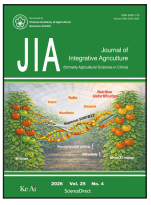
Journal of Integrative Agriculture 2026, 25(4): 1330–1342

Available online at ScienceDirect

Journal of Integrative Agriculture



REVIEW

High-fidelity gut metagenome: A new insight of identification of fuctional probiotics

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Highlights

- It systematically reviews the multiple beneficial effects of probiotics on host health by regulating the structure and metabolic functions of the gut microbiota, clarifies the strain-specific characteristics of their efficacy, and highlights the core value of functional probiotic identification in the fields of human health and livestock farming.
- It comprehensively analyzes the core advantages and inherent limitations of existing functional probiotic identification technologies, including fecal microbiota transplantation (FMT), 16S rRNA sequencing, transcriptome/metabolome analysis, and shotgun sequencing, and identifies the key bottleneck that traditional technologies struggle to break through in strain-level resolution.
- It systematically elaborates on the advances in metagenomic assembly technologies based on next-generation sequencing (NGS), third-generation sequencing (TGS), hybrid sequencing, and Hi-C assisted assembly, with a focus on emphasizing the high-fidelity assembly strategy combining PacBio HiFi and Hi-C, which provides a novel perspective for the precise identification of functional probiotics.

Abstract

Probiotics are considered to exert beneficial effects in humans and animals by modulating the structure and metabolic functions of the gut microbiota. Therefore, the identification of functional probiotics and in-depth exploration of the characteristics and applicability of probiotics are of paramount significance for the development of more effective probiotic products and the formulation of personalized probiotic treatment strategies in the fields of human health and livestock farming. However, due to current limitations in sequencing technologies and considering that microbial communities may encompass closely related lineages, rendering metagenome assembly complex, the generation of complete metagenome-assembled genomes (cMAGs) is hindered. This limitation constrains our comprehensive resolution at the probiotic strain level. In this review, we summarized the effects of probiotics on gut microbiota balance and host health from a functional perspective. The technical methods of functional probiotics identification were summarized from the technical point of view. Furthermore, we introduced methods for microbial metagenome assembly to elucidate the associated progress and advantages and disadvantages of these approaches. Finally, we highlight more advanced metagenomic assembly techniques that may help us assemble high-fidelity intestinal metagenomes, providing powerful tools for the identification of functional probiotics.

Keywords: host health, gut microbiota, probiotics, gut metagenome, metagenomic assembly

Received 7 January 2024; Received in revised form 24 February 2024; Accepted 14 March 2024; Available online 13 May 2024
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doi: 10.1016/j.jia.2024.05.011

1. Introduction

The gut microbiota, often referred to as a "superorganism", constitutes a complex ecosystem comprising a myriad of interacting bacteria, archaea, phages, eukaryotic viruses, and fungi (Lynch *et al.* 2016). It plays a crucial regulatory role in the health and disease of the host, involving various aspects such as diet, disease development, drug metabolism, and immune modulation. Its influence extends to promoting health as well as being associated with the occurrence and maintenance of diverse gastrointestinal and non-gastrointestinal diseases (Xu *et al.* 2013; Lynch *et al.* 2016; Marchesi *et al.* 2016). As reported, the gut microbiota actively participates in the catabolism of dietary fibers, glucose, short-chain fatty acids, branched-chain amino acids, aromatic amino acids, and drugs. Additionally, the gut microbiota is involved in the synthesis pathways of methane, vitamins, and neurotransmitters. And beneficial gut microbiota possess the ability to eliminate exogenous toxins (Fig. 1) (Strozzi and Mogna 2008; Pennisi 2010; Arumugam *et al.* 2011; Haiser *et al.* 2013; Devlin and Fischbach 2015; Wu *et al.* 2020). In addition, the gut microbiota can influence the host in various ways, such as impacting host immune system function (Fulde and Hornef 2014), changing central nervous system (Yano *et al.* 2015), regulation of intestinal endocrine function (Neuman *et al.* 2015), influencing host cell proliferation (Ijssennagger *et al.* 2015), participation in vascular formation (Reinhardt *et al.* 2012), prevention of excessive growth of harmful microorganisms (Kamada *et al.* 2013), and provision of biological energy (Canfora *et al.* 2015) (constituting 5–10% of the host's daily energy requirements). These pathways have a significant impact on the host's psychological, physiological, and immune health (Fig. 1). When there is an imbalance in the gut microbiota, it can lead to a cascade of associated diseases, including gastrointestinal disorders, obesity, type 2 diabetes, cancer, hepatic steatosis, metabolic syndrome, malnutrition, cardiovascular diseases, autoimmune disorders, as well as neurological or psychiatric conditions (Sekirov *et al.* 2010; Lynch *et al.* 2016; Manor *et al.* 2020). Therefore, the gut microbiota is one of the key factors that help regulate host health, and understanding and maintaining the balance of the gut microbiota is essential to promote host health (de Vos *et al.* 2022).

