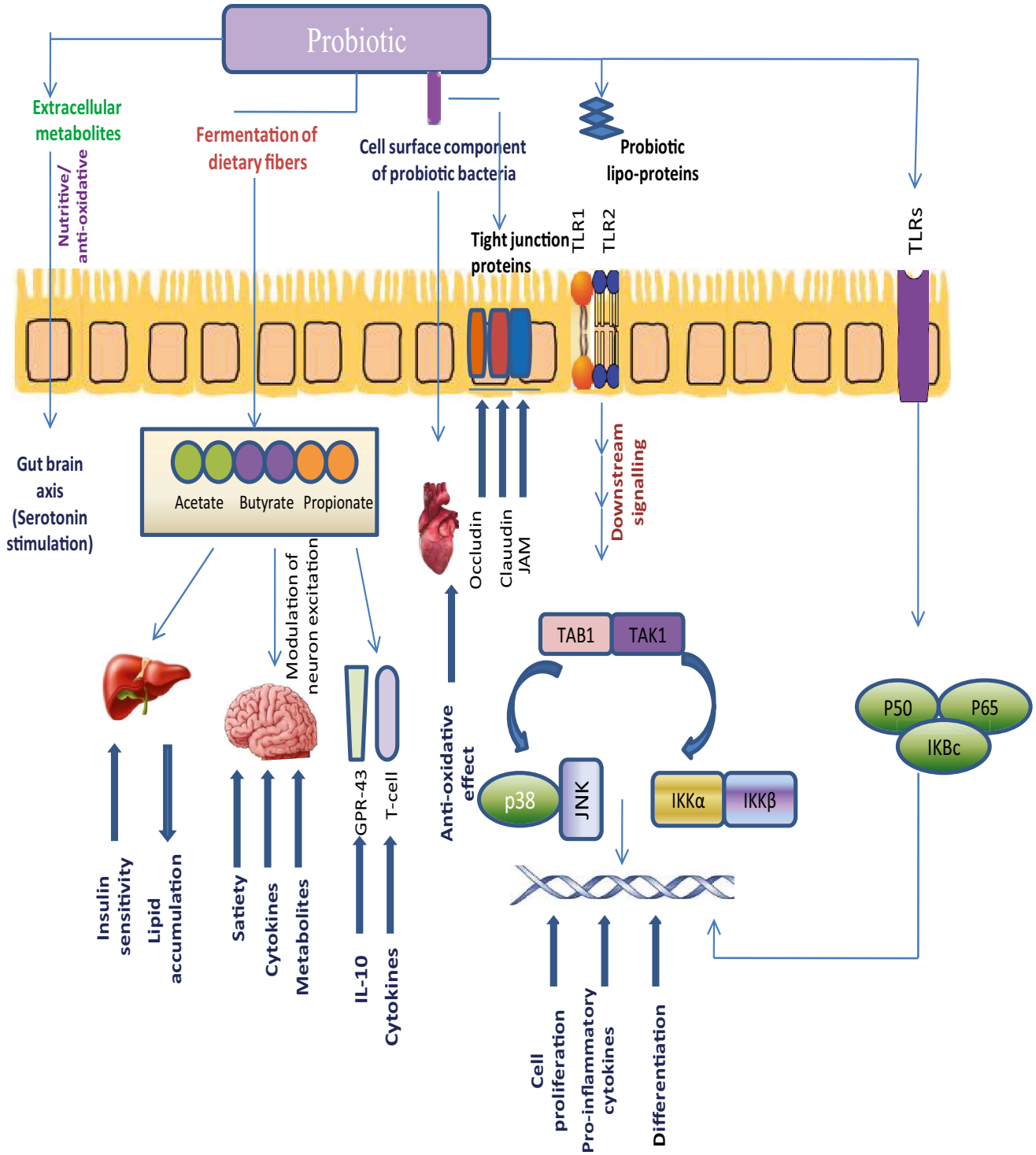

Fig. 1 **a** Mechanism of action of probiotic bacteria; (i) probiotic bacteria can be used to cure depression by enhancing beneficial bacteria population which further improve mood through gut-brain axis; (ii) probiotic-produced SCFAs (acetate, butyrate, propionate) confers beneficial effects in the gut, brain, and also enhace the relese of IL-10 and cytokines; (iii) probiotic bacteria also alters the tight junction protein that forms a seal between adjacent epithelial cells near apical surfaces; (iv) probiotic bacteria interact with epithelial cells through TLR1/TLR2 and induces an increase in downstream signalling that are involved in immune response, and other processes like cell-proliferation, pro-inflmmatory cytokines productions, and differentiations. **b** Transport of probiotic and their active metabolites/ factors from intestinal lumen to mucosa. Probiotic bacteria and their active metabolites confers several beneficial effects by (1) strengthening the epithelial barrier, (2) antimicrobial productions, (3) enhancement of adhesion protein to mucosa, (4) inhibiting the pathogen attachement, (5) activating the dendritic cells, and (6) competitive exlude the pathogens

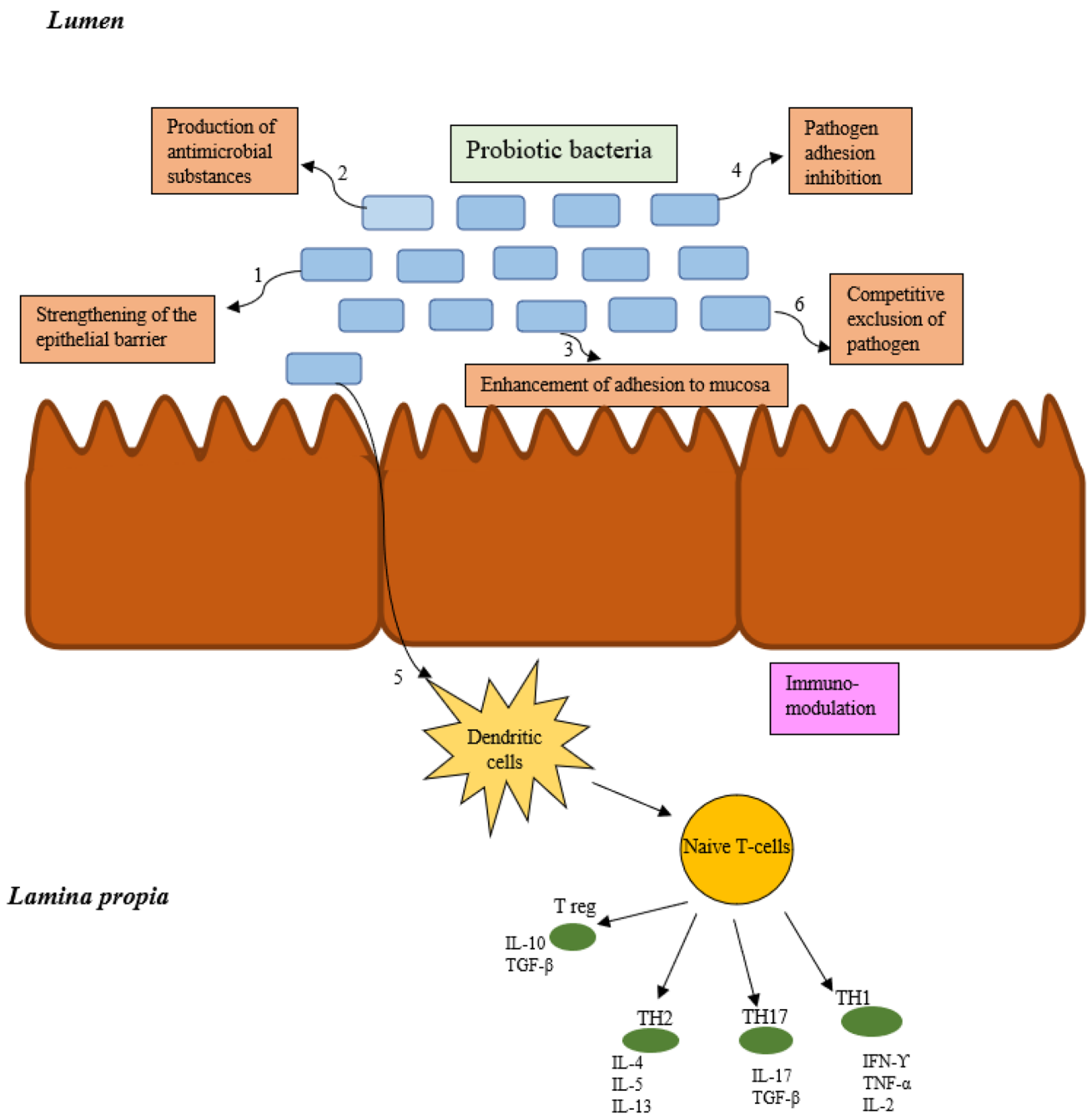

Fig. 1 (continued)

of Nag A/Nag B proteins, maintaining the redox balance and preventing oxidative damages [99, 100].

