REVIEW

Pro-biomics: Omics Technologies To Unravel the Role of Probiotics in Health and Disease

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ABSTRACT

The comprehensive characterization of probiotic action has flourished during the past few decades, alongside the evolution of high-throughput, multiomics platforms. The integration of these platforms into probiotic animal and human studies has provided valuable insights into the holistic effects of probiotic supplementation on intestinal and extraintestinal diseases. Indeed, these methodologies have informed about global molecular changes induced in the host and residing commensals at multiple levels, providing a bulk of metagenomic, transcriptomic, proteomic, and metabolomic data. The meaningful interpretation of generated data remains a challenge; however, the maturation of the field of systems biology and artificial intelligence has supported analysis of results. In this review article, we present current literature on the use of multiomics approaches in probiotic studies, we discuss current trends in probiotic research, and examine the possibility of tailor-made probiotic supplementation. Lastly, we delve deeper into newer technologies that have been developed in the last few years, such as single-cell multiomics analyses, and provide future directions for the maximization of probiotic efficacy. *Adv Nutr* 2021;00:1–19.

Keywords: probiotics, microbiome, metagenomics, transcriptomics, proteomics, metabolomics, lactic acid bacteria

Introduction

Probiotics are defined as "live microorganisms that when administered in adequate amounts confer a health benefit on the host" (1). plethora of studies has revealed the positive impact of probiotics on blood cholesterol concentrations (2), inflammation-related disorders (3), metabolic disorders such as obesity and diabetes (4), and even cancer (5). To evaluate the effectiveness of probiotics and elucidate their mechanisms of action, several preclinical and clinical models are commonly employed. For example, models of intestinal or systemic inflammation are used to study inflammation-related pathogenesis (6), models of high-fat diets have been developed to study metabolic disorders (7), and cancer models are used for the study of oncogenesis (8). Moreover, clinical trials that recruit healthy volunteers or patients, with the aim of correlating clinical outcomes to specific molecular changes caused by the probiotic supplementation, are also performed (9).

The evolution of high-throughput platforms and the development of robust bioinformatic tools have boosted our understanding of probiotic action, promoted the in-depth study of their biology and probiotic-induced cellular responses, and further validated their health-promoting properties. Genomic analyses have aided in the mining of probiotic features, such as acid tolerance or bacteriocin production (10), whereas metagenomic studies have contributed to the elucidation of probiotic–microbiota interactions (11). Transcriptomic, proteomic, and metabolomic platforms have supported the profiling of host–microbe crosstalk and have contextualized the holistic effects induced by the supplementation (12). Furthermore, recent comparative studies firmly support the species-, disease-, sex-, and host-specific probiotic actions, underlining the need for targeted interventions (13). The purpose of this review is to weigh the use of multiomics high-throughput platforms in preclinical and clinical studies, investigating the effects of probiotic supplementation on common pathophysiological conditions, such as immune and metabolic disorders. Furthermore, we critically discuss

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Abbreviations used: AI, artificial intelligence; CRC, colorectal cancer; DEG, differentially expressed gene; DNBS, dinitrobenzene sulfonic acid; DSS, dextran sodium sulfate; HFD, high-fat diet; HPLC-ECD, high-pressure liquid chromatography-electrochemical detection; KEGG, Kyoto Encyclopedia of Genes and Genomes; LGG, *Lactobacillus rhamnosus* GG; NGP, next-generation probiotic; NGS, next-generation sequencing; RNA-Seq, RNA-sequencing; TIFA, TNF receptor-associated factor (TRAF) interaction protein with a forkhead-associated domain; TLR, Toll-like receptor; WGS, whole genome sequencing.

current trends in probiotic research, newly developed technologies, such as single-cell multiomics analysis, and provide future directions for the implementation of personalized probiotic interventions. To this aim, a comprehensive search of current literature on the PubMed database, using the term "probiotics" combined with "transcriptomics," "proteomics," "metabolomics," "metagenomics," "comparative genomics," and "microbiome" was performed. Filters were applied to restrict results to animal and clinical studies. Finally, only articles published in English were included.

Current Status of Knowledge

Transcriptomic and proteomic studies of the immunomodulatory properties of probiotic bacteria

Characterization of the immunomodulatory effects of probiotic bacteria at the transcriptome level is performed either by RNA-sequencing (RNA-Seq) or microarray gene expression platforms. Accordingly, protein chips with antibodies, other proteins, or nucleic acids that bind specifically to protein targets are used to evaluate probiotic-induced changes at the proteome level. Animal studies are usually conducted on models of chemically induced colitis. The animals are treated with dinitrobenzene sulfonic acid (DNBS) or dextran sodium sulfate (DSS), which provoke strong inflammatory responses or induce colonic cell death, ultimately leading to the development of colitis (14). Preclinical and clinical studies employing these methodologies are listed in **Table 1**. For example, administration of *Lactobacillus rhamnosus* CNCM I-3690 to DNBS-induced chronic microinflammation mice, induced downregulation of systemic inflammation markers IL-6, IFN-β, and IFN-γ and restored gut permeability (15). Moreover, transcriptomic analysis with microarrays, targeting the sum of known mouse transcripts, demonstrated that the probiotic strain blocked the activation of the canonical NF-κB pathway and upregulated the expression of TNF receptor associated factor (TRAF) interaction protein with a forkhead-associated domain (TIFA). Validation of the microarray analysis for 7 representative genes was performed by qRT-PCR (15). In another study, colitis was induced by DSS in C57BL/6 mice that were also injected with the carcinogen azoxymethane (16). The simultaneous supplementation with Bifico, a commercially available probiotic cocktail, comprised of *Bifidobacterium longum*, *L. acidophilus*, and *Enterococcus faecalis*, managed to preserve the intestinal architecture (crypt morphology, infiltration of immunological populations). Notably, a reduction of tumor formation was also recorded. To analyze further the clinical outcomes, and elucidate the molecular pathways involved, global transcriptome analysis with cDNA microarrays, was performed. Two-dimensional hierarchical clustering revealed that 300 genes were differentially expressed; 166 genes were upregulated, and 134 genes were downregulated in the probiotic-treated group. The interpretation of the bulk of data derived from this analysis was performed by the Database for Annotation, Visualization, and Integrated Discovery (DAVID). It was found that Bifico influenced the expression of transcripts involved in tight junctions and cytokine–cytokine receptor interaction (16). Similarly, to investigate the global changes in the transcriptomic profile of BALB/c mice orally inoculated with *Bifidobacterium bifidum* PRL2010, the 90k CombiMatrix array was employed (17). It was demonstrated that specific genes coding for proinflammatory proteins, such as kallikrein B, plasma 1 protein (*Klkb1*), were downregulated in the probiotic group, whereas genes encoding tight junction proteins, including catenin α-like 1 (*Ctnna1*) and claudin 10 (*Cldn10*), were upregulated. Mechanistic studies in human colon adenocarcinoma cell lines revealed that these effects were likely attributed to the activation of the NF-κ pathway (17). Finally, to elucidate the holistic effect of *L. plantarum* JDFM LP11 consumption on weaned piglets, RNA-Seq was performed in ileum tissues. Comparative transcriptomic analysis revealed 25 differentially expressed genes (DEGs) involved in the downregulation of intestinal inflammatory responses (18). Similarly, RNA-Seq revealed that *Saccharomyces boulardii* administration to C57BL/6 mice stimulated systemic inflammatory responses (19) (Table 1).

Studies from our laboratory utilizing the dorsal air pouch model of inflammation showed that certain probiotic strains can also exert extraintestinal proinflammatory and immunostimulatory effects. Sterile air was subcutaneously injected to the back of BALB/c mice and after 6 d the intervention group received an injection with the probiotic strain *L. paracasei* K5. Targeted protein microarray analysis of cytokines and chemokines in the exudates revealed that *L. paracasei* K5 upregulated CC and CXC chemokines and cytokines of the IL-1 family, supporting the activation and migration of leukocytes on site. The expression of these factors, alongside the expression of Toll-like receptor (TLR)-2, TLR-4, TLR-6, and TLR-9, were validated using qPCR. These effects could be attributed to the probiotic-induced stimulation of p38 mitogen-activated protein kinase signaling pathway (20). Additional preclinical studies utilizing protein microarrays to investigate the immunoregulatory effects of probiotics are presented in Table 1 (21–23).