According to the effects of the gut microbiota on the host, it can be categorized into three major groups: beneficial bacteria, neutral bacteria, and pathogenic bacteria (Qiang 2021). Among them, the role of beneficial bacteria in maintaining host health has prompted researchers to identify these beneficial microorganisms in the gut microbiota (Lukic *et al.* 2017). *Bifidobacteria* and *Lactobacilli* are currently the most extensively studied beneficial bacteria in the gastrointestinal tract of humans and other mammals and are referred to as probiotics (Lukic *et al.* 2017). The existence of probiotics was first discovered in the early 20th century, and subsequently, various definitions have been proposed by researchers (Juhi *et al.* 2013; Mackowiak 2013). The Food and Agriculture Organization of the United Nations and the World Health Organization define probiotics as "live microorganisms that, when administered in adequate amounts, confer health benefits to the host". This definition is currently the most widely used, scientifically sound, and widely accepted (Bermudez-Brito *et al.* 2012). Research indicates that probiotics can influence the structure and gene expression of the gut microbiota through various pathways, thereby impacting host health. These mechanisms include the production of antimicrobial substances (such as lactic acid, antimicrobial peptides, and hydrogen peroxide) to inhibit the growth of harmful microorganisms, increase the abundance of beneficial microorganisms, regulate gut

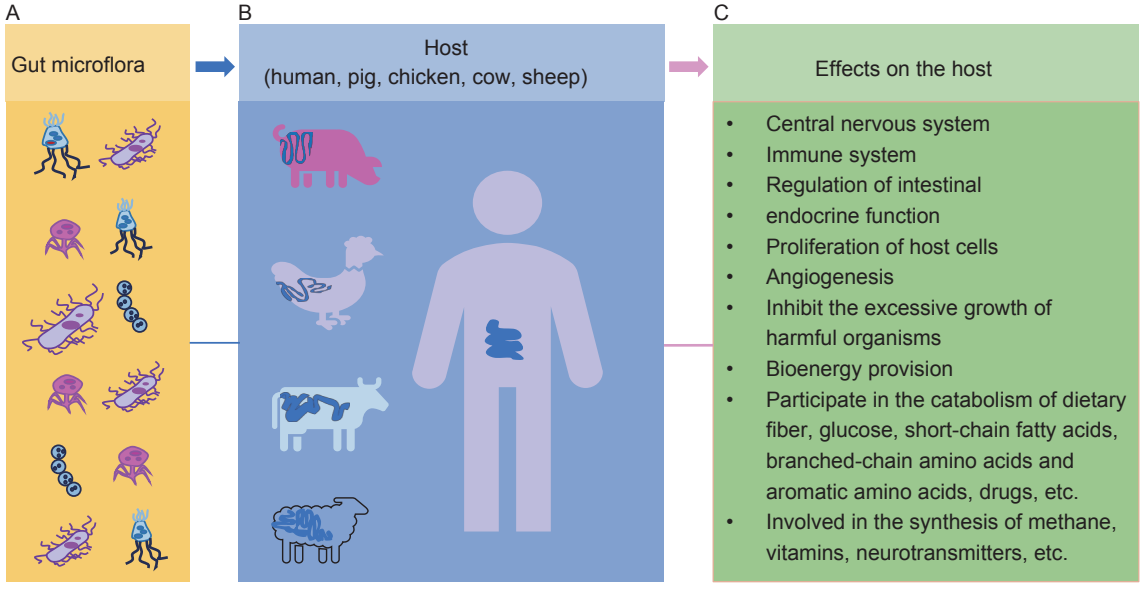


Fig. 1 Effects of gut microbiota on host. A, the gut microbiota in the host. B, different kinds of hosts, including human, pig, chicken, cow and sheep. C, effects of gut microbiota on host.

microbiota metabolism, exert immunomodulatory effects, and promote the barrier function of intestinal epithelial cells. These pathways contribute to maintaining gut health and, consequently, promoting overall host health (Fijan 2014; Martinez *et al.* 2015; Liévin-Le Moal 2016; Lau and Chye 2018). Clinical trials and studies using animal models indicate that probiotics exhibit various characteristics and hold clinical potential for many diseases (Yan and Polk 2011). Moreover, the effects of probiotics are often strain-specific, and the therapeutic potential of different strains varies under specific conditions. All observed effects can only be attributed to the individual strains or combinations of strains tested (Yan and Polk 2011). Table 1 presents the beneficial effects exerted by known probiotic strains through the modulation of the gut microbiota on the host. These effects include enhancing immune function, promoting intestinal function, anti-inflammatory actions, regulating metabolic pathways, reducing antibiotic-associated diarrhea, and lowering the risk of obesity. For example, *L. plantarum* PZ01, *L. salivarius* JM32, and *Pediococcus acidilactici*JH231 have demonstrated effective suppression of the quantity and activity of *Salmonella* in the intestinal tract, thereby enhancing the immunomodulatory effects in broiler chickens (Feng *et al.* 2016). *Sterptococcus thermophilus* KLDS 3.1003 and *Lactobacillus bulgaricus* KLDS 1.0207 have been shown to effectively inhibit the foodborne pathogens *E. coli* ATCC 25922 and *S. aureus* ATCC 25923, thereby enhancing the host's immune response (Evivie *et al.* 2019). *Lactobacillus rhamnosus* GG ATCC 53103 has the ability to facilitate the interactions between key components of the gut microbiota and the host epithelium, thereby further promoting the functionality of the gut microbiota (Eloe-Fadrosh *et al.* 2015). *Akkermansia muciniphila* can improve the intestinal function decline and inflammatory response caused by aging in mice by regulating the structure of intestinal microbiota (Shin *et al.* 2022). The combination of probiotics (*Lactobacillus casei*:*Lactobacillus acidophilus*:and *Bifidobacterium lactis*, in a ratio of 1:1:2) can enrich the glycolysis, gluconeogenesis, and glycerophospholipid metabolism pathways in the ceca of broiler chickens, thereby enhancing the growth performance of broiler chickens (Wang *et al.* 2022). Ingestion of *L. casei* Zhang induced the expression of ABC transporter gene and amino acid metabolism gene in the host on day 14, and activated sugar and SCFA metabolism genes on day 28, thereby promoting host health (Wang *et al.* 2021). *Lactobacillus rhamnosus* GG ATCC 53103 protects against antibiotic-associated diarrhea in children (Esposito *et al.* 2018). *Bifidobacterium breve* B-3 can increase the levels of *bifidobacteria* in the mouse intestine and upregulate the expression of genes associated with fat metabolism and insulin sensitivity, effectively reducing the risk of obesity (Kondo *et al.* 2010). UB0316 (*L. salivarius* UBLS-22 *L. casei* UBLC-42, *L. plantarum* UBLP-40, *L. acidophilus* UBLA-34, *B. breve* UBBr-01, *Bacillus coagulans* Unique IS2 5×10^9 cfu each and fructo-oligosaccharide, 100 mg) can balance the gut microbiota, effectively reducing body weight in overweight/obese adults and improving quality of life (Sudha *et al.* 2019). The administration of *L. plantarum* Dad-13 leads to a reduction in the *Firmicutes* phylum and an increase in the *Bacteroidetes* phylum, particularly the *Prevotella* group, in the human intestine. This subsequently effectively reduces body weight in overweight/obese adults (Rahayu *et al.* 2021). In addition to this, probiotics may have other health-promoting effects (Camille *et al.* 2021). For example, accelerated healing of infected wounds (El-Ghazely *et al.* 2016; Mohseni *et al.* 2018; Sun *et al.* 2023), prevention of neonatal sepsis (Panigrahi *et al.* 2017), anti-infective effects (Zhao *et al.* 2019), antiviral effects (Seo *et al.* 2012), and antioxidant effects (Lamari *et al.* 2016; Shakibaie *et al.* 2017).