An in-vitro proteomic study identified the major proteins associated with adhesion processes as well as the probiotic-host effect was interestingly shown by cell appendages/or envelopes [101]. Furthermore, proteins such as enolase (ENO), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and elongation factor Tu (EF-Tu) promote probiotic adhesion to GIT. However, these proteins are also used by pathogens to bind to the mucous layer of GIT, illustrating the key role of probiotics in preventing enteropathogenic bacteria to attach to the intestinal mucosa [102]. Moonlighting proteins such as GroEL and EF-Tu, present in the probiotic strain *Lactobacillus johnsonii* NCC533, enhance IL-8 secretion in macrophages, illustrating their role in immune modulation. Izquierdo and associates suggested that differences in the surface proteome of *Lactobacillus* strains were responsible for their diversity in adhesion ability to

Table 1 Probiotic strains and their use in metabolic disorder and disease prevention

Strain	Disease prevention	References
*L. rhamnosus*GG	Allergy and Immune Response	[33]
Enterococcus mundtii ST4SA	Antibiotic removal	[34]
Lactobacillus strains		[35]
L. brevis KB290		
Bifidobacterium strains		
L. plantarum 299v	Cardiovascular disease	[36]
E. coli 1917	Colitis	[37]
E. faecalis	Colon cancer	[38]
L. plantarum	Diarrhea	[39]
*L. casei*DN-114 001		[40]
B. bifidum	Eczema	[41]
B. lactis		[42]
Escherichia coli	Food allergies	[43]
L. casei	Gastroenteritis	[44]
E. faecium M-74	Hypercholesterolemia	[45]
Propionibacterium freudenreichii		[46]
L. reuteri	Infant colic syndrome	[47]
Lactobacillus strains	Intestinal dysbiosis	[48]
*L. johnsonii*N6.2		
*B. infantis*35624	Irritable bowel syndrome	[49]
B. bifidum		[50]
*E. coliDSM*17252		[51]
Lactobacillus strains		
Saccharomyces cerevisiae CNCM I-3856		[52]
L. acidophulus	Lactose intolerance	[53]
S. cerevisiae	Pain relief	[54]
L.acidophulus	Peptic ulcer disease	[55]
L. rhamnosus GR-1	Urinary tract infection	[56]
L. reuteri RC-14		
L. acidophilus	Ulcerative colitis	[57]
E. coli		
Bifidobacterium		[58]
L. rhamnosus GR-1	Vaginal candidiasis	[59]
L. pentosus B281	Inhibition of cancer cell proliferation	[60]
L. plantarum B282	and cell cycle arrest	
L. casei ATCC 393	Induction of apoptosis	[61]
Bacillus polyfermenticus KU3		[62]
Enterococcus faecium RM11		[63]
L. fermentum RM28		
L. acidophilus SNUL	Suppressed proliferation of tumor cells	[64]
L. casei YIT9029		
B. longum HY8001		
L. reuteri PTCC 1655	Prevention of gastric cancer	[65]
L. kefiri P-IF		[66]
Bacillus natto,	Prevention of colorectal carcinoma	[67]
L. acidophilus		
Propionibacterium freudenreichii	Prevention of liver cancer	[68]
Streptococcus thermophilus	Decreased the risk of colorectal cancer	[69]
Lactobacillus delbruckii		
L. acidophilus L1	Decreased the risk of bladder cancer	[70]
L. casei Shirota (LcS)	Minimized the human papilloma virus (HPV) associated infection	[71]

Table 2 Microorganism employed as probiotics

Species	Response	References
A. muciniphila	Enhance the immunity and gut barrier function, production of Vitamin B12	[75]
B. uniformis	Intestinal homeostasis and immune	[75]
B. coagulans, *B. subtilis*, B. laterosporu	Control of infections and increased survival of host	[76]
B. longum, *B. catenulatum*, B. breve, B. animalis, B. bifidum	Enhanced maturation of DCs and production of IL-10 & IL-12, upregulation of c-myc and il-6 genes	[77]
E. faecium	Prevention of diarrhea and minimizing the chronic sinusitis and bronchitis	[78]
L. plantarum, *L. paracasei*, *L. acidophilus, L. casei, L. reuteri,L. rhamnosus, L. curvatus, L. crispatus*	Induction of mucin secretion, induction of mucosal, humoral and cellular immune responses	[79]
L. lactis	Activate the innate immune response and protection against pathogen infection	[80]
P. productus	Improvement of nervous system functioning	[75]
P. jensenii, *P. freudenreichii*		[75]
S. boulardii	Prevention of *Clostridium difficile* infection and antibiotic associated diarrhea	[81]
S. sanguis, *S. oralis*, *S. mitis*, *S. thermophilus, S. salivarius*	Inhibition of fungal infection, prevention of biofilm formation by pathogenic bacteria	[82]

mucous of GIT [103]. A higher ratio of proteins like GroEL and DnaK responsible for mucin binding was present in highly adhesive *L. plantarum* WHE 92, when compared to less adhesive *L. plantarum* 299v and CECT 4185. The proteomic map of the *L. plantarum* 299v identified 29 proteins, highlighting their role in mucin binding [101]. Ashida et al. [104] reported the surface layer protein (SlpA) of *L. acidophilus* in adhesion to Caco-2 cells. The immuno-stimulatory activity and protective role of SlpA in *L. helveticus* against *Salmonella typhimurium* have been reported [105]. A mutational study featuring the SlpA of *L. acidophilus* confirmed the SlpA's role in binding to dendritic cells for enhanced pro-inflammatory cytokine production [106]. Candela et al. [107] reported that exposure to bile acids upregulates Plg receptors such as ENO/DnaK and phosphoglycerate mutase in *Bifidobacterium animalis* BI07. Similar changes were observed in the surface layer proteome of *B. longum* under bile stress [108]. These findings illustrate the role of bile as signaling trigger molecule to enhance the probiotic-host cross-talk and further colonization processes.

Recently, *Caenorhabditis elegans* has attracted attention as an Ideal model for testing the efficacy of suitable probiotic candidates [109]. As compared to cell culture, *C. elegans* has a certain advantage of being an entire organism with different cell and tissue structures. Additionally, the absence of ethical issues and lower cost allows a safe orientation of future experiments on mammals and also decrease the number of animals to use/or sacrifice. A study showed that probiotic administration decreased the bacterial load in its intestine and also increased its lifespan. Some *Lactobacilli* and *Bifidobacterial* strains (*B. infantis*, *B. longum*) increased the survival of *C. elegans* from 17% to a maximum of 46% [110–112]. A recent study observed that probiotic bacterium *L. paracasei* D3-5 and *L. plantarum* SK-9 administration increased the function of *C. elegans* with an improvement in physical functions and high muscle mass [112]. Probiotic bacteria enhance the longevity of *C. elegans* via modulating the p38 mitogen-activated protein kinase (p38 MAPK) signaling pathway and through induction of the SKN-1 genes encoding antioxidant proteins. A study showed that probiotic intake increased the longevity of *C. elegans* through involving the pmk-1 and NHR (nuclear hormone receptor) family; however, the detail mechanism is still unclear [113]. Recently, it was observed that probiotic *B. infantis* administration significantly increased the lifespan of *C. elegans* through mutation of Toll-like receptor homolog TOL-1 [114]. These shreds of evidence clearly indicate that *C. elegans* model represents a cheap, easy-to-handle and rapid tool to screen the new probiotic microorganisms and also to explore the molecular aspects of interactions. Additionally, in the future *C. elegans* will emerge as powerful, bioethical and pertinent model host between *in-vitro* and mammalian models.