The employment of high-throughput platforms in clinical studies for the investigation of probiotic-induced immunomodulatory effects has been limited. *L. casei* Shirota or skim milk was administered to healthy elderly subjects in a randomized, placebo-controlled, single-blind crossover study, for a period of 4 wk, followed by a 4-wk washout, and a crossover to the other treatment. At the end of each treatment peripheral blood and saliva were analyzed in antibody microarrays for the presence of immunological markers. It was found that probiotic supplementation promoted the activity of NK cells and increased the ratio of IL-10 to IL-12. Overall, the intervention led to a reduction of the inflammatory status of the host, while supporting innate immunity (24). In a randomized, placebo-controlled, crossover study, 7 healthy volunteers were assigned to receive *L. acidophilus*, *L. casei*, *L. rhamnosus*, or a maltodextrin solution (placebo group), for 6 wk (25). At the day of intervention, the participants consumed the probiotics in maltodextrose solution every

TABLE 1 Transcriptomic and proteomic preclinical and clinical studies of the immunomodulatory activities of probiotics[1]

Type of study	Treatment	Sample	Omics platform	Molecular mechanisms	Clinical outcome	Reference
Animal study	*Lactobacillus rhamnosus* CNCM I-3690 (5×10^9 CFU)	Blood, MLN, spleen, colon, and ileum	Transcriptomics (microarrays)	Inhibition of F-κB pathway	↓Systemic inflammation, restored colon and ileum permeability	Martín et al. (15)
DNBS-induced chronic microinflammation (SPF male C57BL/6 mice) ($n = 30$)	10-d gavage regimen					
Animal study	Bifico $>1.2 \times 10^7$ CFU/d (*Bifidobacterium longum*, *L. acidophilus*, *Enterococcus faecalis*)	Colon	Transcriptomics (microarrays)	↓Proinflammatory chemokines (Cxcl-1, -2, -3, and -5, Ccl-7)	↓Tumor formation	Song et al. (16)
AOM/DSS C57BL/6J mice ($n = 14$)	Gavage, daily, 3 cycles of DSS treatments				↓Colonic inflammation	
Animal study	*B. bifidum* PRL2010 (10^9 CFU)	Colon	Transcriptomics (microarrays)	Upregulation of tight-junction genes and β-defensin, downregulation of immune modulators	Modulation of innate immune responses	Turroni et al. (17)
Female BALB/c mice ($n = 5$)	5-d oral inoculation					
Animal study	*L. plantarum* JDFM LP11 (2.5×10^7 CFU/ml)	Ileum	Transcriptomics (RNA-Seq)	↓Bpi, ↓Rsad2,	Morphological changes in the epithelial layers of the intestines	Shin et al. (18)
Female crossbred piglets ($n = 6$)	Liquid and solid probiotics via drinking and feeds, daily for 4 wk			↓Slpi, ↓Lum ↓Olfm4, ↓Dmbt1		
Animal study	*Saccharomyces boulardii* (10^8 CFU)	Draining mesenteric lymph nodes	Transcriptomics (RNA-Seq)	Modest transcriptional changes	Induced systemic immune response	Hudson et al. (19)
C57BL/6 mice ($n =$ NS)	Gavage the day of analysis (single dose)					
Animal study	*L. paracasei* K5 (5×10^8 CFU)	Air pouch exudate	Proteomics (microarrays)	Modulation of chemokine expression	↑Leukocyte recruitment (induced by all strains)	Chondrou et al. (20)
BALB/c mice (air pouch model) ($n = 10$)	*L. casei* ATCC 393 (5×10^8 CFU) LGG (5×10^8 CFU) Subcutaneous injection in air pouch					
Animal study	*L. pentosus* 281 (5×10^8 CFU)	Air pouch exudate	Proteomics (microarrays)	↑sICAM	↑Leukocyte recruitment (induced by all strains)	Saxami et al. (21)
BALB/c mice (air pouch model) ($n = 10$)	*L. plantarum* 282 (5×10^8 CFU) *L. casei* ATCC 393 (5×10^8 CFU) LGG (5×10^8 CFU) Subcutaneous injection in air pouch			↑IL-16 ↓CXCL-13 ↓TIMP-1 ↓M-CSF		

(*Continued*)

TABLE 1 (*Continued*)

Type of study	Treatment	Sample	Omics platform	Molecular mechanisms	Clinical outcome	Reference
Animal study	*L. casei* ATCC 393 (10^9 CFU)	Tumor tissue	Proteomics (microarrays)	Modulation Th1 responses	↓Mean tumor volume	Aindelis et al. (22)
Colon CT26 syngeneic tumor mice model ($n = 10$)	Oral administration, daily for 13 d					
Animal study	*L. acidophilus* (6×10^8 CFU)	Serum and colonic tissue	Proteomics (microarrays)	Elevation of TGF-β	↓Mean tumor number	Mendes et al. (23)
AOM/DSS male C57BL/6 mice ($n = 29$)	*L. rhamnosus* (6×10^8 CFU)					
	B. bifidum (6×10^8 CFU)					
	Oral gavage, daily for 3 cycles of DSS treatments					
Randomized placebo-controlled, single-blind crossover study	*L. casei* Shirota (1.3×10^{10} CFU)	Venous blood, saliva	Proteomics (microarrays)	↑NK cell activity	Immunomodulation	Dong et al. (24)
Healthy volunteers (55–80 y) ($n = 16$)	Per os, daily for 4 wk followed by a 4-wk washout period and 4 wk crossover to the other treatment			Increased IL-10/IL-12 ratio		
Randomized, placebo-controlled, crossover study	*L. acidophilus* (5.2×10^{10} CFU)	Duodenum tissue	Transcriptomics (microarrays)	Modulation of the IFN and IL signaling	Species- and host-specific immunomodulation	van Baarlen et al. (25)
Healthy volunteers ($n = 7$)	*L. casei* (3.2×10^{10} CFU) *L. rhamnosus* (1.68×10^{10} CFU) Per os, daily for 6 wk					
Phase I open-label study	LGG (10^{10} CFU)	Venous blood	Transcriptomics (RNA-Seq)	Modulation of the expression of immune cell trafficking and inflammatory response gene-sets	Modulation of inflammation	Solano-Aguilar et al. (26)
Elderly healthy volunteers (65–80 y) ($n = 11$)	Per os, 2 capsules daily for 28 d					

[1] AOM, azoxymethane; *Bpi*, bactericidal permeability increasing protein; *Ccl*, chemokine (C-C motif) ligand; CFU, colony-forming units; *Cxcl*, chemokine (C-X-C motif) ligand; *Dmbt1*, deleted in malignant brain tumors 1; DNBS, dinitrobenzene sulfonic acid; DSS, dextran sodium sulfate; LGG, *Lactobacillus rhamnosus* GG; *Lum*, lumican; M-CSF, macrophage colony-stimulating factor; MLN, mesenteric lymph node; NS, not specified; *Olfm4*, olfactomedin 4; *Rsad2*, radical *S*-adenosyl methionine domain-containing 2; sICAM, soluble intercellular adhesion molecule; *Slpi*, secretory leukocyte peptidase inhibitor; SPF, specific pathogen free; Th1, T helper cell type 1; TIMP-1, metallopeptidase inhibitor 1; ↓, statistically significant downregulation; ↑, statistically significant upregulation.

30 min for 6 h, leading to duodenum biopsy. Affymetrix microarrays were utilized to analyze the transcriptomic profile of the duodenum. Results analysis was carried out by the generation of regulatory nodes (protein–protein and protein–DNA interactions) using the Bibliosphere software. The results demonstrated that *L. casei* induced the expression of TLR-3 and TLR-9, *L. acidophilus* upregulated various ILs and interfered with IL-23 signaling, whereas *L. rhamnosus* modulated IFN-signaling pathways. Interestingly, these transcriptomic changes were found to be highly variable among participants (25). The calculation of the CV revealed that genes encoding factors with predominant roles in cellular signaling, such as the transcriptional factor signal transducer and activator of transcription 3, demonstrated the lowest expression variability. In contrast, chemokines of the CC family exhibited the highest expression variability (25). Finally, RNA-Seq technology was used to investigate the effect of *L. rhamnosus* GG (LGG) on whole blood cell expression in elderly healthy volunteers (26). Differential expression and ingenuity pathway analysis showed that molecular networks involved in crucial processes, such as immune cell trafficking, inflammatory response, and cell-to-cell signaling, were modulated by the probiotic strain. These effects could be attributed to inhibition of NF-κB complex activation and CCL2 expression (26).