In summary, probiotics play a crucial role in maintaining gut health and promoting host well-being. However, as mentioned earlier, the efficacy of probiotics is strain-specific, and different strains may have distinct mechanisms of action and clinical applications. Therefore, the identification of functional probiotics, in-depth exploration of their characteristics and applicability, is of significant importance for the development of more effective probiotic products and the formulation of personalized probiotic treatment strategies

Table 1 Beneficial effects of probiotic bacteria

Properties	Probiotic bacteria strains[1)]	References
Immunomodulatory effect	*L. plantarum* PZ01	Feng *et al.* (2016)
	L. salivarius JM32	Feng *et al.* (2016)
	P. acidilactici JH231	Feng *et al.* (2016)
	S. thermophilus KLDS 3.1003 and *L. bulgari*cus KLDS 1.0207	Evivie *et al.* (2019)
Promote intestinal function	*L. rhamnosu*s GG ATCC 53103	Eloe-Fadrosh *et al.* (2015)
Anti-inflammatory effect	*A. muciniphila*	Shin *et al.* (2022)
Regulatory metabolic pathway	*L. casei*, *L. acidophilus*, and *B. lactis*, in a ratio of 1:1:2 *L. casei* Zhang	Wang *et al.* (2022)
		Wang *et al.* (2021)
Prevention of antibiotic-associated diarrhea in children	*L. rhamnosus* GG ATCC 53103	Esposito *et al.* (2018)
Helping overweight and obese people lose weight	UB0316	Sudha *et al.* (2019)
	B. breve B-3	Kondo *et al.* (2010)
	L. plantarum Dad-13	Rahayu *et al.* (2021)

[1)] UB0316 is a compound probiotic that includes *Lactobacillus salivarius* UBLS-22, *L. casei* UBLC-42, *L. plantarum* UBLP-40, *L. acidophilus* UBLA-34, *Bifidobacteria breve* UBBr-01, *Bacillus coagulans* Unique IS2.

in the fields of human health and livestock farming.

2. Research progress on identification methods of functional probiotics

Currently, the techniques employed for the identification of functional probiotics both domestically and internationally primarily include fecal microbiota transplantation (FMT), 16S rRNA analysis, transcriptome analysis, metabolome analysis, and shotgun sequencing analysis.

2.1. FMT

FMT is a method of directly altering the recipient's gut microbiota to normalize its composition and obtain therapeutic benefits, that is, by performing FMT through a series of active symbiotic bacteria extracted from healthy adults, if these microbial combinations can lead to positive effects, They can be thought of as a mixture of multiple probiotics (Wang *et al.* 2019). Mcnulty *et al.* (2011) found that when fermented *Lactobacillus* microbiome was added, the microbiome of mice with a human gut microbiome model showed significant changes in the expression of enzymes involved in multiple metabolic pathways, especially enzymes related to carbohydrate metabolism. The findings can provide some guidance for the development of more effective probiotic preparations. In another randomized clinical trial, rectal infusion of *Lactobacillus reuteri* ATCC 55730 into the intestines of patients with ulcerative colitis was found to reduce the production of mucosal inflammatory cytokines (Oliva *et al.* 2012). FMT can comprehensively restore a healthy gut microbiota ecosystem and be utilized for identifying functional probiotics. However, due to concerns such as the risk of disease transmission, patient acceptance, adverse outcomes, and uncertain effects on the recipient's immune system, clinicians and researchers are compelled to seek alternative effective therapies to replace FMT (Yeh and Morowitz 2018; Wang *et al.* 2019).