Probiotics: Important Source of Bioactive Molecules

Probiotics are important sources of various bioactive compounds (Table 3). Among these bioactive compounds, bacteriocins are proteinaceous toxins produced by a wide variety of bacteria [129]. Target-specific bacteriocins are non-toxic in nature and limit the progression of pathogens to neighboring cells. Many LAB-produced bacteriocins are emerging as antibiotics with proven potential in in-vitro and in-vivo

Table 3 Bioactive compounds of probiotic bacteria and its effect on health system

Probiotic strain	Bioactive compounds	Health effects	References
*B. coagulans*RK-02	Exopolysaccharides (EPS)	Amelioration of toxic oxidative free radicals	[115]
B.adolescentis DSM 18,350	Folate vitamins	Biosynthesis of nucleic acid	[116]
B. pseudocatenulatum			
Bifidobacterium spp.	Pyridoxine (Vitamin B6)	Amino acid metabolism	[117]
Clostridium spp.	Amino acids	Provides essential amino acid	[118]
E. casseli flavus MI001	Enterocins	Antimicrobial activity	[119]
Enterobacteriaceae	Metabolite like amino acids	Nutrient support	[118]
Enterococcus spp.	Aromatic amino acids	Improve the male reproductive system	[118]
Fusobacterium varium	amino acids (arginine)	Improve the male/female reproductive system	[118]
F. prausnitzii,	Butyric acid	Energy source for colonocytes	[120]
Lactobacillus spp.	Lactic acid	Improve the metabolism	[121]
Lactobacillus spp.	Vitamin B1	Synthesis of nucleic acid/fatty acids	[122]
Lactobacillus sp. *G3*	Amylase	Starch hydrolysis	[123]
L. gasseri strains DSM 20,604 and 20,077	Inumins/Levans	Minimize fat/cholesterol absorption	[124]
L. fermentum E-3	Superoxide dismutase/Catalase	Antioxidant activity	[125]
L. fermentum E-18			
L. fermentum CECT 5716	Vitamin B9	Energy metabolism	[126]
*L. reuteri*JCM1112	Vitamin B12	Improves the RBC formation	[127]
P. freudenreichii	Propionic acid	Play a role in gluconeogenesis	[128]
P. freudenreichii	β-galactosidase	Hydrolysis of β-galactoside	[128]

systems [130, 131]. LAB-bacteriocins are very specific to their target species, displaying bacteriostatic and bactericidal activity and do not affect their neighboring bacteria. Therefore, simultaneous applications of bacteriocins and antibiotics are appearing as possible therapeutic agents [132–135]. Members of the LAB-group belong to the heterofermentative group and have the ability to produce various organic acids. The produced organic acids act on the outer surface of pathogenic bacteria and hamper the protein synthesis machinery resulting in increased fatalities [136]. The lactic acid produced by LAB completely inhibits the growth of pathogens, such as *E. coli*, *Salmonella*, and *L. monocytogenes* even at 0.5% (v/v) concentration. Additionally, the mucous layer of epithelial cells provides protection against damages caused by these acids. Similarly, diacetyl produced by many LAB-groups, interacts with arginine receptors of Gram-negative bacteria, leading to reduced arginine availability [137]. Some LAB-groups have the ability to produce H_2O_2, which inhibits pathogenic bacteria through a toxic oxidation mechanism [138]. The reuterin produced by *L. reuteri* inhibited DNA replication and was shown to compromise the survival of *E. coli* and *Candida* spp. [139].

Probiotic bacteria have the ability to produce EPSs in bulk [140]. Microbial EPSs have caught attention for their cumulative health effect. These have been shown to promote an immunostimulatory response, antitumor and antioxidant activity, and also lower blood cholesterol [141]. Enzymes such as glycosyltransferase and glycantransferase can metabolize sugar nucleotides into EPSs. EPSs contain multiple sources of oligosaccharides, which are cross-linked polymers, composed of monomer units. Probiotics together with other gut residing bacteria ferment non-digestible oligosaccharides to monosaccharides, therefore, providing nutrients for the proliferation of gut microflora [142]. Dietary oligosaccharides boost the immunoglobulin-A (IgA) level, which plays a natural role in the host's defense. In animal studies, fructooligosaccharide (FOS) induced the synthesis of IgA [143]. Similarly, FOS and galactooligosaccharides (GOS) have been used for nutritional therapy in case of constipation [142].

Probiotics are a source of various important enzymes like lactase, amylase, esterase, and lipases. Lactase hydrolyses lactose to glucose and galactose, which are further converted to SCFAs [144]. LAB reduces the amount of lactose in yoghurt through β-galactosidase, lowering lactose intolerance [145]. Similarly, amylase and peptidase are also probiotically produced enzymes that severely affect the biochemical reaction of the host, modulating its metabolism. Probiotic bacteria also play an important role in flavor development through the biochemical conversion of amino acids to aldehydes, alcohols, and acids. Small molecules like acyl-homoserine lactones produced by gut bacteria play an important role in quorum sensing and biofilm formation [146]. Many LAB produces small peptides and amino acids by proteolysis of casein [147].

Many probiotic bacteria produce water and fat-soluble B-group vitamins. Water-soluble vitamins are absorbed in the intestine, whereas fat-dissolvable vitamins are ingested as micelles in the intestinal tract [148]. They are required for various metabolic processes of carbohydrates, fats, amino acids, and nucleic acid synthesis. Many *Bacillus* and *E. coli* strains have been shown to produce riboflavin [149]. Similarly, *Bifidobacteria* strains produce B-complex vitamins, which help in the maintenance of the overall health of the gut [150]. Interestingly, few bacterial strains are sufficient sources of B_{12}, which is not synthesized by plants and is important for blood formation and nervous system function. The LAB probiotic strain *L. reuteri* is able to produce the cobalamin. *Propionibacterium shermani* also produces B_{12}, propionic acid, and other useful metabolites for industrial application. Another group of vitamins, thiamine, is produced by *Bifidobacterium* [150].

Metabolic and Functional Potential of Probiotics

Gut bacteria ferment the undigested carbohydrates to produce energy which can be used for multiple body functions. These probiotic bacteria maintain a healthy symbiotic relationship with the host's gut flora. Under optimal intestinal conditions, they modulate their metabolic pathway machinery to metabolize undigested sugar into SCFAs, such as acetate, butyrate, and propionate [121]. It is estimated that most of the produced SCFAs (up to 95%) are utilized by the gut microbiota for their energy-dependent processes. However, their profile varies with different prebiotic sources. The produced SCFAs act in a beneficial way, specifically as an energy source and also counteracting pathogenic microorganisms [151]. In humans, they enhance signaling pathways like AMP kinase in muscle, and promote fatty acid oxidation, thereby decreasing lipid accumulation [152]. Among SCFAs, acetate crosses the blood–brain barrier and is associated with regulatory neuropeptides that favor appetite suppression [153]. Butyrate is used as an energy source for the propagation of colonocytes and enterocytes. Additionally, butyrate has shown therapeutic potential in diseases like diarrhea, colon cancer, cardiovascular disease, and other inflammatory responses [154, 155]. Propionate diffuses into the portal vein system to be utilized for hepatic gluconeogenesis processes [156]. Moreover, SCFAs reduce the secretion of cytokines/or chemokines by lowering the local infiltration of macrophages, and enhance adipogenesis in a G-protein coupled receptors such as the GPR43-dependent mechanism, by activating the peroxisome proliferator receptor [152]. To regulate carbohydrate and fat metabolism, probiotic bacteria produce many amino acids and their derived molecules (Supplementary Figure 1) [144]. The amino acids produced in the gut are further fermented by bacteria to produce phenol and indoles, maintain the energy balance, and inhibit pathogen infections. Moreover, the fermentation of amino acids generates several chains of esters, alcohols, and organic acids, which form the aroma and flavor of various food products [157].

Few probiotic bacterial strains contain exopolysaccharide-metabolization enzymes such as glycosyltransferases and glycantransferases, which convert sugars to EPSs. Besides EPS-producing, probiotic bacteria offer further advantages in antitumor and antioxidant activity, immune-stimulatory behavior, and also lower blood cholesterol [158]. The human body develops intolerance to lactose due to a deficiency in the enzyme lactase; however, LAB possesses the enzyme β-galactosidase which decreases the amount of lactose in food products, especially yoghurt [159]. Additionally, LAB also produces enzymes like amylase and peptidase that improve the metabolism of their host [160]. The biological effect of administrated probiotics is severely affected by the enzymatic activities in the gut, being estimated that *Lactobacillus* and *Bifidobacteria* exhibit over 20 different enzymatic functions. *B. longum* administration changes the intestinal microbiota, lowering the β-glucuronidase level [161]. In addition, *B. longum* increases ATP production through the phosphoketolase pathway leading to enhanced acetic acid production [162]. Similarly, *B. animalis* activates the pathway of intermediate metabolites like formate, resulting in enhanced oxalate consumption [99].

Furthermore, clinical trials using probiotics to treat non-alcoholic fatty liver disease (NAFLD), showed a reduction in liver aminotransferase activity [163]. A random clinical study, featuring 30 healthy individuals fed with yogurt containing two probiotic *Lactobacillus* strains, compared with healthy individuals fed with standard yogurt, detected nineteen enzymatic activities in the feces of probiotic-treated volunteers. The activity profile tested by both groups was constant; however, the naphthol-AS-BI-phosphohydrolase and leucine arylamidase activities were higher in the feces of the probiotic administrated group [164]. In another study, probiotic-treated group received *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711, showing higher fecal butyrate levels, when compared to the control group. Changes in fecal acid contents could be used as biomarkers for identifying individuals who benefited from probiotic treatment; however, the impact of the probiotic intake on microbiota and metabolites production is associated with the gut microbial ecosystem [165].