Transcriptomic and proteomic studies of the effect of probiotics on host metabolic pathways

Many studies have correlated mono- or multispecies probiotic interventions with distinct effects on the expression of genes associated with carbohydrate and lipid metabolic pathways. The introduction of multiomics approaches has supported the thorough investigation of these alterations in models of metabolic disease (**Table 2**). Transcriptomic microarray analysis of hepatic tissues extracted from high-fat-diet (HFD) mice supplemented with *B. pseudocatenulatum* CECT 7765, exhibited decreased expression of CD36 (*Cd36*), a fatty acid transporter that regulates lipid accumulation in the liver, and upregulated expression of phosphatase 1 regulatory subunit 3B (*Ppp1r3b*), early growth response 1 (*Egr1*), and insulin growth factor binding protein 2 (*Igfbp2*), counteracting the effects of the HFD (27). For in-depth analysis of the molecular events associated with *L. acidophilus* SJLH001 supplementation in HFD mice, RNA-Seq technology was used. It was shown that the intervention resulted in 844 DEGs, of which 275 DEGs were correlated with lipid metabolism and ion transport (28). Validation of the downregulated expression of glucose transporter type 4 (*Glut4*), scavenging receptor *Cd36*, and *Tlr-2*, and upregulated expression of cholesterol synthesis-related genes, such as apoA4 (*ApoA4*) and 3-hydroxy-3-methylglutaryl-CoA reductase (*Hmgcr*), was performed by qRT-PCR. Notably, the probiotic-treated mice exhibited significant reductions of total cholesterol and oral glucose concentrations (28). Similarly, RNA-Seq analysis was performed to study the modulation of lipid metabolism of broilers treated with *L. johnsonii* BS15 (29) and of weaned piglets fed with *L. reuteri* (30) (Table 2). Protein microarrays can also be used to investigate probiotic-induced alterations of host metabolic pathways. For example, it has been demonstrated that *L. plantarum* Ln4 administration in HFD obese mice significantly downregulated the expression of adipokines, leptin, lipocalin-2, and angiopoietin-like protein-L3, which are correlated with insulin resistance and obesity (31). Similarly, adipokine arrays showed a decrease in leptin concentration in mice supplemented with *L. plantarum* strains DSR M2 and DSR 920 (32).

Global transcriptomic analysis has also been employed in clinical studies focusing on the effects of probiotics on postprandial metabolism (Table 2). Healthy, young male volunteers participated in a randomized, double-blind, crossover study to investigate the effect of probiotic yoghurt (Thermophilic Yoflex Culture and LGG) on gene expression after meals (33). The volunteers received the probiotic yoghurt or acidified milk for 2 wk, followed by a 2-wk washout period and then a crossover to the other group. Distinct changes in the peripheral blood transcriptome between the probiotic and control groups were recorded. Specifically, RNA-Seq analysis revealed changes in the expression of gene sets involved in glycolysis, oxidative phosphorylation, and inflammation, such as aryl hydrocarbon receptor and epiregulin, with distinct kinetic characteristics in the probiotic group. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis indicated a significant positive enrichment of the insulin signaling pathway, after consumption of the probiotic yoghurt (33). The KEGG pathway database can facilitate the meaningful analysis of transcriptomic data, because it supports the visualization of results and determination of their biological significance (34).

In another study, cDNA and protein microarrays were used for the study of time-dependent effects of *L. plantarum* WCFS1 supplementation on healthy volunteers. To this aim, the probiotic was infused to the pylorus of the participants for 1 or 6 h and tissue samples were collected for transcriptomic (1 h) and proteomic (6 h) analysis. Transcriptomic analysis of samples from the duodenal mucosa after short-term stimulations revealed the abrupt inhibition in fatty acid metabolism and cell proliferation. Proteomic analysis showed that genes correlated to lipid and fatty acid metabolism, as well as genes involved in oxidative stress protection, were upregulated. Moreover, 1-h probiotic treatments led to modulation of genes encoding for innate immunity factors, whereas 6-h stimulations modulated the expression of members of the human leukocyte antigen family (35).

RNA-Seq and microarray technologies are employed to elucidate the molecular mechanisms of probiotic action. Both platforms can efficiently detect DEGs and provide a high-resolution snapshot of gene expression at a specific time-point and under set conditions. Additionally, RNA-Seq can identify noncoding transcripts such as microRNAs and long noncoding RNAs, important epigenetic regulators of the cell, offering a more realistic picture of physiological conditions (36, 37). Recent comparative studies have shown that RNA-Seq exhibits higher sensitivity and wider dynamic range than

TABLE 2 Transcriptomic and proteomic preclinical and clinical studies of the effect of probiotics in metabolic signaling[1]

Type of study	Treatment	Sample	Omics platform	Molecular mechanisms	Clinical outcome	Reference
Animal study HFD C57BL/6J mice ($n = 5$)	*Bifidobacterium pseudocatenulatum* CECT 7765 (10^9 CFU) oral gavage, daily for 7 wk	Liver tissue	Transcriptomics (microarrays)	↓Lipid transport-related genes ↑Carbohydrate metabolism-related genes	Attenuated HFD-related hepatic steatosis and hyperlipidemia	Moya-Pérez et al. (27)
Animal study HFD C57BL/6J mice ($n = 8$)	*Lactobacillus acidophilus* La-JLH001 (10^9 CFU), oral gavage, daily for 20 wk	Gastrointestinal and adipose tissue	Transcriptomics (RNA-seq)	Glucose metabolism-related genes: ↓Slc10a2, ↑Slc9a3 Cholesterol synthesis-related genes: ↑Hmgcr	Limited HFD-induced hypercholesterolemia and hyperglycemia	Sun et al. (28)
Animal study Male chicks (Cobb 500) ($n = 100$)	*L. johnsonii* BS15 (10^6 CFU), oral consumption for 15 d	Hepatic tissue	Transcriptomics (RNA-seq)	↑Fabp2, ↑Acsbg1 ↑Plin-1, Plin-2, ↓Pla2g4a	Altered hepatic metabolism	Qing et al. (29)
Animal study Crossbred Landrace × Large White) ($n = 9$)	*L. reuteri* ZLR003 (2×10^9 CFU), oral consumption for 10 d	Jejunum tissues	Transcriptomics (RNA-seq)	Enrichment of GOs related to arachidonic and linoleic acid metabolism	Altered colonic metabolism	Zhang et al. (30)
Animal study HFD C57BL/6J mice ($n = 5$–7)	*L. plantarum* Ln4 (5×10^8 CFU), oral gavage, daily for 5 wk	Plasma, epididymal fat tissue	Proteomics (microarrays)	Epididymal fat tissue: ↓CRP, ↓ANGPT-L ↓IGFBP-1, -3, -5, ↓leptin, ↓lipocalin-2	Attenuated diet-induced obesity and modulated the expression of T2D biomarkers	Lee et al. (31)
Animal study HFD C57BL/6J male mice ($n = 5$)	*L. plantarum* DSR M2 (10^9 CFU), *L. plantarum* DSR 920 (10^9 CFU), oral gavage, daily for 12 wk	Serum, liver tissue, and epididymal fat	Proteomics (multiplex bead array)	Lipogenesis-related proteins: ↓PPARα Inflammation markers: ↓MCP-1, ↓TNF-α	Improved obesity parameters	Lee et al. (32)
Randomized, double-blind, crossover study design. Healthy young male volunteers ($n = 7$)	Probiotic yoghurt (Thermophilic Yoflex Culture and LGG) per os, daily for 2 wk	Blood	Transcriptomics (RNA-seq)	Differentially expressed KEGG insulin signaling gene-sets	↑Postprandial insulin response	Burton et al. (33)
Randomized placebo-controlled crossover study. Healthy young volunteers ($n = 7$–8)	*L. plantarum* WCFS1 (10^{11}–10^{12} CFU) pylorus infusion for 1 or 6 h	Pylorus tissue	Transcriptomics (microarrays)	↓Degs1, ↑Fabp1, ↑Cd36, ↑Idh1	Global transcriptomic changes	Troost et al. (35)

[1] *Acsbg1*, acyl-CoA synthetase bubblegum family member 1; ANGPT-L, angiopoietin-like protein-L; *Cd36*, clusters of differentiation 36; CFU, colony forming units; CRP, C-reactive protein; *Degs1*, Δ4-desaturase, sphingolipid 1; *Fabp*, fatty acid binding protein; GO, Gene Ontology; HFD, high-fat diet; *Hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *Idh1*, isocitrate dehydrogenase (NADP(+)) 1; IGFBP, insulin-like growth factor-binding protein; KEGG, Kyoto Encyclopedia of Genes and Genomes; LGG, *Lactobacillus rhamnosus* GG; MCP, methyl-accepting chemotaxis protein; *Pla2g4a*, phospholipase A2 group IVA; *Plin*, perilipin; PPARα, peroxisome proliferator-activated receptor α; Slc, solute carrier family; T2D, type 2 diabetes; ↓, statistically significant downregulation; ↑, statistically significant upregulation.

microarrays (36). However, high cost, and complexity of data analysis correlated with this approach, must be taken into consideration. Conclusively, both platforms can contribute greatly to probiotic research by providing high-quality data.