2.2. Microbial 16S rRNA (16S rDNA) sequencing

Microbial 16S rRNA sequencing is mainly to extract the whole DNA of the microorganism, and then use the designed primer for 16S rRNA (16S rDNA gene) to amplify the sequence of the V4 region of 16S rRNA specific to each microorganism. The sequence information was then obtained by Sanger sequencing or Illumina NGS for comparison. The advantages of this method are quick and simple operation and low cost. It can be used for targeted studies of known flora in the microbiome (Frese *et al.* 2015). Angelescu *et al.* (2019) based on (GTG)_5-PCR fingerprinting and 16S rRNA gene sequencing, identified lactic acid bacteria (LAB) at the species level in traditional fermented beverages such as water kefir and braga (a Romanian fermented beverage made of cereals). The strains selected from these beverages also exhibited probiotic potential. Shin *et al.* (2022) described how oral administration of *A. muciniphila* probiotics during aging and four regenerative processes restored intestinal function and reduced age-related inflammatory changes in mice through 16s rRNA sequencing and metagenomic sequencing datasets. The authors believe that this dataset provides an understanding of the relationship between aging and the gut microbiome and will pave the way for the development of therapeutic strategies. Liu *et al.* (2014) utilized 16S rDNA sequencing technology to investigate the significant impact of oral administration of the probiotic *L. fermentum* I5007 on altering the composition and diversity of the intestinal microbiota in piglets. This intervention was found to promote the development of the piglet intestinal tract, increase intestinal surface area, and enhance the number of intestinal glands. Ren *et al.* (2023) conducted a 16S rRNA sequencing analysis to investigate the changes in the composition and structure of the mouse intestinal microbiota after a 14-d treatment with *L. acidophilus*, *L. plantarum*, *Bacillus subtilis*, and *Enterococcus faecalis*. The findings provide a solid foundation for the theoretical research on the preparation of probiotics and their targets. Although 16S rRNA sequencing can analyze the gut microbiota, at a relatively low cost, its disadvantage is that it can only be accurate to the level of the genus and above the genus, and can not distinguish the species and below the species level. Moreover, only the 16S rRNA sequences of each microorganism in the microbial community were obtained, and the complete genomic information of the microorganisms could not be acquired. The identification in this method relies on information from known microbial databases, and it can only align with a limited number of known microorganisms. It cannot identify unknown microorganisms within the microbial community, thus impeding the development of microbial genomics (Janda and Abbott 2007).

2.3. Transcriptome analysis

The transcriptome is the collection of all RNA transcripts in an individual or cell population, both coding and non-coding. Transcriptome analysis is employed to gain in-depth insights into processes such as cell differentiation, carcinogenesis, transcriptional regulation, and the discovery of biomarkers (Wang *et al.* 2009). Eloe-Fadrosh *et al.* (2015) used transcriptomic analysis to describe how the probiotic bacterium *L. rhamnosus* GG ATCC 53103 promotes interactions between key components of the human gut microbiome and the host epithelium. Specifically, transcriptomic analysis revealed that the expression of the genome of *L. rhamnosus* GG ATCC 53103 occurs only during the consumption of the probiotic. Additionally, analysis of the host's transcriptome found that during the consumption of the probiotic, there was an increase in the expression of genes involved in adhesion and a large number of genes involved in bacterial motility. These results provide evidence for the discrete, transient functional effects exhibited by specific individual probiotics (Eloe-Fadrosh *et al.* 2015). Wang *et al.* (2021) utilized metagenomic and metatranscriptomic techniques to reveal the *in vivo* expression patterns of *L. casei* Zhang. Initial analyses of the transcriptomes of both the probiotic *L. casei* Zhang and the resident gut microbiota indicated that the intake of the probiotic does not alter the overall composition of the resident gut microbiome, and the transcription of ingested *L. casei* Zhang is different from its *in vitro* transcription patterns. Furthermore, analyses of the resident gut microbiota transcriptome showed significant

differences in transcription patterns on the 14th and 28th days of intake, with ABC transporter genes and amino acid metabolism genes being induced on the 14th day, and genes related to sugar and short-chain fatty acid (SCFA) metabolism being activated by the 28th day. This study suggests that ingested probiotics must alter their transcription patterns to survive and adapt within the human gut (Wang *et al.* 2021). Through transcriptome technology, we can not only gain a deep understanding of the genomic functional elements and molecular components of probiotics but also unravel biological processes and mechanisms of disease occurrence (Wang *et al.* 2009; Costa *et al.* 2010). However, transcriptome analysis typically generates a vast amount of data, including extensive gene expression data and bioinformatics analysis results. Processing and interpreting this data require specialized bioinformatics skills and computational resources, which can be a challenge for non-experts. Moreover, transcriptome data typically necessitates gene annotation to determine which genes are expressed under different conditions. However, gene annotation still has some limitations, such as unknown gene functions, transcriptome variations, and distinguishing between gene families, among others (Wang *et al.* 2009). These may limit the interpretation and understanding of transcriptome data.

2.4. Metabolomic analysis

Metabolomics is an analytical approach that studies the metabolites present in biological fluids, cells, and tissues. It is often used to identify potential biomarkers (Johnson *et al.* 2016). In the context of metabolomic techniques, measuring the concentration and types of metabolites produced by the gut microbiota can help reveal the molecular mechanisms through which probiotics regulate the metabolism of the gut microbiota. Westfall *et al.* (2021) introduced an artificial model of the human gastrointestinal tract called ABIOME (Artificial Bioreactor Imitating Microbiome Environment) and employed machine learning tools to predict the metabolic activity of probiotic formulations. The results indicated that probiotics might contribute to alleviating inflammatory responses Westfall *et al.* (2021). Wang *et al.* (2022) utilized a combined transcriptomic and metabolomic approach, revealing the regulatory effects of probiotics on cecal metabolism in broiler chickens. This study provides important clues for exploring the mechanisms of probiotic action. Metabolomic analysis can directly detect metabolites produced by microorganisms, providing information about microbial functionality. However, metabolomic analysis faces challenges in accurately annotating many metabolites, leading to a susceptibility to false positives. Compared to using metabolomics alone, integrating metabolomics with other omics technologies maximizes the advantages of each technique, allowing the discovery of unknown yet biologically significant metabolites (Tokarz *et al.* 2017).