Another clinical study featuring 33 healthy individuals of different age groups (26 to 76), were given an oral dose of *L. plantarum* strain Lp-8, showed higher levels of acetate and propionate when compared to butyrate, illustrating that the production of fecal metabolites is bacteria-dependent [166]. A 4-week random clinical trial involving 20 human volunteers fed with *Bifidobacterium* reported higher levels of metabolites like acetate, butyrate, isobutyrate, and succinate

when compared to control groups [167]. Probiotic bacteria either by themselves or in symbiosis upon administration to elderly people could be able to improve their metabolic functions [168]. In a trial of 96 days with probiotic yeast *Saccharomyces boulardii*, the strain was found to be helpful for decreasing the occurrence of diarrhea receiving the total enteral nutrition. The level of fecal butyrate was found to be lower in patients as compared to healthy individuals. A trial with *S. boulardii* boosted the SCFAs content in patients and increased fecal SCFAs, potentially contributing to protection against diarrhea [169]. Probiotic *L. plantarum* strain 299v enhanced the fecal SCFAs content especially butyrate in patients suffering from *C. difficile* infection, which contributed to minimizing the effects of antibiotics. The SCFAs level was found to be decreased in the group treated with the antibiotic metronidazole when compared to the probiotic-treated group. However, after the study, the total SCFAs level regressed to pre-antibiotic treatment levels [169].

A clinical study with *L. casei* featuring 77 subjects of 84 years of age showed a decrease in norovirus-gastroenteritis processes, while a significant amount of fecal acetic acid content was observed [170]. Considering the microbiome population, *Bifidobacterium* and *Lactobacillus* are the dominant genera, while the population of *Enterobacteriaceae* decreased in the fecal content of probiotic-treated individuals. Recently, Nagata et al. [171] documented those individuals treated with probiotic *L. casei* strain Shirota, recorded higher fecal acetate content with significantly lower fever incidence and enhanced bowel movement. Additionally, the *C. difficile* count was lower in the probiotic-supplemented group. Likewise, *L. paracasei* Lpc-37 and *B. lactis* HN019 consumption reduced the risk of diarrhea in children, and the concentration of fecal SCFAs and branched-chain fatty acids (BCFAs) were higher in probiotic-treated subjects. *L. paracasei* Lpc-37 intake led to increased *Bifidobacterium* population, isobutyrate, and isovalerate levels. *B. lactis* HN019 administration was found to be positively correlated with the microbiota population and negatively correlated with the propionate level [172]. Consumption of probiotic *L. salivarius* CRCT5713 increased the fecal *Lactobacilli* population and the fecal amino acid content, especially of butyric acid [173]. Oral application of probiotic *B. lactis* Bb12 lowered the fecal pH in newborn infants and is positively correlated with higher acetate and lactate content [174]. Among other probiotic strains, *B. bifidum* W23 and *B. animalis* W52 exhibited higher SCFA and lactate concentrations and lower succinate/lactose concentrations in children with eczema. The observed outcome emphasizes the role of SCFAs and other metabolites in the regulation of the immune system [161]. The use of probiotics provided beneficial effects in terms of improving carbohydrate metabolism and fasting blood glucose levels, reducing metabolic stress, and influencing microbial ecology and immunity [175]. A probiotic preparation containing *Bifidobacterium*, *Lactobacillus*, and *S. salivarius* attenuated NAFLD, primarily at the level of amino acid metabolism, nucleic acid degradation, and microbial-mediated amino acids metabolism. In addition to lowering liver lipids concentration, probiotics also play an important role in NAFLD and some of the fecal metabolites act as biomarkers to evaluate the response [176].

Genetic Engineering of Probiotics: a Promising Way Forward

In the human body, cellular metabolism and complex biological processes are regulated by various enzymes. Therefore, any enzyme deficiency leads to several metabolic disorders, where the accumulated toxic product produces abnormal responses, in some cases leading to death [177]. Thus, engineering the beneficial probiotic bacterial strains with specific enzymes or metabolic pathways resulted in protection against metabolic disorders [27, 178]. Additionally, the engineering of probiotics improves their performance in terms of their site-specificity and multi-host functionality [179]. In the present scenario, the interest is increasing in engineering the probiotics in combination with synthetic biology approaches to enhance the delivery of target molecules [178, 180], the sensing ability [181, 182], and antipathogenicity [183–186]. Additionally, various molecular biology tools targeting the multiple regulatory systems can be employed to engineer the beneficial probiotic strains (Fig. 2). Engineered probiotic strains have advantages over microbiota-mediated therapy by displaying specific functions that are not shown by gut microbiota. Individuals with diabetes may develop abnormal levels of blood glucose due to insufficient production of pancreatic insulin. Currently, researchers are designing recombinant probiotic strains for the treatment of diabetes. For example, *Lactococcus lactis* was engineered to secrete an immunoregulatory cytokine IL-10 and the oral delivery of this strain was found promising for the treatment of type 1 diabetes [187] (Fig. 3).

In another study, a recombinant strain of *L. gasseri* was developed to include the glucagon-like peptide (GLP)-1 gene, which is known to induce insulin production both in vitro and in vivo. Oral delivery of the *L. gasseri* GLP-1 strain in diabetic rats resulted in enhanced insulin production from pancreatic beta cells as well as in the reduction of blood glucose (10 to 20%) [188]. Similarly, engineered *L. lactis* strains are able to secrete the autoantigen GAD65370–575, leading to a decrease in IL-10-induced pancreatic islet inflammation under hyperglycemic conditions [189]. Deficiency in phenylalanine hydroxylase (PAH) is the root cause of the metabolic disorder phenylketonuria, where the organism accumulates phenylalanine resulting in several health issues [190]. By recombinant development, phenylalanine ammonia-lyase (PAL) gene from *Anabaena*