The application of metabolomics to probiotic interventions

Metabolomic approaches are utilized for the identification and characterization of the sum of metabolites in cells, tissues, organs, or organisms, produced under set conditions (38). GC-MS is the gold standard for metabolomic studies of volatile compounds with biological relevance (39). In this context GC-MS is widely applied for accurate detection and determination of SCFAs in probiotic animal studies (40–42) (**Table 3**). SCFAs are produced by gut microbiota and play a major role in host metabolism, inflammation, intestinal homeostasis, and microbiota-gut-brain communication (43, 44). For example, GC-MS recorded the enhanced production of SCFAs in C57BL/6J mice, after oral administration daily for 7 wk of the probiotic strain *B. animalis* ssp. *lactis* GCL2505. Notably, the probiotic-treated mice exhibited higher cecal and plasma concentrations of acetate and propionate that were positively correlated with improved glucose tolerance and reduced fat accumulation and adipocyte volume (40).

Variations of the GC-MS platform can be applied for targeted and untargeted metabolomic studies, and the identification of specific metabolites or the sum of metabolites in a sample (45). In this regard, GC coupled with a flame ionization detector is a reliable technique to assess changes in fatty acid profiles in animals following administration of probiotic bacteria (46–48) (Table 3). Furthermore, GC-time-of-flight-MS has been used to capture changes in fatty acid content of plasma samples, derived from infants who consumed cereal fortified with the probiotic strain *L. paracasei* LF19. Among the 288 differentially produced metabolites, concentrations of the MUFA palmitoleic acid were significantly lower in the probiotic group (49).

LC-MS is commonly preferred for studies investigating nonvolatile lipid compounds in complex samples (Table 3). In this respect, ultra-high-performance LC was employed to profile the fecal lipid content of infants who consumed LGG during the first months of life (50). It was demonstrated that probiotic treatment differentially altered the production of various fatty acids and led to the promotion of immunological tolerance, which could be beneficial in the context of allergy or asthma prevention (50). Similarly, newborns who were recruited to consume a polybiotic mix, consisting of *Bifidobacterium bifidum*, *B. breve*, *B. longum*, and *B. longum* ssp. *infantis*, showed significant changes in the concentrations of fecal fatty acids, sterol lipids, and glycerophospholipids compared with breastfed infants or newborns who consumed regular whey-based formula (51). Another important application of LC-MS in probiotic research is for the measurement of bile acids in plasma or feces of preclinical or clinical experimental models (52–54) (Table 3). Ultra-performance LC analysis showed an increase in fecal unconjugated bile acid content in mice fed the probiotic mix Lab4 (*L. acidophilus* CUL21 and CUL60, *B. bifidum* CUL20, and *B. animalis* ssp. *lactis* CUL34), and *L. plantarum* CUL66 (52). Moreover, alterations in amino acid metabolism and decreased plasma concentrations of primary and secondary conjugated bile acids were recorded in overweight adults who received *B. animalis* ssp. *lactis* 420 alone or in combination with Litesse Ultra polydextrose. These metabolic changes were accompanied by a clinical reduction of fat mass (54).

NMR spectroscopy is a commonly applied analytical method in metabolomics for the identification and quantification of compounds in nontargeted approaches. It can be used for the identification of low molecular weight compounds that are present in high concentrations in complex samples (55). NMR analysis showed that conventionalized gnotobiotic mice fed *Bifidobacterium longum* BB536 had significantly elevated fecal concentrations of butyrate, acetate, and pimelate, a biotin precursor, compared with control mice (56). In the same manner, children with a high risk of eczema who were enrolled in a double blind, randomized, placebo-controlled trial and consumed the polybiotic mix of strains *B. bifidum* W23, *B. animalis* ssp. *lactis* W52, and *Lactococcus lactis* W58, showed enhanced fecal total SCFA and acetate concentrations and temporary protection against the development of eczema (57).

Finally, metabolomic platforms are also used to investigate changes in the concentrations of neurotransmitters and hormones linked to probiotic supplementation (Table 3). For the study of monoamine neurotransmitters that could be present in nanomolar concentrations in samples, HPLC-electrochemical detection (HPLC-ECD) is commonly used (58). For example, intestinal tissues extracted from early-life-stressed maternally separated C57B1/6J male breastfed pups, gavaged with *B. pseudocatenulatum*, were analyzed with HPLC-ECD for the evaluation of monoamine content. It was found that probiotic supplementation normalized dopamine and adrenaline concentrations. These effects were accompanied by the amelioration of stress and depression-like behaviors (59). Similar findings were presented in an independent study investigating the psychotropic effects of *L. plantrarum* PS128 on maternally separated mice (60) (Table 3).

The use of metagenomics in probiotic research

Metagenomics are employed for the investigation of the structure (taxonomic metagenomics) and function (functional metagenomics) of complex microbial communities, present in any environment with specific conditions, without the need of isolation and propagation of the microbes in laboratory settings (61). Metagenomic studies utilize 2 approaches: amplicon-based detection of specific sequences, such as hypervariable regions of the 16S rRNA gene in bacteria; or whole-genome shotgun sequencing (WGS) (62). WGS provides higher sensitivity and higher yield in variant detection; however, in many cases amplicon sequencing is more accessible, due to lower cost and less complicated

TABLE 3 Metabolomic preclinical and clinical studies of the beneficial effects of probiotic supplementation[1]

Type of study	Treatment	Sample	Omics platform	Molecular mechanisms	Clinical outcome	Reference
Animal study	*Bifidobacterium animalis* ssp. *lactis* GCL2505 (BlaG),	Blood, cecum	GC-MS	Cecum:↑SCFAs (acetate, propionate)	BlaG: improvement of clinical parameters of metabolic disorder BlaJ: NE	Aoki et al. (40)
ale C57BL/6J mice ($n = 23–26$)	*B. longum* ssp. *longum* JCM1217T (BlaJ (10^9 CFU) oral administration, daily for 7 wk			Plasma: ↑acetate		
Animal study	*B. bifidum* TMC3115 (10^9 CFU)	Cecum	GC-MS	↑SCFAs	Lower risk of IgE-mediated allergies in adulthood	Cheng et al. (41)
Pregnant (day 13 of gestation) SPF BALB/c mice and their offspring (OVA immunization) ($n = 18$)	Oral gavage, daily from birth to day 21					
Animal study	SIMFORT- VITAFOR (10^7 CFU)	Cecum	GC-MS	Neonates and adults: ↑butyrate	Protection against experimental asthma in adulthood	Nunes et al. (42)
Neonatal and adult C57BL/6 mice (OVA immunization) ($n = NS$)	Oral gavage, 3 times a week for 21 d					
Animal study	*B. animalis* IPLA R1 ($\sim 5 \times 10^8$ CFU)	Portal blood, liver, subcutaneous adipose tissues	GC-FID	↓Triglycerides	Protection against DIO metabolic outcomes	Salazar et al. (46)
HFD male C57BL/6J mice ($n = 8$)	Oral intake of 10% skim milk containing the strain for 10 d			↓Stearic acid ↓Arachidic acid ↓Palmitoleic acid		
Animal study	*Lactobacillus fermentum* CECT5716 (10^{10} CFU) oral gavage for 5 wk	Whole blood and gastrointestinal tissues	GC-FID	Dams: ↓EPA, ↑γ-linoleic acid	Immunomodulation	Azagra-Boronat et al. (47)
Wistar rat dams and offspring ($n = 48$)	(3 wk of pregnancy and 2 wk of lactation)			Pups: ↓total saturated fatty acids, ↓palmitic acid		
Double-blind, randomized, placebo-controlled, parallel trial in healthy volunteers ($n = 35$)	*L. plantarum* Q180 (4×10^9 CFU)	Feces	GC-FID	↑SCFAs	↓Postprandial TG	Park et al. (48)
	Oral intake, daily for 12 wk			↑Indole and phenol concentrations	↓ApoB-48 ↓ApoB-100 concentrations	
Double-blind, placebo-controlled, randomized intervention trial	*L. paracasei* ssp. *paracasei* F19	Venous blood	GC-TOF	↑Putrescine	NA	Chorell et al. (49)
Healthy term infants ($n = 84$)	(10^8 CFU) oral consumption, daily from 4 to 13 mo			↓Palmitoleic acid		