2.5. Shotgun sequencing technology

The shotgun sequencing method involves sequencing and exploring the genomes of microbial communities in specific environmental samples (especially those diverse and difficult-to-culture microorganisms). This approach aims to analyze the genomic composition and functional genes of microbial populations, interpret the diversity and abundance of microbial communities, and discover and study new genes with specific functions. Li *et al.* (2016) investigated the control effect of probiotic feeding on liver cancer and its mechanisms of inhibiting tumor progression through a mouse model and Shotgun metagenomic sequencing. The relevant research results highlight the remarkable potential of probiotics in extraintestinal cancers. Although shotgun metagenomic sequencing technology has been widely employed in microbial metagenome assembly, microbial community identification, and related studies on structural reconstruction, it relies on NGS. The substantial amount of redundant sequences generated as short fragments cannot be classified at the species and strain levels (Bickhart *et al.* 2022).

2.6. Technical limitations

The limitations of the aforementioned techniques result in the wastage of sequencing data resources and the loss of genomic information for many strains. Addressing this issue is particularly crucial for obtaining the genomic information of unknown strains and strain identification. It can also aid in conducting more in-depth and comprehensive research on the impact of probiotics on the gut microbiota and host health. Therefore, we can consider approaching from a metagenomic assembly perspective to provide a novel approach to complete metagenomic assembly, enabling a more thorough and comprehensive study of probiotics and gut microbiota.

3. High-fidelity gut metagenome assembly and annotation would support a future guidance of studying probiotics impact on gut microbiome

Meta-genome assembly involves the analysis and assembly of metagenomic sequencing data from microbial communities to obtain the complete genome of individual microorganisms. This process is of crucial significance in microbial community studies, including research on probiotics, as it provides valuable guidance by revealing the full genomic content of microbial individuals (Yang *et al.* 2021). During the meta-genome assembly process, diverse sequencing technologies and platforms can be employed to carry out the sequencing procedure., such as second-generation sequencing (next generation sequencing, NGS), third-generation sequencing (TGS) and Hi-C assistance, Subsequently, specific assembly algorithms and software are employed to process and assemble the sequencing data. Fig. 2 is a fishbone diagram of the metagenomic assembly analysis process. This work faces several challenges, including sample complexity, species abundance imbalance, the presence of duplicate sequences, and horizontal gene transfer. To overcome these challenges, researchers are dedicated to developing and improving sequencing technologies, enhancing assembly algorithms, and constructing new analytical tools. These efforts aim to enhance the quality and accuracy of meta-genome assembly.

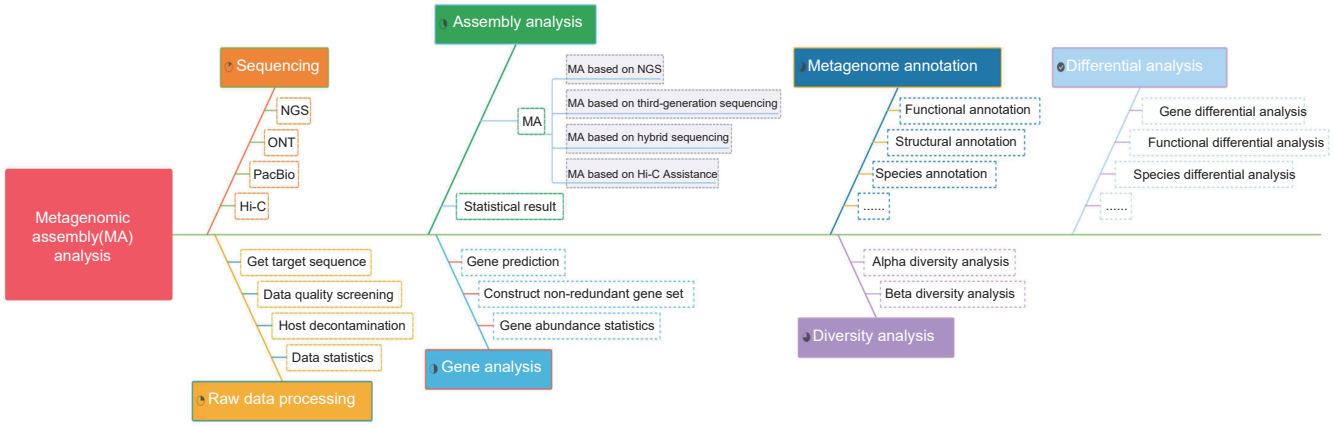


Fig. 2 Metagenome assembly analysis workflow. Metagenomic assembly analysis includes quality control processing of the original data from off-machine sequencing to screen out high-quality sequences for splicing and assembly. According to different sequencing technology, the appropriate assembly method was selected. This is followed by genetic analysis, including steps such as gene prediction and construction of non-redundant gene sets. The non-redundant gene sets were annotated at species and function levels, and diversity and difference analysis were carried out.