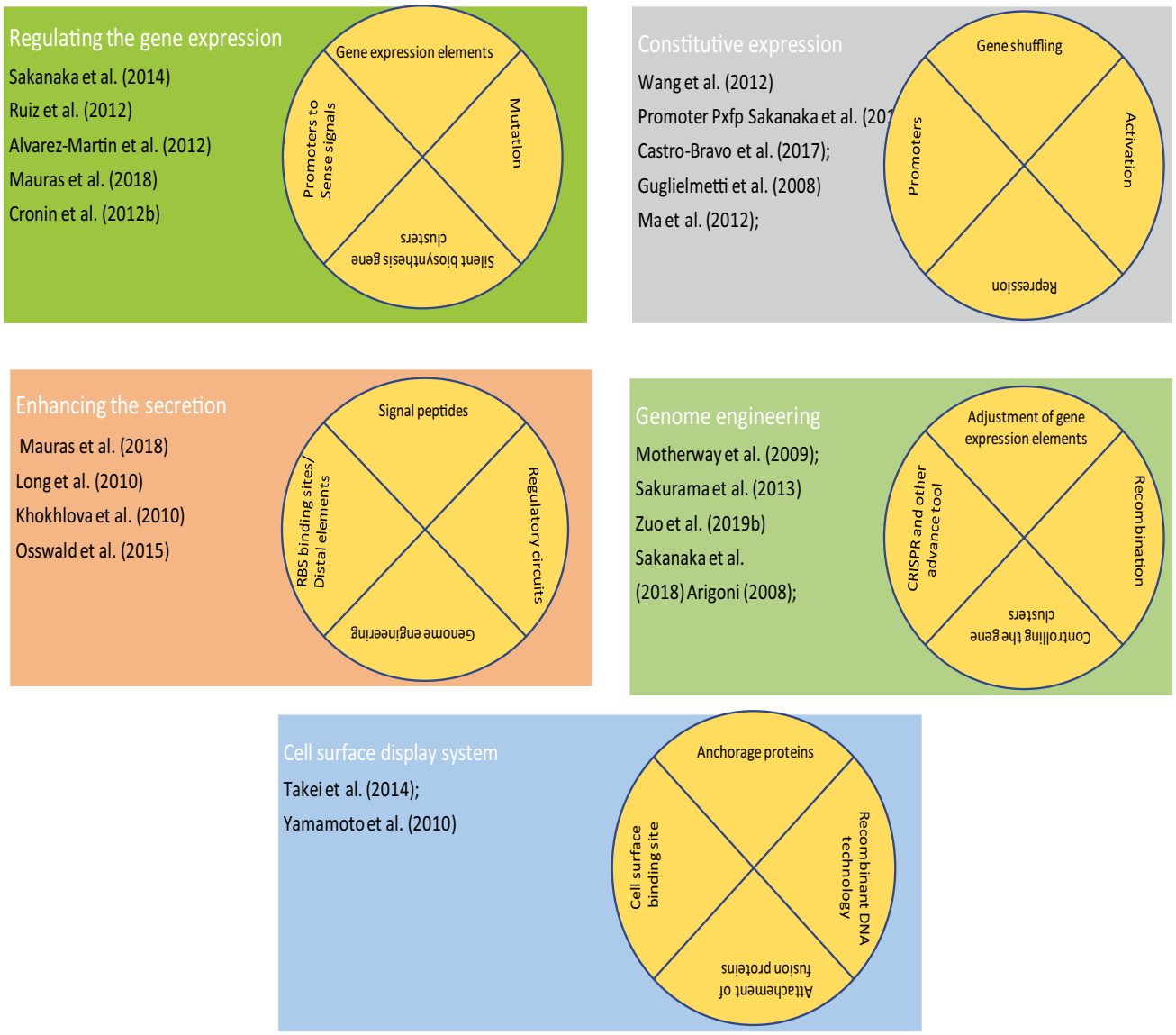

Fig. 2 The use of molecular biology tools in combination with synthetic biology approach to engineer the probiotic bacteria to enhance their functionalities; the improvement can be done by **a** regulating the gene expression, **b** constitutive expression of gene under suitable promoters, **c**. enhancing the secretion of target enzyme by addition of signal peptides, **d**. edition of genes through genome engineering, and **e**. through development of cell surface display system

variabilis was introduced into *L. reuteri*, which reduced the blood phenylalanine level in an in-vivo mice study [191]. Similarly, the probiotic strain SYNB1618 was developed to secrete PAL and L-amino acid deaminase (LAAD), which greatly reduced the blood phenylalanine up to 38% [178]. Recently, strain SYNB1618 (*thyA*$^{-}$, *argR*$^{-}$) was developed by genomic insertion of argR215, and its oral administration greatly reduced the ammonia level, thereby enhancing the survival rate up to 50% [27].

Moreover, in recent years, various mutagenesis approaches like single-crossover-insertion [192], double deletion [193], homologus recombination [194], and inducible plasmid self-destruction (IPSD)-mediated genome engineering have been well established for probiotic *Bifidobacterial* strains. Many of these approaches possess several drawbacks like low-transformation efficiency, unstable mutations, loss of plasmid, and extensive screening of a large pool of colonies (Supplementary Figure 2) [195]. However, the CRISPR-Cas genome editing tool can be employed for rapid and efficient genome modifications [196, 197]. This newly discovered tool can be used for transcriptional regulation and control of the genetic circuits [196]. The CRISPR-Cas systems for genome engineering have been applied to various lactic acid bacterial groups like *Lactobacillus*, *Lactococcus*, and *Streptococcus*

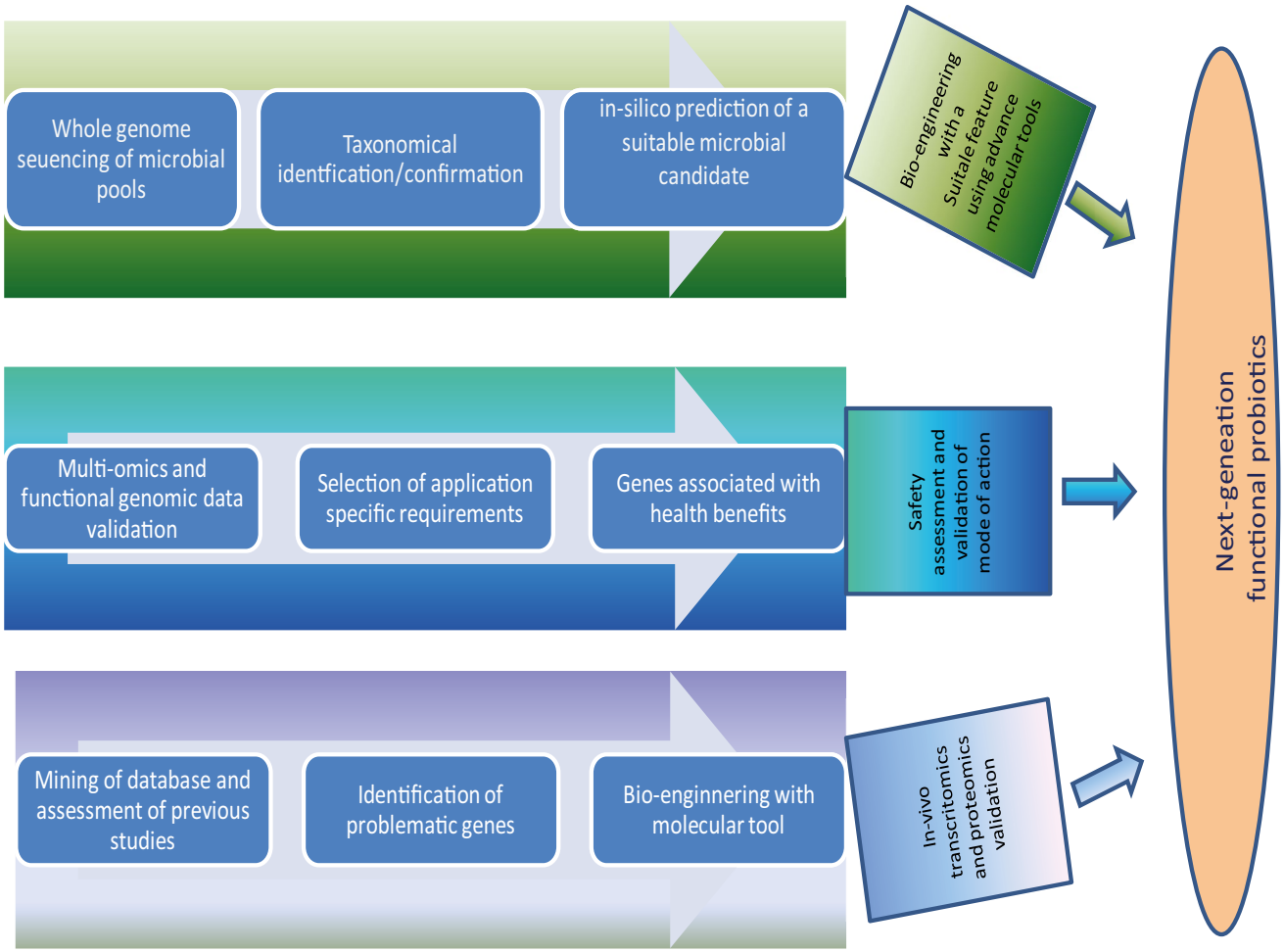

Fig. 3 Schematic diagram summarizing the each route for development of next-generation probiotics; the probiotic bacteria can be engineered for enhancing their suitable feature using adavanced molecular biology tools, further its efficacy can be tested by in-vivo evaluation followed by evaluating the safety aspects, and validating the mode of action

[196, 197]. The CRISPR-Cas in combination with IPSD will further improve the genome engineering in probiotic bacterial groups [198]. Therefore, the development of genetic tools including the plasmid expression systems, genome engineering approaches, etc. will promote the synthetic biology applications of probiotics. A number of broad-host range plasmids such as pSH71, pAMβ11, pBC1, and pTB6 have been characterized for gene expression in LAB [199, 200]. However, their functionality in other probiotic strains has not been well established.

Various approaches like transformation, conjugation, electroporation or phage transduction, etc. can be used to transfer plasmid DNA to other microbial strains. However, evolved bacterial host defence systems like CRISPR-Cas, and restriction-modification systems prevent the invasion of foreign DNA [201–203]. To overcome these issues, conjugation is an alternative tool to transfer plasmid DNA in a single-stranded form from donor to recipient bacteria [204]. Higher success was obtained by transferring the plasmid from *E. coli* to a variety of *Bifidobacterial* strains using a conjugative transfer system [205]. On the other hand, for efficient expression of the foreign gene products in a host, promoters, regulatory elements such as ribosome binding sites (RBS), cell-surface anchoring elements, and various secretion and localization signals need to be considered [206]. Promoters such as *hup* encoding a histone-like protein and *gap* encoding glyceraldehyde-3-phosphate dehydrogenase, induce the expression of genes in *Bifidobacterial* strains at high levels [207]. The promoter *Pgap* has been used for efficient homo and/or heterologous expression and for the production of various fluorescent proteins and therapeutic factors in *Bifidobacteria* [208, 209]. In response to certain signals, genetic circuit elements reprogramme the gene expression in a tightly controlled manner [210]. Bacterial one/two-component system, and quorum sensing are used as a tool to construct the genetic circuit to diagnose

disease [181, 182], bacteriocidal activity [185, 186, 211], and to deliver therapeutic molecules [180]. Additionally, the gene expression system sensing the GI-signals for pH, bile salts, inflammation, etc. can be used to construct a genetic circuit for various diagnostic, and therapeutic purposes [182].