(*Continued*)

TABLE 3 (*Continued*)

Type of study	Treatment	Sample	Omics platform	Molecular mechanisms	Clinical outcome	Reference
Randomized, placebo controlled infant study. Infants with high risk of asthma (birth to 6 mo) ($n = 10$)	LGG (10^{10} CFU) Per os, daily from birth to 6 mo	Feces	UPLC-MS/MS	↑PUFA, ↑DPA, ↑ETA	Immunological tolerance	Durack et al. (50)
Double-blind, randomized, placebo-controlled, early-life study. Newborns (birth to first year of life) ($n = 49$)	Polybiotic mix (10^7 CFU/g) (*B. bifidum*, *B. breve*, *B. longum*, *B. longum* ssp. *infantis*) per os, from birth to 1 y	Feces	UPLC/Q-TOF	Modulation of fecal fatty acids and sterol lipids	NA	Bazanella et al. (51)
Animal study	Lab4 mix (5×10^8 CFU) (*L. acidophilis* CUL21 and CUL60, *B. bifidum* CUL20, *B. animalis* ssp. *lactis* CUL34) and *L. plantarum* CUL66	Fecal, blood, liver, and intestinal tissue samples	UPLC-MS	↓Total cholesterol	Lowering of total plasma cholesterol, reduction of weight gain	Michael et al. (52)
Male C57BL/6J mice ($n = 6$)	Oral intake for 14 d			↑Fecal unconjugated bile acids		
Randomized, double-blind crossover study	*Bacillus subtilis* R0179 (2.5×10^9 CFU)	Plasma	LC-MS/MS	↑Deconjugated bile acids	NE	Culpepper et al. (53)
Healthy adults ($n = 18$–19)	*L. plantarum* HA-119 (2.5×10^9 CFU) *Bifidobacterium animalis* ssp. *lactis* B94 (2.5×10^9 CFU) oral intake, daily for 6 wk					
Double-blind, randomized, parallel, placebo-controlled clinical trial. Overweight or obese individuals ($n = 25$)	*B. animalis* ssp. *lactis* 420 (10^{10} CFU) + Litesse Ultra polydextrose. Oral intake, daily for 6 mo	Plasma	UHPLC-MS/MS	↓Deconjugated bile acids	Improved gut barrier and obesity-related markers	Hibberd et al. (54)
Animal study	*Bifidobacterium longum* BB536 (10^9 CFU)	Feces	NMR	↑Pimelate	Modulation of gut luminal metabolism	Sugahara et al. (56)
GF female BALB/c mice ($n = 6$)	Oral intake, daily for 14 d			↑Butyrate		
Double-blind, randomized, placebo-controlled trial	*B. bifidum* W23, *B. animalis* ssp. *lactis* W52 and *L. lactis* W58, Ecologic Panda (10^9 CFU per strain)	Feces	^{1}H-NMR	↑Lactate	Temporary prevention of eczema onset	Kim et al. (57)
Infants with family history of allergic disease ($n = 60$)	Oral intake, daily for 12 mo			↑SCFAs		
				↓Succinate		
				↓Lactose		
Animal study MS C57Bl/6J male mice ($n = 18$)	*B. pseudocatenulatum* CECT 7765 (10^8 CFU) gavage, daily for 21 d	Ileum tissue	HPLC-ECD	↑Dopamine	Modulation of stress responses	Moya-Pérez et al. (59)
				↑Noradrenaline		
Animal study	*L. plantarum* PS128 (10^9 CFU) Gavage, daily for 4 wk	Prefrontal cortex tissue	HPLC-ECD	↑Dopamine	Amelioration of anxiety and depression-like behaviors	Liu et al. (60)
MS C57Bl/6J male mice ($n = 10$)						

[1] BlaG, *Bifidobacterium animalis* ssp. *lactis* GCL2505; BlaJ, *Bifidobacterium longum* ssp. *longum* JCM1217T; CFU, colony-forming units; DIO, diet-induced obesity; DPA, docosapentaenoic acid; ECD, electrochemical detection; ETA, eicosatetraenoic acid; FID, flame ionization detector; GF, germ-free; HFD, high-fat diet; LGG, *Lactobacillus rhamnosus* GG; MS, maternally separated; NA, not available; NE, no effect; NS, not specified; OVA, ovalbumin; SPF, specific pathogen free; TOF, time of flight; UHPLC-MS/MS, ultra-high-performance liquid chromatography-tandem mass spectrometry; UPLC-MS, ultra-performance liquid chromatography-mass spectrometry; UPLC/Q-TOF, ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry; ↓, statistically significant downregulation; ↑, statistically significant upregulation.

bioinformatic needs (63). Both platforms have been used for the investigation of probiotic-induced modulations in the gut microbiome, and the effects on inflammation, gut–brain communication and neurological disorders, and host metabolism (**Table 4**).

Metagenomics to study the effect of probiotic-induced microbiome modulation in intestinal inflammation.

Gut dysbiosis has been implicated as a contributing factor in inflammation, because it supports the proliferation of inflammation-promoting bacteria, often leading to acute colitis. Several preclinical studies have aimed at evaluating the potential of probiotic and symbiotic treatments to reverse these structural microbiota changes (64, 65) (Table 4). To this end, administration of the probiotic mix *L. acidophilus*, *L. rhamnosus*, and *B. lactis* alone or in combination with inulin, to DSS-treated C57BL/6J mice, resulted in the increase of beneficial bacterial populations of *Bifidobacterium, Lachnospiraceae*_NK4A136, and *Lachnospiraceae*_UCG-006, and in the decrease of bacteria belonging to the *Alistipes* genus, as demonstrated by 16S rRNA sequencing. Additionally, these microbial alterations were accompanied by attenuation of gut inflammation, as evidenced by histological examinations (64). Accordingly, shotgun metagenomics reported that administration of *L. plantarum* LP-Onlly to IL-10$^{-/-}$ mice, an inflammatory bowel disease model, induced the proliferation of *Bacteroides* and *Akkermansia muciniphila*. These changes could be correlated with the improved inflammation profile observed (65). The dysbiotic gut is also characterized by epigenetic modifications in the colonic tissue that are associated with increased risk of carcinogenesis. These alterations mainly involve promoter hypermethylation of tumor suppressors, such as the Wnt inhibitory factor 1 (WIF1) and other genes involved in the Wnt signaling pathway (66). Recent metagenomic studies of human colon cancer tissues have correlated these epigenetic changes with high abundance of specific tumorigenic bacteria, such as *Peptostreptococcus* and *Fusobacterium* species (67). Furthermore, high abundance of these bacterial species has been linked with the onset and lower survival rate of patients with colorectal cancer (CRC) (68). Metagenomic analysis was employed in 2 clinical studies to investigate the potential inhibitory effect of probiotic supplementation against the proliferation of these bacterial species (69, 70) (Table 4). In the first study, CRC patients were assigned to receive a mixture of *B. longum*, *L. acidophilus*, and *E. faecalis* or placebo, for 5 d. Colonic tissue samples were collected, and their microbial load was analyzed by 16S rRNA pyrosequencing. Metagenomic analysis revealed a significant reduction in *Peptostreptococcus* and *Comamonas* populations and a decrease from 10% to 2% in *Fusobacterium* species (69). In the second study, 15 CRC patients were assigned to receive daily probiotic tablets containing *B. lactis* Bl-04 and *L. acidophilus* NCFM or placebo, from the day of diagnosis until surgery. The intervention resulted in elevated *Clostridiales* spp. and butyrate-producing *Faecalibacterium*, *Roseburia*, and *Eubacterium* bacteria, whereas the CRC-associated *Fusobacterium* and *Peptostreptococcus* species were decreased in the probiotic group, as evidenced by16S rRNA gene sequencing (70).