3.1. Metagenome assembly methods based on NGS

In the beginning, metagenome assembly based on NGS technology gained widespread applications. For instance, in 2022, a research team from Inner Mongolia University, China conducted a study on the restoration of antibiotic-disrupted gut microbiota using *L. reuteri* strain Probio-M9 and its therapeutic effects in tumor-bearing mice undergoing immune checkpoint blockade (ICB) therapy. The researchers performed metagenomic analysis using NGS data and found that Probio-M9, in combination with ICB therapy, significantly suppressed tumor growth compared to the group without probiotic treatment. Moreover, this synergistic effect effectively restored the antibiotic-disrupted fecal microbiota. The researchers emphasized the significance of Probio-M9 in investigating collaborative tumor therapy through microbial intervention and provided evidence affirming the safety of probiotics in adjuvant ICB therapy (Gao *et al.* 2021). In a research paper published in 2022 by Beck *et al.* (2022) researchers conducted long-term metagenomic analysis on fecal samples from preterm infants who were either exposed to or not exposed to probiotic products. They found that probiotic products exerted strain-specific effects that influenced the development of the gut microbiota in preterm infants and had important implications for host-microbe interactions. These research findings contribute to our comprehensive understanding of the impact of probiotics on the host gut microbiota and demonstrate that short-read sequencing can be used to characterize complex microbial communities. In another study, researchers investigated the role of probiotics in bioremediation and analyzed the dynamic changes of the gut microbiota during intestinal repair processes and their impact on host metabolism. Using 16S rRNA amplicon sequencing, microbial metagenomics analysis, and metabolomics analysis, the authors found that probiotics aided in the reconstruction of the gut microbiota, and revealed that gut microbiota and protective effect of GR-1 on the antioxidative role of the intestine against copper (Feng Y *et al.* 2022). While NGS technology offers advantages such as high-quality sequencing, high accuracy, and low cost, one limitation is that the complexity of metagenomic datasets poses challenges for many assembly algorithms and software, leading to slow computation and significant resource requirements (Table 2). In this context, the introduction of Minia software as a short sequence assembly tool based on the de Bruijn graph principle has gained attention for its high speed (El-Baky *et al.* 2020). Compared to previous tools like ABySS (Birol *et al.* 2009) and SOAPdenovo2 (Luo *et al.* 2012), Minia not only achieve faster assembly but also consumes fewer computational resources, making it an efficient tool in terms of memory resource utilization. Another highly efficient metagenome assembly tool is MEGAHIT, developed by the University of Hong Kong and BGI Genomics (HKU-BGI). This tool is based on the k-mer iterative De-Bruijn graph method and utilizes NGS *de novo* assembly algorithms, allowing for efficient time and cost savings. It is particularly suitable for handling complex environmental samples and large, complex datasets, such as those encountered in the analysis of biological gut environments (Dinghua *et al.* 2015). The following Table 2 lists mainstream assembly methods based on NGS data. Despite the great development achieved, due to persisting challenges in metagenomic assembly with NGS, such as the presence of relatively short sequencing fragments and excessively fragmented results, these limitations can compromise the reliability of probiotic-related or other studies. Therefore, it is imperative to enhance the accuracy and efficiency of assembly software or methods to positively impact research outcomes.

3.2. Metagenome assembly methods based on TGS

Due to certain limitations in NGS-based metagenomic sequencing and assembly methods, including poor genome assembly quality, fragmented assemblies, limited identification of populations, and incomplete analysis of

Table 2 Metagenome assembly methods based on next-generation sequencing (NGS)[1)]

Assembly method	Applicable sequencing technology	Principle	Applicable scenarios	References
MEGAHIT	NGS	De Bruijn graph-based	Suitable for multi-sample co-assembly and handling complex environmental samples and large complex datasets	Dinghua *et al.* (2015)
Minia	NGS	De Bruijn graph-based	Suitable for low memory requirements, fast speed, and similar accuracy to other tools	El-Baky *et al.* (2020)
SOAPdenovo2	NGS	De Bruijn graph-based	Suitable for large plant and animal genomes	Luo *et al.* (2012)
IDBA-UD	NGS	De Bruijn graph-based	Suitable for handling data sets with highly uneven depth, capable of accurately reconstructing longer overlaps	Peng *et al.* (2012)
MetaSPAdes	NGS	De Bruijn graph-based	High accuracy, suitable for single-sample assembly	Nurk *et al.* (2017)
ABySS	NGS	Bloom filter-based	Capable of assembling large genomes on a single machine, suitable for large genome assembly	Birol *et al.* (2009)

[1)] The table presents several mainstream metagenome assembly methods based on NGS technology and provides an overview of their underlying principles and applicable scenarios.

core genomic functions. Moreover, there are challenges in achieving comprehensive assembly of bacterial genomes and in distinguishing microbial members with close phylogenetic relationships but different lineages. These limitations and challenges have driven the demand for complete metagenome assembly. With advancements in sequencing technology, the application of long-read sequencing techniques has greatly enhanced the richness of metagenomic and microbial genomic information mining. As a result, significant advancements have been made in pertinent research on the impact of probiotics on gut microbiota, unveiling deeper insights into the mechanisms through which probiotics exert their effects on the gut microbiota. For example, Suzuki *et al.* (2019) utilized nanopore long-read sequencing technology and discovered a higher number of mobile genetic elements in the human gut microbiome genomes compared to existing data in public databases. They also identified widespread plasmid distribution from *Bacteroides* species and elucidated the functional genes clustered on mobile genetic elements. Recently, Kruasuwan *et al.* (2023) conducted a study evaluating the quality and safety of animal probiotic products with long-read sequencing data and found that metatomic data was more helpful in recovering high-quality metatomic assembly genomes to explore bacterial and yeast populations, antimicrobial resistance genes, and functional genes in detail. Compared to short fragment sequence assembly in NGS, the use of long-read sequencing technologies (such as TGS) can significantly improve the quality and accuracy of genome assembly (Zhou *et al.* 2021). In this context, numerous assembly software and methods tailored for TGS have emerged successively (see the Table 3 below for mainstream assembly software based on TGS). Among them, Chin *et al.* (2013) elucidated a novel method called Hierarchical Genome Assembly Process (HGAP) for genome assembly using PacBio long-read sequencing data. This method employs a hierarchical assembly strategy that involves multiple rounds of assembly and correction to eliminate errors and contaminants from the sequencing data, resulting in high-quality, clean, and complete genome sequences. The authors validated the effectiveness of the HGAP method using genome data from various microorganisms, including *E. coli* O104:H4 strain, *Yersinia pestis* strain, and *Staphylococcus aureus* strain. Subsequently, in 2018, the assembly software called miniasm was introduced. This software is capable of assembling data from both the PacBio and Oxford Nanopore Technologies (ONT) platforms. Based on the Overlap-Layout-Consensus principle, miniasm is an ultra-fast *de novo* assembly software designed specifically for handling long-read data with high noise levels (Michael *et al.* 2018). In 2020, a software called metaFlye was introduced for the assembly of TGS data. The authors evaluated metaFlye using simulated and real microbial communities and found that the assembly produced by metaFlye exhibited excellent sequence integrity and outperformed other assembly software such as FALCON (Moritz *et al.* 2019), miniasm (Michael *et al.* 2018), and OPERA-MS (Bertrand *et al.* 2019) in terms of performance. The authors also utilized metaFlye to assemble the microbiome of sheep. In this case study, metaFlye successfully assembled 63 contigs representing nearly complete bacterial genomes (Kolmogorov *et al.* 2020). In 2022, the Li Heng Research Group at Harvard Medical School developed a third-generation HiFi metagenome assembly software called hifiasm-meta. In simulated data, hifiasm-meta demonstrated superior strain-level resolution compared to metaFlye, and in real data, hifiasm-meta was able to assemble a larger number of mega-sized and circular contigs compared to other software. The researchers used hifiasm-meta to process a large-scale sheepB dataset and obtained 438 near-complete metagenome-assembled genomes and 245 circular contigs, demonstrating the advantage of hifiasm-meta in assembling high-quality circular metagenomic assembly genome (Feng X *et al.* 2022).