In recent years, engineered probiotics to treat bacterial infections and inhibit pathogen proliferation in the gastrointestinal tract have received great attention. These "programmed" probiotics are a safer therapy to efficiently target multidrug-resistant bacteria, through the secretion of antimicrobials, immunomodulation, inhibition of adhesion, and toxic protein production from pathogens [184, 212]. Among pathogens, biofilm-forming bacteria cause severe chronic infections leading to immune system imparity. Researchers across the globe have focused on engineering probiotics to inhibit biofilm formation [213, 214]. Attempts were made to develop an engineered *E. coli* strain to inhibit the *Pseudomonas aeruginosa*, through the modulation of quorum sensing. The engineered *E. coli* was developed with the addition of E7 lysis protein and pyocin S5 which minimized the *P. aeruginosa* growth by 99%, also reducing biofilm formation by 90% [214]. During an *in-vivo* study, dispersin B (anti-biofilm protein) was introduced to the constructed strain and the engineered strain showed higher efficacy in *C. elegans* and mice infection studies [184]. Furthermore, microcin H47 was introduced in *E. coli* N 1917 to counteract the *Salmonella* infections [215]. To counteract the overgrowth of *Salmonella*, atetrathionate sensing system was incorporated, which induced the production of H47 and was found to be effective against *Salmonella* [216]. Similarly, strain N 1917 was engineered to secrete the antimicrobial peptide Mccj25 and the administration of this strain was effective to decrease the population of *Salmonella* by 97%, while the homeostasis of the native microbiome was maintained.

On the other hand, intestinal infection with *Vibrio cholera* causes acute diarrheal disease and if it remains untreated, it can lead to shock and even death [217, 218]. Infections by this pathogen are regulated by the quorum-sensing system in a density proportional manner. Therefore, attempts were made to disrupt the sensing system of *V. cholerae*, using an engineered probiotic *E. coli* strain. Administration of the strain was found to be quite effective to decrease the toxin formation in a mouse model [219]. Additionally, oral delivery of *L. lactis* could prevent the *V. cholerae* infection through lactic acid secretion. Pursuing engineered probiotics, researchers focused on developing a strong sensing system to identify molecules or produce strong signals, making them an effective diagnostic device. In that sense, a diagnostic circuit was introduced in *L. lactis* through the fusion of CqsS (*V. cholera*) and NisK (*L. lactis*) domains. The constructed *L. lactis* strain was orally delivered to cholera-infected mice, enhancing their β-lactamase secretion, which was recorded by colorimetric shift [185]. Similarly, Sedlmayer et al. [220] coupled the formyl peptide sensor with quorum sensors to eliminate pathogenic infections. Expression cassettes encoding luciferase (lux CDABE) and β-galactosidase (lac Z) were introduced into *E. coli* to develop the strain PROP-Z, to detect liver metastasis by luminescent analysis [221, 222]. High levels of nitric oxide (NO) serve as a signaling device, as well as an inflammatory indicator in inflammatory bowel disease [223, 224]. Probiotics were engineered to detect NO levels which serve as markers for inflammation.

Therefore, the development of metabolic engineering in parallel with synthetic biology enabled the creation of novel probiotic strains with valuable features. The use of engineered probiotic strains should precede rigorous clinical trials, required to minimize the exposition of patients to any potential risks [225]. Gene encoding as a novel effector function must be evaluated in vitro and in vivo in terms of genetic stability during the production processes. Nonetheless, further research is needed to provide the adequate colonization of anecological niche in the gut by delivered strains.

Probiotics and COVID-19

Coronaviruses are a large family of viruses that have crown-like appendages on their surface. Human coronaviruses (CoV) were identified in the mid-1960s, and currently, the following seven types of CoV have been identified (https://www.cdc.gov/coronavirus/types.htm). Viral infections in the respiratory tract lead to disturbance in the gut microbiota [226]. COVID-19 infection resulted in an increase in disorders of the human stomach and intestine, lymphopenia, acute respiratory distress, and multi-organ failure [227]. Additionally, the overproduction of proinflammatory cytokines termed as "cytokine storm" was observed in COVID-19 patients [228]. The increase in cytokine can damage the lung, brain, liver, cardiovascular system, etc. [229].

A recent study demonstrated that COVID-19 infection reduced the count of *Bifidobacterium* spp. and *Lactobacillus* spp. [230]. Initially, the treatment option applied for coronavirus disease 2019 (COVID-19) included artificial ventilation and antiviral agents. In addition to the development of vaccines, the use of biological agents to target viral infections using immunomodulation has received great attention [231]. Baud et al. [232] listed the bacteria such as *B. bifidum*, *B. breve*, *L. casei*, *L. gasseri*, *L. plantarum*, *Pediococcus pentosaceus*, etc. as potent probiotics with the ability to decrease the burden of COVID-19 pandemic. Similarly, patients suffering from other complications like antibiotic-related diarrhea showed a higher proportion of COVID-19 infection. These recent findings further indicate that there is a requirement for

microbiota balance in patients suffering from COVID-19 infection using some probiotics [230]. COVID-19 infection also causes severe hypoxia, whereas a reduction in probiotic species leads to an increase in the number of pathogens like *Actinobacteria* spp., *Corynebacterium* spp., and *Ruthenibacterium* spp. [233]. Available theoretical evidence suggests the biological role of probiotics in fighting the cytokine storm related to COVID-19 infection; however, there is an urgent need for clinical and laboratory evidence [232, 234].

Experimental evidence suggests that *L. paracasei* and *L. plantarum* have the capability to reduce the immune-inflammatory response [235]. The biological activity of probiotic bacteria belonging to *Lactobacillus* and *Bifidobacterium* controls the gastrointestinal dysbiosis associated with COVID infection [236]. Similarly, biofilm-forming probiotic bacteria like *L. Reuteri* and a few *Lactobacillus* spp. produces biologically active molecules that showed anti-inflammatory properties [237, 238]. Bottari et al. [239] illustrated the immune benefits of probiotics in COVID infection by priming mucosal immunity through stimulation of IgA secretion, improvement of biological functions of macrophages, and stimulation of regulatory cells. Experimental evidence suggests that probiotics also shape the T cell subsets [240], enhance the antimicrobial peptide production [241], and also direct the Th17 cells' differentiations in the small intestine [242]. Probiotics belonging to *Lactobacilli* have been reported to produce peptides that interact with the ACE2 receptor, the host entry receptor of SARS-CoV-2 viruses [243]. A recent study by Minato et al. [244] find that *Paenibacillus* bacteria naturally produce carboxypeptidase homologous to the ACE2 receptor.

Recently, it was observed that acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike proteins binds to human angiotensin-converting enzyme 2 (ACE2). To counteract this problem, biongineered probiotics expressing ACE2 were developed to neutralize SARS-CoV-2 [245]. Probiotic *L. paracasei* (LP) expressing secretory human ACE2 (sACE2) controlled the viral spread by sequestering the virus or blocking the spike protein interaction with host cell-associated receptors. The sACE2 produced by probiotic bacteria confers systemic effects to control the viral entry at multiple organs including the lungs. Additionally, the bio-engineered bacteria enhance the innate immunity and confer beneficial effects to control dysbiosis in the SARS-CoV-2 infected patients [245].

Probiotics and Cancer

Besides treating various metabolic diseases and disorders, probiotics also modulate cancer signaling [246], through induction of apotosis [247], and autophagy [248], inhibition of mutagenic and kinase activity [249], activation of tumor suppressors [250], and prevention of metastasis [251]. The structural components of probiotic bacteria, their secreted metabolites, and various other signaling molecules can alter the metabolic and regulatory reactions associated with the host [252].