Metagenomics to study the effect of probiotic-directed microbiome modulation in neurological disorders.

Probiotic administration can affect neurodevelopment, behavior, and mood disorders by modifying the gut microbiome (71–76) (Table 4). It has been demonstrated that *Bifidobacterium breve* CCFM1025 altered the gut microbiota composition of chronically stressed C57BL/6J male mice, after a 5-wk oral administration regimen (73). 16S rRNA sequencing of fecal samples revealed the increased abundance of SCFA-producing *Allobaculum* spp., *Coprococcus* spp., and *Bifidobacterium* spp. Higher stool SCFA concentrations are positively correlated with elevated colonic 5-hydroxytryptophan and 5-hydroxytryptamine hippocampal concentrations, resulting in antidepressant effects (73). Similarly, administration of a probiotic formulation containing *L. plantarum* LP3, *L. rhamnosus* LR5, *B. lactis* BL3, *B. breve* BR3, and *Pediococcus pentosaceus* PP1 for 8 wk to mice, reversed stress-induced microbiota structural changes by stimulating proliferation of *Actinobacteria*, *Cyanobacteria*, *Lactobacillus*, and *Bifidobacterium* populations. These compositional alterations could have supported attenuation of serum corticosterone concentrations and subsequent alleviation of depressive-like manifestations (74). Metagenomics was also employed in a randomized, double-blind, placebo-controlled, multicenter clinical trial to investigate the effect of probiotic capsules consisting of *B. bifidum* BGN4 and *B. longum* BORI to individuals aged >65 y for a 3-mo period (75). Stool samples were collected and their microbial load was examined with 16S rRNA gene sequencing. Significant reductions of *Eubacterium*, *Clostridiales*, *Prevotellaceae*, and *Allisonella* abundances in the gut were recorded. The intervention resulted in favorable outcomes in cognitive function and mental stress (75). Another clinical study indicated that administration of heat-killed *L. gasseri* CP2305 to medical students prior to national examination alleviated stress manifestations and positively influenced their sleep patterns, while it also reversed microbiota changes related to stress. Indeed, the probiotic managed to normalize the abundances of *Bifidobacterium* spp. and *Streptococcus* spp. populations that were disrupted due to stress. Furthermore, the authors postulated that this probiotic could directly signal to the brain via the vagus nerve and stimulate the gut–brain axis (76).

Metagenomics to study the impact of probiotics on host metabolic control.

A disrupted gut microbiome negatively influences host metabolism, which can contribute to the development of metabolic syndrome (77). Metagenomics has revealed probiotic-directed modulation of gut microbiota for the management of metabolic disease (78–81) (Table 4). Amplicon sequencing of feces sampled from HFD mice gavaged with *Bifidobacterium pseudolongum* showed significant

TABLE 4 Metagenomic studies of the effects of probiotic supplementation on the gut microbiota and beneficial health outcomes[1]

Type of study	Treatment	Sample	Omics platform	Microbiota composition changes	Clinical outcome	References
Animal study DSS-treated C57BL/6J mice ($n = 60$)	*Lactobacillus acidophilus* (5×10^8 CFU) *L. rhamnosus* (5×10^8 CFU) *Bifidobacterium lactis* (5×10^8 CFU) Gavage, daily for 1 wk	Feces, rectum mucosa	16S rDNA sequencing	↑Bifidobacteria, ↑*Lachnospiraceae*_NK4A136 ↑*Lachnospiraceae*_UCG-006 ↓*Alistipes*	↓Colonic inflammation	Wang et al. (64)
Animal study IL-10 knockout mice ($n = 12$)	*L. plantarum* LP Onlly (10^9 CFU) Gavage, daily for 4 wks	Feces	Shotgun sequencing WGS	↑*Bacteroides* ↑*Akkermansia muciniphila*	Prevention of gut inflammation	Chen et al. (65)
Single-center, prospective, randomized, controlled study CRC patients ($n = 11$)	*B. longum*, *L. acidophilus*, and *Enterococcus faecalis* (1:1:1) (6×10^7 CFU) Oral intake, daily for 5 d	Colorectal mucosa tissue	16S rRNA sequencing	↓*Fusobacterium* ↓*Peptostreptococcus*	NA	Gao et al. (69)
Prospective randomized study CRC patients ($n = 28$)	*B. lactis* Bl-04 (1.4×10^{10} CFU) *L. acidophilus* NCFM (7×10^9 CFU) and inulin. Oral consumption for 31±28 d	Feces, tumor tissue	16S rDNA sequencing	Fecal samples: ↑*Firmicutes* Tumor microbiota: decrease of *Fusobacterium* and *Peptostreptococcus*	NA	Hibberd et al. (70)
Animal study C57Bl6/J mice with autism spectrum disorder ($n = 10$)	*L. reuteri* MM4-1A (10^8 organisms/mouse/d) Oral administration, daily for 4 wk	Feces	16S rRNA sequencing	↑*L. reuteri*	Restoration of social behavior	Buffington et al. (71)
Animal study C57BL/6J male mice ($n = 10$)	*L. reuteri* ATG-F4 (10^7 CFU/mL) Oral administration, daily for 4 wk	Feces	16S rRNA sequencing	↑*Bacteroidetes* ↑*Bacteroidales* S24–7 ↑*Prevotellaceae*	↑Serum dopamine concentrations	Beck et al. (72)
Animal study Chronically stressed male C57BL/6J mice ($n = 10$)	*B. breve* CCFM1025 (10^8 CFU/10g) oral gavage, daily for 6 wk	Feces	16S rRNA sequencing	↓*Allobaculum* spp. ↓*Bifidobacterium* spp. ↑*Coprococcus* spp. ↑*Oscillospira* spp.	Antidepressant effects	Tian et al. (73)
Animal study	*L. plantarum* LP3, *L. rhamnosus* LR5, *B. lactis* BL3, *B. breve* BR3, and *Pediococcus pentosaceus* PP1 (2×10^8 CFU/mL), oral administration, daily for 8 wk	Feces	16S rRNA sequencing	↑*Bifidobacterium* spp.	Antidepressant effects	Liu et al. (74)

(*Continued*)

TABLE 4 (*Continued*)

Type of study	Treatment	Sample	Omics platform	Microbiota composition changes	Clinical outcome	References
ICR stressed mice ($n = 10$) Randomized, double-blind, and placebo-controlled multicenter trial. Healthy elders (>65 y)	*B. bifidum* BGN4 (10^9 CFU/d)	Feces	16S rRNA sequencing	↓*Eubacterium*, ↓*Clostridiales*	↑Cognitive function	Kim et al. (75)
	B. longum BORI (10^9 CFU/d)			↓*Prevotellaceae*, ↓*Allisonella*		
	Oral intake, daily for 12 wk					
Double-blind, placebo-controlled, parallel-group clinical trial. Healthy students ($n = 29$)	Heat-killed *L. gasseri* CP2305 (10^{10} CFU) per os, 2 tablets daily for 24 wk	Feces	16S rRNA sequencing	↑*Bifidobacterium* spp. ↓*Streptococcus* spp.	Ameliorated sleep disturbance and anxiety	Nishida et al. (76)
Animal study HFD C57BL/6J mice ($n = 7$)	*L. rhamnosus* GG (10^7 CFU), oral administration, daily for 10 wk	Feces	16S rRNA sequencing	↓*Oscillospira* spp.	↓Weight gain ↓Organ weight	Ji et al. (78)
Animal study HFD C57BL/6J mice ($n = 6$)	*B. pseudolongum* (10^{11} CFU), gavage, daily for 6 wk	Feces	16S rRNA sequencing	↑*Butyricimonas*, ↑*Bifidobacterium* ↑*Odoribacter* ↓*Firmicutes/Bacteroidetes* ratio	↓Body mass and visceral fat ↓Plasma triglycerides	Bo et al. (79)
Animal study HFD C57BL/6J mice ($n = 15$)	*Bacillus amyloliquefaciens* SC06 (10^8 CFU), oral consumption, daily for 8 wk	Feces	16S rRNA sequencing	↓*Firmicutes/Bacteroidetes* ratio	↓Subcutaneous fat weight, ↓insulin resistance	Wang et al. (80)
Animal study HFD SPF Kunning mice ($n = 8$)	*L. plantarum* FZU3013 (10^9 CFU/mL), oral consumption, daily for 8 wk	Feces	16S rRNA sequencing	↑*Alistipes*	Potential role in prevention of NAFL and hyperlipidemia	Chen et al. (81)