However, despite the advantages of long-read sequencing and real-time sequence acquisition, TGS especially ultralong ONT currently has a higher random error rate, requiring repetitive sequencing for error correction. Although PacBio HiFi is more accurate than ONT and NGS, PacBio is more expensive and also produces relatively little valid data and shorter fragments. This issue significantly increases sequencing costs and adds challenges to metagenome assembly.

3.3. Metagenome assembly methods based on hybrid sequencing

Due to the advantages and limitations of NGS and TGS

Table 3 Macrogenome assembly methods based on third-generation sequencing (TGS)[1)]

Assembly method	Applicable sequencing technology	Principle	Applicable scenarios	Reference
HGAP	PacBio	Overlap-layout-consensus	Mainly used for small genomes, such as common bacteria and microbial species	Chin *et al.* (2013)
FALCON	PacBio	Overlap-layout-consensus	Mainly used for relatively larger animal and plant genome assemblies	Moritz *et al.* (2019)
miniasm	PacBio, ONT	Overlap-layout-consensus	Fast assembly speed, suitable for handling noisy long-read data	Michael *et al.* (2018)
metaFlye	PacBio	De Bruijn graph-based	Suitable for assembly of single genomes	Kolmogorov *et al.* (2020)
hifiasm-meta	PacBio	Read overlap-based algorithm	Assembly software developed for PacBio's HiFi metagenomic data, provides better assembly quality and speed	Feng X *et al.* (2022)
Shasta	ONT	Overlap-layout-consensus	Suitable for continuous assembly of complex regions and significantly reduces genome assembly time	Shafin *et al.* (2020)
HiCanu	PacBio, ONT	Overlap-layout-consensus	Handles complex fragments, repetitive sequences, and large data samples	Nurk *et al.* (2020)

[1)] Table 3 provides an overview of several mainstream macrogenome assembly methods based on TGS technologies. It includes their principles and applicable scenarios. In this table, ONT represents Oxford Nanopore Technologies, and PacBio represents Pacific Biosciences, both of which belong to TGS technologies.

technologies, researchers have combined these two approaches for macrogenome assembly analysis to fully leverage their respective strengths (Zhou *et al.* 2021). On 3/14/2020, a team led by Lu Zhang from Hong Kong Baptist University, China published a recent study. The study utilized 32 macrogenome sequencing datasets generated by different sequencing technologies, including short-read sequencing data from mainstream platforms like Illumina and BGISEQ, long-read sequencing data from 10× Genomics, and long-read sequencing data from PacBio and Oxford Nanopore. The research team conducted a comprehensive evaluation and benchmark testing of 19 commonly used assembly tools on simulated datasets as well as macrogenome sequencing datasets from simulated microbial communities or the human gut microbiome. Their research findings indicated that by adopting hybrid assembly tools and combining short-read and long-read sequencing data for heterozygous assembly, it is possible to improve overall assembly length and approach near-cMAGs numbers. This approach holds tremendous potential (Zhang *et al.* 2023). Another example is a research team from Stanford University School of Medicine, which successfully assembled 20 circular genome contigs from 13 human fecal samples using long-read assembly, short-read correction, and error detection with subsequent gene circularization (Moss *et al.* 2020). Subsequently, the research team at Inner Mongolia Agricultural University used three sequencing and assembly strategies to study the benefits of probiotic yogurt: assembly using only third-generation sequences, second-third-generation hybrid assembly, and assembly using only second-generation sequences. The assembly results show that two-third generation hybrid assembly is a powerful strategy to assemble circular and more accurate and complete genomes directly from the complex human gut metagenomic database, and from these sequencing data, the research team successfully assembled 802 cMAGs. This study provides further evidence of the benefits of consuming probiotic yoghurt for maintaining human gut health, and the active probiotics in probiotic yoghurt can maintain the healthy state of the human gut by improving the balance of gut microflora (Jin *et al.* 2023).