The structural components constitute the microbe-associated molecular pattern and include the S-layer proteins, flagella, pili, capsular polysaccharides, lipoteichoic acid, and lipopolysaccharides [253]. The metabolites include the secreted proteins, extracellular vesicles, short-chain fatty acids, hydrogen peroxides, and bacteriocins [254]. Previous reports showed that probiotic bacteria induce apoptosis to inhibit tumorigenesis and progression via altering the tumor necrosis factor, inhibitors of apoptosis proteins, B cell lymphoma (Bcl)-2, caspases, and p53 gene [255, 256]. The bacteriocin produced by probiotic bacteria shows anticancer activity through the formation of minute pores on the plasma membrane which induce apoptosis and cell cycle arrest at the G1 phase [257]. The probiotic-derived ferrochrome act as tumor-suppressive molecule to inhibits the colon cancer progression via c-jun N-terminal Kinase (JNK)-mediated apoptosis [258]. Similarly, linoleic acid produced by probiotic *L. plantarum* initiate the apoptosis in breast cancer cells via downregulation of the NFκB pathway [259]. On the other hand, *L. acidophilus* and *B. bifidum* increased the cytotoxic activity against breast and colon cancer cells by upregulating IFN-γ and TNF-α expression, and downregulating Bcl2 expression [260]. Probiotic bacterium *L. acidophilus* induces apoptosis via increasing the mRNA expression of surviving [261]. Another study revealed that *Propionibacterium* enhanced apoptosis in colorectal carcinoma cells through the action of SCFAs on mitochondria [262].

The surface protein from *L. acidophilus* induced HCT116 cell death via altering the level of autophagy-linked protein (microtubule-associated protein 1) [263]. The exopolysaccharides of probiotic bacteria activate the autophagy in colon cancer cells via stimulating the Beclin1/GRP78 and apoptotic pathways Bcl-2 and Bak proteins [248]. A previous study demonstrated that probiotic-produced SCFAs target the tumor cell through epigenetic regulation of the tumor suppressor and oncogenes [264]. The probiotic bacterium *L. acidophilus*, *L. rhamnosus* MD14, and *L. rhamnosus* GG enhanced the expression of tumor suppressor genes in an experimental colon carcinogenesis model [265]. Similarly, probiotic bacterium *B. longum* induced the expression of tumor suppressor miR-15 and miR-145 in an experimental murine colorectal cancer [266]. Some studies demonstrated the probiotic-mediated tumor-suppressive effect by downregulation of oncogenes [267, 268]. The probiotic *L. crispatus* and *L. rhamnosus* alter the cancer progression by affecting the expression of mTOR-related genes and altering the proto-oncogenes (Wnt/β-catenin) pathways [269]. Similarly, probiotic bacteria also downregulate the KRAS proto-oncogene, thereby decreasing the progression of colon cancer [265].

Probiotic bacteria also modulate the kinase and phosphatase enzymes that play a major role in tumor biology such as cell propagation and metastasis etc. [270]. Previous studies showed that probiotic bacteria and their produced metabolites act as kinase inhibitors to treat diarrhea after cancer therapy. A study showed that probiotic secretory proteins modulate the protein kinase C (PKC) to protect the intestinal epithelial tight junctions from H_2O_2-induced damage [271]. Probiotic bacterium *L. plantarum* induces apoptosis via downregulating the MAP-kinases and upregulating the phosphatases [272]. These shreds of evidence clearly show the multifarious effects of probiotics against cancer. Although, researchers have discovered the apoptotic potential of probiotics on cancer, their molecular mechanisms still need to be discovered. Further, in-depth investigation and clinical trials w.r.t probiotic-mediated autophagy and its role in cancer elimination are urgently required.

Probiotics and Neurodegenerative Disease

Neurodegenerative diseases are serious disorders especially in the aging population and are characterized by degeneration of structure and function of the central nervous system (CNS) and peripheral nervous system (PNS). These diseases will impose an increasing socio-economic burden and incidences are also increasing year by year [273]. At present, the most common neurodegenerative diseases are Alzheimer's disease (AD) and Parkinson's disease (PD) and steadily progress due to the loss of neurons in the brain [274]. The etiology of these diseases is still unknown; however, inflammation may be involved in the progressive development of these diseases [275]. A recent study showed a decrease in the population of intestinal microbes and a higher abundance of pro-inflammatory bacteria in AD patients as compared to healthy ones [276]. Another study showed an increased population of gram-negative Bacilli in AD patients may lead to increased distribution of lipopolysaccharides from the gut to the systemic circulation and it enhances the pathology of AD through neuroinflammation [277]. Similarly, in PD patients, a higher population of hypothetical pathogens such as *E. coli*, *Enterococcus*, *Proteus*, and *Streptococcus* was observed [278]. The influence of the brain-gut axis has attracted attention especially in neurodegenerative diseases and gut microbiota affects immunity, inflammation, and neuroregulation through the brain-gut axis [279]. These evidences illustrate that maintaining a healthy microbiome benefits normal brain functioning, and boosts the immune response etc.

Probiotic intake helps the proliferation of intestinal microflora, ameliorating gastrointestinal function, reducing gut leakiness, and helping fight against pathogens by regulating the immune system [280]. A recent study showed that probiotic intake decreased the inflammatory cytokines IL-6 and TNF-α in serum and improved the anti-inflammatory cytokines IL-10 in serum and brain in PD mice [281]. Probiotic administration reduced the expression of pro-inflammatory IL-1, TNF-α, and increased the expression of anti-inflammatory TGF-β, and PPAR-γ in PD patients [282]. Additionally, the CBM (total bowel movement), spontaneous bowel movements (SBM), very low-density lipoproteins (VLDL), triglyceride, and Bristol Stool Scale improved in PD patients.

A recent in-vitro study with probiotic *Lactobacillus* and *Bifidobacterium* reduced oxidative stress, and pro-inflammatory cytokines in peripheral blood mononuclear cells isolated from PD patients [283]. A recent investigation showed that a mixture of probiotics conferred a neuroprotective response on dopaminergic neurons to counteract motor impairments in a PD mouse model [284]. Probiotic formulation VSL#3 controls the expression of various genes in the brain cortex, minimizes inflammation, and improves neuronal performance [285]. The innovative probiotic formulations SLAB51 (known as Sivomixx) effectively control the 6-hydroxydopamine-induced deleterious effects both in in-vivo and in-vitro PD models. The impairment involves the protection of dopaminergic neurons, restoration of pro-survival and neuroprotective pathways, induction of anti-inflammatory pathways, and amelioration of behavioral impairments [286]. These evidences clearly illustrates that probiotics intake modified the gut microbiome and influenced the protective response to CNS disease via the gut-brain axis, mediating different pathways involving neural, hormonal, inflammatory, antioxidant, and immune signaling [287, 288].

Next-Generation Probiotics

Probiotics are live microorganisms, usually isolated from gut bacteria; however, until their safety and health effects are fully characterized, they cannot be given the term "Probiotics." Among various nomenclatures, probiotics are referred to as "functional food" or "beneficial bacteria" that are thought to prevent disturbances and balance the host's system [289]. Previous research trials showed that the gut microbiota has limited therapeutical potential, therefore there is a high demand for novel and cost-effective formulations [290, 291]. Promising results have been observed in bacterial species *Lactobacillus* and *Bifidobacterium* in the prevention of diverse metabolic and inflammatory diseases. The information generated from recent clinical studies showed a positive outcome for inflammatory and metabolic disease, therefore making the path for selection of next-generation probiotics including the members of *Akkermansia muciniphila*, *Bacteroides uniformis*, *Clostridium* spp., and *F. prausnitzii* [292, 293]. *A. muciniphila* are well-known for mucin degradation and were linked to the maintenance of a healthier metabolic status. Under a high-fat diet, microbiota composition especially *A. muciniphila* decreased and was associated with

several abnormalities like high-level blood glucose, insulin resistance, and increased plasma triglycerides [294]. In a clinical study with different diets, Dao et al. [295] found that an optimal or even increased population of *A. muciniphila* was associated with blood glucose maintenance as well as minimizing hypercholesterolemia. In another study, it was observed that the presence of *A. muciniphila* restored the gut barrier and also reduced the inflammation [296].

Among all microbes comprised in the gut, *Bacteroides* constitute approximately 25% of the total population. These commonly behave as commensal microorganisms and are transmitted from mother to child during delivery. *Bacteroides* are primarily responsible for the fermentation of complex sugars, producing volatile fatty acids in the gut. Among *Bacteroides*, *B. fragilis* and *B. thetaiotamicron* can efficiently metabolizes carbohydrates. Polysaccharides produced by *B. fragilis* activate the T cell-mediated immune response and therefore, strengthen the host immunity [297]. Round et al. [298] reported that *B. fragilis*-produced polysaccharide signals through TLR2 and activates Toll-like receptor (TLR) pathways. However, *B. fragilis* are benefitted from the presence of several virulence factors on their capsules and thereby avoiding the host immunogenic responses. Additionally, the presence of enterotoxins and proteases are responsible for the destruction of tight junctions in the intestinal epithelium and brush border enzymes, respectively. Capsular polysaccharides of *B. fragilis*s showed a positive effect on the host, reducing host inflammation and pathogenesis. *B. uniformis* isolated from the feces of infants were considered a potential probiotic strain. Administration of this strain improved the lipid profile, reduced glucose-insulin and leptins, and increased phagocytosis under a high-fat diet. Therefore, this strain ameliorated the diet-induced metabolic disorders and immunological dysfunctions in obese mice [299]. Butyrate-producing *Eubacterium hallii* are natural residents of the gut, demonstrated to lower mucosal inflammation, strengthening the epithelial barrier function, and providing the energy for colonocytes [300].