[1] CFU, colony forming units; CRC, colorectal cancer; DSS, dextran sodium sulfate; HFD, high-fat diet; ICR, Institute of Cancer Research; NA, not available; NAFL, nonalcoholic fatty liver; SPF, specific pathogen free; WGS, whole genome sequencing; ↓, statistically significant downregulation; ↑, statistically significant upregulation.

changes in the structure and function of the gut microbiota. The ratio of *Firmicutes*/*Bacteroidetes* was significantly reduced, whereas *Butyricimonas* and *Bifidobacterium* abundances increased. KEGG analysis for the identification of functional microbiota alterations showed that probiotics modulated pathways involved in lipid, carbohydrate, and amino acid metabolism. These findings were accompanied by the clinical decrease of body mass, visceral fat, and gross energy intake (79). Furthermore, hyperlipidemic HFD mice gavaged with *L. plantarum* FZU3013 had increased populations of *Alistipes,* a genus that contains several SCFA-producing species, and *Ruminococcus*. The administration of *L. plantarum* FZU3013 also minimized the gut population of *Desulfovibrio*, which is positively correlated with obesity and type 2 diabetes (81).

Genomics and comparative genomics of probiotics

The genome of novel potentially probiotic isolates should be fully sequenced and available in public databases to facilitate accurate taxonomic identification, investigation of functional traits, and evaluation of the safety profile (82). The advancement of next-generation sequencing (NGS) technologies has made WGS of probiotic strains more affordable and accessible. Furthermore, the numerous available bioinformatics tools support assembly, annotation, and phylogenetic analysis of the genomic sequences (83, 84). Computational mining of whole genomes is used to construct phylogenetic trees, provide more accurate insights into the evolutionary relations between strains, and facilitate molecular taxonomy. In this light, the genus *Lactobacillus* has recently been reclassified as 25 genera, based on shared physiological and metabolic properties. The new taxonomic classification could enhance our understanding of common probiotic mechanisms of action (85, 86).

Comparative genomics is also employed to investigate the range of phenotypic variability between strains, as well as to identify the entire set of strain-specific genes in their pangenome (the entire set of genes of all strains within a monophyletic group) and to highlight shared properties (87). Moreover, it can elucidate conserved sequences in the genome of probiotics that code for essential cellular functions, and regulatory elements that modulate their expression. Cluster of orthologous genes analysis can classify these findings into distinct categories, such as clusters involved in transcription, metabolism, cell motility, and signal transduction, among others (88). Furthermore, genomic comparison of novel isolates with already characterized probiotics can reveal functional properties, such as adhesion to epithelial cells, autoaggregation, stress response mechanisms, and defense mechanisms, including the presence of virulence factors and resistance to antibiotics (89).

The rapid spread of antibiotic resistance is a major threat to global health. Probiotic bacteria with intrinsic resistance to antibiotics are generally recognized as safe because they present a minimal risk of spreading drug-resistant genes to other more harmful species. However, in probiotic stains with acquired resistance genes, mainly carried on mobile genetic elements, such as plasmids, transposons, and integrons, the possibility of horizontal transmission is considered to be high, thus representing a serious safety issue (90). Indeed, the transfer of a vancomycin resistance gene from enterococci to the probiotic strain *L. acidophilus* has been recorded in vitro and, more importantly, in the gut of mice at high frequencies (91). Assessment of antibiotic resistance of novel probiotic strains is performed phenotypically by the determination of minimal inhibitory concentrations (92), and genotypically by PCR-based techniques and sequencing (93, 94). For the identification and localization of previously undetected antibiotic resistance genes, DNA microarrays, as well as WGS platforms, might also be used (94, 95). Furthermore, multiomics technologies and other advanced molecular tools, such as live imaging, can accurately represent probiotics–microbiome interactions and evaluate the risk of antibiotic resistance gene transfer, as well as risks linked to long-term consumption (96). To date, horizontal gene transfer from probiotics to other bacteria has not been recorded in the human host; however, further research is needed.

Future probiotics and the road to success

Recent breakthroughs in the study of the microbiome, supported by multiomics and systems biology approaches, have highlighted the role of the gut microbiota in the host's health. It has also been demonstrated that the gut microbiota, alongside other host- and sex-specific traits, contributes significantly to the efficacy of probiotic supplementation. Consequently, the idea of personalized, tailor-made probiotics has been developed, setting the stage for a new era of probiotic research (97). Evidently, personalized probiotic supplementation should consider anthropometric and immunological features, as well as the microbiome and genetic fingerprint of the host (**Figure 1**). Indeed, several studies have associated genetic diversity with specific gut microbial populations (98, 99). The observed variation was pinpointed in loci coding for factors involved in host–microbial interactions, such as the pattern recognition receptor and chemokine signaling, and in metabolic pathways. These data could allude to the fact that the genetic makeup of the host could affect probiotic action. Furthermore, a number of studies have shown that the human gut microbiota exerts a host-specific resistance to probiotic colonization. In a recent elegant study, individuals who consumed the commercial probiotic mix Supherb Bio-25 (*B. bifidum*, *L. rhamnosus*, *L. lactis*, *L. casei* ssp. *casei*, *B. breve*, *S. thermophilus*, *B. longum* ssp. *longum*, *L. casei* ssp. *paracasei*, *L. plantarum*, and *B. longum* ssp. *infantis*) exhibited host- and site-specific patterns of colonization. Interestingly, germ-free mice that were conventionalized with fecal microbiota from individuals resistant to probiotic colonization, were less receptive to probiotic supplementation, whereas mice that were fed with fecal microbiota of permissive individuals showed a better response. These findings underline the fundamental role that the gut microbiota plays in the efficiency of probiotic colonization (13). Similarly, it has

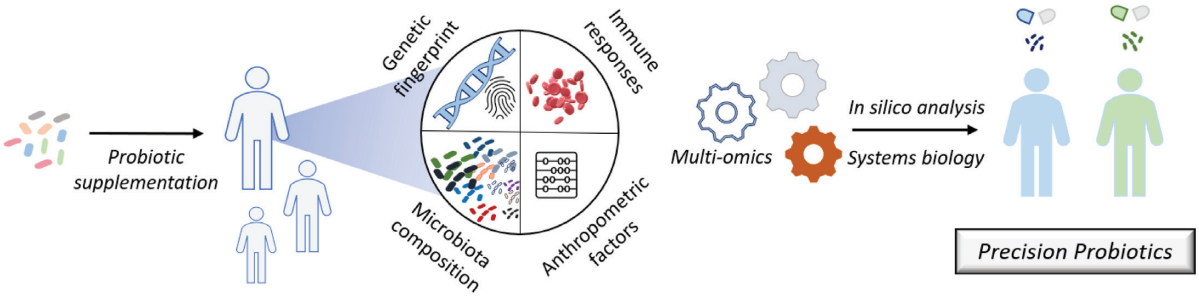

FIGURE 1 The road to precision probiotics. The unique host genetic and microbiota signatures, alongside anthropological and immune parameters, can determine the efficiency of probiotic supplementation. The identification of these factors using high-throughput multiomics analysis and systems biology approaches can set the basis for tailor-made probiotic interventions.

been shown that probiotic resistance to colonization could be associated with the diversity of the residing microbial populations. In this respect, *L. helveticus* MTCC 5463 colonization was more efficient in healthy individuals, who had a higher gut microbiome α-diversity (100). It has also been demonstrated recently that antibiotic treatments can alleviate this innate resistance to probiotic colonization. Indeed, individuals who were treated with wide-spectrum antibiotics prior to supplementation with Supherb Bio-25, were more permissive than naïve individuals (101). Comparative analysis of metagenomic data from permissive and nonpermissive individuals could reveal species that could act as predictive markers for the success of probiotic supplementation. For example, the microbiome of the responders to *L. helveticus* MTCC 5463 supplementation had decreased abundance of *Clostridium* and increased *Eubacterium* populations (100).

Probiotic research needs to gravitate toward the understanding of strain-specific mechanisms of action, by delving deeper into their specific genetic and metabolic signatures. The advancement of the field of comparative genomics has facilitated the prediction of probiotic characters and attributes. Additionally, global transcriptome and proteome analyses are being employed to investigate the production of proteins and other small molecules that implement probiotic action, and posttranslational modifications that can modulate their activity (102). Metabolomic studies have aided in the characterization of produced metabolites, termed "postbiotics." Postbiotics are soluble, bioactive compounds secreted by probiotic microorganisms in their growth medium (103), that can originate from both intracellular and extracellular compartments and can be cell surface proteins, secreted proteins and peptides, exopolysaccharides, teichoic acids, organic acids such as SCFAs, enzymes, neurotransmitters such as γ-aminobutyric acid, or vitamins (104). They can act alone or synergistically and induce immunomodulatory or epigenetic effects in the host, whereas some of them can exert antimicrobial activities against pathogenic bacteria (105). The fractionation of cell-free supernatants, and enzymatic (proteinase K, trypsin, catalase, α-chymotrypsin), heat, and pH treatments, can provide valuable information about their molecular weight and chemical identity. However, only high-throughput analyses can provide definitive answers about the identity and physicochemical properties of these compounds. Such analyses can be carried out in platforms like LC-MS or GC-MS and NMR. Importantly, metabolomics can decipher the context in which these molecules can be produced and secreted. Several studies have shown that probiotics can produce a wide array of metabolites, based on the environmental stimuli that they receive. More specifically, it has been shown that gastrointestinal tract conditions (106), available nutrients (107), as well as proinflammatory microenvironments (108), can determine the production and secretion of certain metabolites. Apart from host-related interactions, bacteria grown in food matrices show different metabolic signatures compared with those cultured in common laboratory media (109). These platforms can also be used to investigate the interactions between different probiotic strains of multispecies supplements, because interbacterial communication can also modulate gene expression and consequent metabolite production. It is known that bacteria utilize an array of language signals, such as quorum sensing, to communicate with other bacteria of the same or different species. The exchanged signals can determine important behaviors such as biofilm formation, production of virulence factors, metabolism, and response to stress (110). These language signals and other metabolites could also determine the probiotic–microbiome interactions. For instance, a recent study supported that lactobacilli contained in the mix Supherb Bio-25 delayed microbiome reconstitution after antibiotic treatments, because they produced uncharacterized antibacterial compound(s) (101). Conclusively, comparative metabolomic studies between different strains of probiotics, or strains grown in different media or simulated host conditions, could be informative about their species- and host-specific actions.

The current trend in probiotic research is the identification of microorganisms with probiotic potential that are not originated from dairy products. These microorganisms, termed "next-generation probiotics" (NGPs), are usually isolated from the gastrointestinal tract of mammals and are solely intended for pharmaceutical use (111). Because they require stringent survival conditions, such as complex growth media and reduced oxygen availability, their propagation in laboratory conditions is quite challenging. The advent of culture-independent platforms has facilitated the study of their probiotic attributes and safety profile in silico. In this context, metagenomics has supported the discovery of novel NGPs, whereas comparative genomics has provided a basis for the characterization of their biological functions, virulence, and antibiotic susceptibility (112). Furthermore, proteomics and metabolomics can be employed for the investigation of temporal and spatial actions on the host and accurately describe their holistic effects. Indeed, several studies have already associated NGPs, such as *Prevotella copri*, *Bacteroides thetaiotaomicron*, *Akkermansia muciniphila*, and *Faecalibacterium prausnitzii*, with favorable health outcomes in intestinal inflammation and metabolic disorders (113). For the commercialization of these strains, it is important to ensure maximum viability during production and shelf life, taking into consideration their unique survival requirements (111). Furthermore, survival during gastric passage should be guaranteed, because these bacteria have evolved to withstand the conditions of the lower, but not the upper, gastrointestinal tract (114). Lastly, it is important to note that appropriate regulatory frameworks should be put in place for the evaluation, characterization, and commercialization of products containing these bacteria (111).

Multiomics analysis has, indeed, broadened our understanding about the global effects of probiotic supplementation. Although these platforms have been extensively used to explore the interaction of probiotics with immune and metabolic signaling, as well as with the residing gut populations, the meaningful interpretation of the generated data remains challenging. The evolving field of systems biology has provided the mathematical and computational tools to create probabilistic models of biological function at the single-cell, tissue, or organ-system scale based on the data derived from these platforms (115). More specifically, systems biology utilizes statistical methods, such as gene set enrichment analysis, and computational units, such as biological networks and dynamic models, to decipher the information flow from gene to transcript, to protein and metabolites. However, the rapid production of experimental data demands more robust tools than mechanistic models. To this aim, artificial intelligence (AI) approaches, such as machine learning, deep learning, and artificial neural networks, can be utilized to manage big numerical datasets. The predictive models produced by AI could be invaluable for precision medicine (115). Systems biology has, indeed, been proven useful in probiotic and microbiome research. More specifically, the compilation of data extracted by high-throughput platforms has aided in the understanding of the genomic and functional properties of probiotics and has supported the strain specificity of their actions (116), whereas systems medicine and microbiome-wide association studies have provided insights about the role of the microbiome in health and disease, paving the way to targeted therapeutics (117). In a representative study, Bisanz et al. (118) characterized the global molecular and cellular events triggered by vaginal probiotic treatments for postmenopausal women (118). Microbial profiling by NGS, microarray analysis of chemokine and cytokine production, and metabolomic GC-MS analysis of the vaginal fluid suggested that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 did induce mild changes that were, however, not reflected in clinical outcomes. The authors postulated that host-specific factors and the small sample size contributed to the lack of statistically significant clinical results and proposed that further studies are needed. Thus, this multifaceted analysis managed to reveal fine molecular changes that would otherwise be overlooked.

Novel technologies that have recently been introduced could complement our understanding about host–microbe interactions. Single-cell multiomics analyses can realize the simultaneous profiling of nucleic acids and proteins at single-cell resolution. Different platforms allow for combined genome and transcriptome sequencing (single-cell genome and transcriptome sequencing), genome, epigenome, and transcriptome sequencing (single-cell triple omics sequencing), or transcriptome and proteome sequencing (RNA expression and protein sequencing) (119). These cutting-edge technologies are highly suitable to study dynamic processes, such as time-dependent host–microbe interactions (120). The 2 major limitations on the implementation of such techniques are cost and data analysis. Data analysis of these platforms includes the integration of a bulk of data generated from different platforms (121, 122), which complicates the meaningful interpretation of the results (123). However, as the use of these technologies and the field of systems biology mature, these obstacles are expected to be overcome.

Conclusions

here is a growing body of evidence that demonstrates the prophylactic and therapeutic potential of probiotics against acute or chronic diseases. Nevertheless, probiotics have not yet been introduced in clinical practice, except for the management of a limited number of gastrointestinal disorders, because several questions related to probiotic production, efficacy, and health benefits remain unanswered. The great variability of probiotic mechanisms of action and host-specific traits has contributed greatly to discrepancies in outcomes presented in preclinical and clinical studies. Clinical protocols differ a lot between probiotic studies, thus meta-analysis often leads to contradictory conclusions. Furthermore, the dynamics of probiotic–probiotic interactions are insufficiently researched; consequently it is difficult to assess the effectiveness of multistrain supplementation because it is unclear whether inhibitory or proliferative relations exist among strains. To this end, multiomics approaches can be applied to systematically characterize and predict host–microbe

and microbe–microbe interactions and evaluate probiotic efficacy. The identification of probiotic genes and the context in which they are expressed, as well as the immense range of metabolites that can be produced by each strain, can contribute to the elucidation of strain-specific actions and interactions with the host and its microbiota. Accordingly, high-throughput analyses of the unique genomic, proteomic, metabolomic, and metagenomic signature of the host can shed light on factors that influence the efficiency of probiotic action. Adding to that, the thorough investigation of NGP and postbiotic attributes can make available an arsenal of bioactive compounds with potential health-promoting effects to people who are allergic to dairy products or to other vulnerable populations, such as the immunocompromised, and are advised not to consume traditional probiotic foods or supplements. In future, routine use of multiomics platforms, single-cell technologies, and the integration of systems biology in probiotic research will contribute to the careful design of tailor-made interventions that would take into consideration species-, host-, and disease-specific factors and hopefully bring probiotic supplementation from bench to bedside.

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