Moreover, various physiological processes related to energy metabolism and improved insulin sensitivity were observed following oral supply of this specific strain to obese and diabetic mice. Additionally, an increased dosage of *E. hallii* did not show any negative effect on physiological parameters and food intake, illustrating the strain as a safe and effective therapeutic agent. Various strains belonging to *Clostridia* clusters were effective in Treg cell differentiation. Atarashi et al. proposed that *Clostridia* spp. are strong metabolites producers including SCFAs [301]. The produced SCFAs regulate the Foxp3 gene, controlling the Treg-cell development. The author proposed that a cocktail of bacterial strains acted more effectively for treating disease as compared to a single strain. *Faecalibacterium prausnitzii* belonging to *Clostridium* cluster IV is one of the most predominant bacteria in human feces (3 to 5%). The absence of *F. prausnitzii* is commonly associated with gut-associated disorders. However, studies with *F. prausnitzii* in humans are still lacking; therefore, further investigations are needed to unravel the safety aspect of this bacterium in humans.

Probiotics and Its Safety Aspects

According to the Food and Drug Administration (FDA), probiotics are classified as dietary supplements, therefore having less stringent requirements in terms of their safety, dosage, and efficacy. Most of the probiotic strains fall into the GRAS category [302]. The use of probiotics has been growing exponentially and shown in a wide variety of applications such as the food industry and health care. Probiotics are live microorganisms gaining popularity because of their beneficial effects on human health; however, they may cause minor infections, especially in adults with secondary conditions (e.g., chronic infections, diabetes, immune deficiency) [303]. A total of 24 fungemia cases have been recorded associated with the probiotic *S. boulardii* [304]. Munoz et al. [305] reported three cases of *S. cerevisiae* infection, in an ICU dealing with *S. boulardii* therapy. However, in clinical trials, no reports of bacterial/fungal infection were observed following probiotic use. All cases of fungemia were observed in immuno-compromised, chronic or debilitated patients. Additionally, probiotic bacteria may be able to transfer antibiotic resistance genes to their neighbor pathogenic microorganisms. Several properties critical for probiotic survival should be properly monitored before commercializing a specific strain, as the evidence clearly shows variations in probiotic strains belonging to the same species. Additionally, various other properties like formulation, stability of the organism, colonization in the intestinal epithelium, anti-pathogenic response, immuneactivation, and various other important functionalities need to be carefully monitored. The industrial application requires the stability and survival of the used strains [306].

Limitation and Future Aspects

As probiotics are live organisms, their count in food items must be kept at an optimum level. According to the National Sanitary Surveillance Agency, approximately 10^8–10^9 CFU of probiotics in food are regarded as safe [307]. However, its level can be affected by bacteriophages that lead to cellular lysis [308]. Moreover, the accurate effect in the host depends on the ingested strain, its quantity, survival rate, and physiological condition of the host [309]. Moreover, methodologies to detect culturable bacteria require specific synthetic media under defined conditions. The selection of phage-resistant strains can be performed via plaque assay, qPCR, flow cytometry, and biosensors [310, 311]. Selective

culture techniques are limited in providing the most holistic definition of viable probiotic bacteria for the dose available in the final product. Reliance on cell-culture techniques does not provide direct cues on microbe-microbe and/or microbe-host interactions, pertaining to the gut environment and is even not consistent in in-vivo trials. Many of the probiotic strains lose their viability during the processing of food products via acidification or in the presence of oxygen in the medium [312, 313]. Viability can be controlled by approaching classical microbiological methods such as growing in a specific cultivation medium [314]. Additionally, previous studies also showed poor colonization efficiency of exogenous probiotics compared to those of humans in the gut [315, 316]. Many quorum sensing disrupting probiotic bacteria have the potential to inhibit the pathogenic microbes; however, their signaling behaviour and complexity may differ in-vivo, when compared to in-vitro experiments. Additionally, manipulation of the quorum-sensing pathway may result in inhibition of commensal bacteria in vivo [317]. Although, the use of probiotics are more often related to the enhancement of beneficial gut microbiota or protecting the host against multiple diseases. However, the exact mechanisms by which probiotics alter the microbiota in in-vivo models are still not properly known.

Nonetheless, many debate that the effect of probiotics might not relate to their interaction with the host microbiome [5]. The systemic review of Kristensen et al. [23] reported the lack of evidence for probiotic-mediated modulation of microbiota in many of the analyzed studies. Similarly, previous studies summarized multiple trials associated with beneficial probiotics of which only 21% showed microbiome alterations [318]. Their establishment for broad function and replication in the host environment is not clear [319]. Further, human clinical studies are required to follow probiotic-mediated cell response to assess possible outcomes and observe the colonization potential of strain-specific probiotics. The identification and characterization of biomarkers linked to probiotic properties could lead to the evolution of novel strains with improved function and health effects [320]. Importantly, in-vivo probiotic effects in animal models do not guarantee effective translation to human subjects and therefore, future research should focus on accurately translating animal trial findings to human therapies. Despite having demonstrated their potential for various clinical and nutritional applications, further studies are required to strengthen the implementation of probiotics for human usage. Future research should be directed towards controlled human studies to determine the strain-specific role of probiotics and which dosages may guarantee effectiveness and safety.

Although the effects of probiotics in health care have been demonstrated in many studies, the molecular approach integrated with proteomics still requires further research efforts to unravel the probiotic-cross talk. Further in-depth studies are still needed to explore probiotics' applications as therapeutic agents. More clinical information is required to provide the evidence to prevent primary and secondary *Clostridium*-derived infection (CDI), including the selection and use of bacterial strains that show better results in immunocompromised or critically ill patients [321]. *Bacteroides* spp. rapidly transfer antibiotic resistance gene among bacteria in the human colon, both within the *Bacteroides* genus and among *Bacteroides* species and Gram-positive bacteria, therefore this characteristic has to be taken into account while developing next-generation probiotics. Information regarding the safety profile of *Faeccalibacterium* and *Akkermansia* is not sufficient; therefore, further animal and human clinical studies are needed [322]. The development of more advanced technical tools will promote the production and formulation of next-generation probiotics, enabling their use as supplements. Using next-generation probiotics in food is limited to their safety and suitability to the target consumer groups when compared to traditional probiotics [320]. Moreover, recently developed marine probiotics showed their potential in the prevention of ecological damages. An integrated effort would help to develop better formulations that improve viability, safety, and higher efficacy. Moreover, the properties of a probiotic strain are important for the efficacy of the treatment, maintenance of their characteristics, and relative efficacy should be verified. The increasing evidences indicated that manufacturing and production procedures may influence the quality and safety of probiotics [323, 324]; thereby, attention should be given to choosing the specific formulation to be administered. Furthermore, before the use of probiotics for the prevention and treatment of disorders, greater investment in clinical trials is necessary.

Conclusions

Probiotics possess several promising attributes which fulfil our prompt nutritional and clinical necessities. Many probiotic microbes have been found to show positive responses against several diseases. The contribution of probiotics to the treatment of severe diseases such as cancer, obesity, diabetes, and infectious diseases is growing, representing a high demand in the research arena. Technological advancements linked to RNA sequencing, culturomics and metabolomics have propelled the field of probiotics, detailing the interaction with the indigenous microbiome. Presently, probiotic administration of food products presents a cost-effective alternative and fruitful source for the assessment of the effects of newly discovered probiotic strains. Moreover, further developments gathered in clinical trials highlighted the role of specific probiotic strains in terms of their safety (for clinical and food applications), antagonistic activity towards

pathogens, and ability to tolerate acid and bile stress. The probiotics-based biotherapy shows enormous potential as a therapeutic agent against neurodegenerative disease; however, extensive clinical trials and characterization of the biochemical effects of probiotics are required to fully elucidate the scope of probiotics against specific diseases. There is still much to explore the connection between probiotics and gut microbiota with neurodegenerative disease, which will open a new possibility for researchers in the largely uncharted territory of neurodegenerative and gut microbiota. The studies on the relationship between gut microbiota and neurometabolites will provide a new concept for the prevention and intervention of neurodegenerative diseases. The development of probiotics demands close interaction between the pharmaceutical industries, research agencies, and regulatory bodies.

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Declarations

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