In the field of hybrid assembly algorithm and software development, SPAdes was introduced in 2012 as a NGS assembly software suitable for small genomes such as bacteria and fungi (Bankevich *et al.* 2012). In recent years, SPAdes has also introduced a feature called hybrid SPAdes, which is capable of assembling both second- and third-generation mixed sequencing data (Antipov *et al.* 2016). Compared to other assembly software, SPAdes has several advantages. Firstly, it has low hardware requirements and incorporates error correction functions to improve assembly accuracy, making it suitable for assembling mixed second-generation and TGS data. Secondly, SPAdes is easy to install and use, providing researchers with convenience and flexibility in genome assembly. In 2019, the research group led by Niranjan Nagarajan at the Genome Institute of Singapore released a hybrid assembly software called OPERA-MS. OPERA-MS adopts a fragment assembly strategy that integrates metagenome clustering and precise scaffolding algorithms. Based on virtual gut microbiota and artificial community sequencing data, researchers assembled near-complete metagenomes using only 9× long-read coverage depth, which is close to the most complete metagenomes currently available (Bertrand *et al.* 2019).

In summary, hybrid assembly methods based on mixed sequencing technologies allow us to leverage the high accuracy and coverage depth of NGS and the long-read lengths of TGS. This enables us to obtain more accurate and complete metagenome assembly results, thereby improving our ability to analyze complex microbial communities. These assembly software and hybrid sequencing technologies provide robust support for our understanding of the mechanisms by which probiotics exert their effects on host organisms.

3.4. TGS and NGS hybrid sequencing with Hi-C assistance would provide the perfect solution for the assembly of complete metagenomes

The Hi-C-assisted genome assembly method is a technique that utilizes NGS short reads for sequencing quality correction and employs Hi-C for assisted genome assembly. Hi-C technology captures the spatial interactions between different regions within the genome, thereby aiding in the exploration of the three-dimensional structure of the genome. This approach would hold significant value in metagenomic research as it helps in assembling high-quality genomes.

For instance, Timothy P. L. Smith and Pavel A. Pevzner, among others, conducted a metagenomic sequencing study on sheep fecal samples using HiFi sequencing technology combined with Hi-C binning. They successfully identified 428 metagenome-assembled genomes with completeness exceeding 90%. To address the challenge of highly similar strains, the researchers utilized the metaFlye assembly algorithm and the MAGPhase algorithm. Additionally, leveraging Hi-C data, they identified 1,400 complete and 350 partially complete biosynthetic gene clusters, as well as 424 potential host-virus (host-plasmid) associations. Using classification with GTDB-TK, these HiFi cMAGs were assigned to 197 different genera and 15 different phyla. These findings demonstrate that with the support of advanced algorithms and the assistance of Hi-C technology, HiFi long-read sequencing can effectively differentiate similar microbes and provide a more comprehensive microbial community classification (Bickhart *et al.* 2022). Kim *et al.* (2022) successfully assembled complete circularized prokaryotic genomes of the human gut using SMRT HiFi and Hi-C data. To obtain as many circularized contigs as possible, the research team used three assembly software: metaFlye, HiCanu, and hifiasm-meta for initial assembly of the sequencing data. Based on the assembly results, the authors recommended further assembly using hifiasm-meta. To obtain high-quality assembly results, the researchers utilized the Human Reference Gut Microbiome (HRGM) database and designed a filtering method based on the consistency between sequences of the same species' genomes. Ultimately, the research team obtained 102 cMAGs. Evaluating the completeness of the cMAGs, these genomes can serve as a complete reference for prokaryotic microbes in the human gut. Furthermore, after classification and annotation using the GTDB, the researchers found that by combining HiFi with the hifiasm-meta software, they successfully assembled cMAGs from both cultivable and uncultivable microbes. The results of this study demonstrate the significance of using SMRT HiFi and Hi-C data, along with appropriate assembly, annotation software, and analysis workflows, to obtain high-quality complete metagenome-assembled genomes, which is crucial for studying the human gut microbiome (Kim *et al.* 2022).

In recent years, compared to traditional Hi-C data, *in situ* Hi-C (*in situ* chromatin conformation capture) technology has become a widely used method in assisting genome assembly, due to its advantages such as a more natural chromatin environment, higher contact frequency, and lower background noise. This is in contrast to the limitations of traditional Hi-C data, which include significant technical noise, a low proportion of effective data, and a high number of false-positive interactions(Kong *et al.* 2020). In our opinion, building upon the existing macrogenome assembly methods, it is possible to develop an efficient and user-friendly high-fidelity methods, such as gut *in situ* Hi-C for gut microbiome assembly, which can be used to study the impact of probiotics on the gut microbiome community. Thus, PacBio HiFi sequencing and *in situ* Hi-C sequencing can be performed on the host gut microbiome before and after probiotic supplementation (e.g., over a period of 3 mon). And utilize data analysis software such as HiCUP, HiClib, or HiC-Pro for data processing. HiC-Pro offers a comprehensive and mature analysis workflow with good quality control (Servant *et al.* 2015), making it recommended for quality control purposes. We can further use hifiasm-meta or hifiasm software to perform initial genome assembly on PacBio HiFi data. Subsequently, we can combine Hi-C assemble software such as LACHESIS (Burton *et al.* 2013; Putnam *et al.* 2016; Dudchenko *et al.* 2017; Ghurye *et al.* 2019), to optimize and assemble the metagenome generated by hifiasm from the scaffold to chromosome level, in order to obtain high-quality metagenome-assembled genomes.

Next, we can use tools such as CTDB and NCBI RefSeq for taxonomic annotation and utilize PacBio's MAGPhase program to distinguish highly similar bacterial strains within individual samples. Additionally, in HiFi assembly, we can identify biosynthetic gene clusters and perform functional annotation of the metagenome using antiSMASH to obtain more accurate and comprehensive information. Complete metagenome annotation encompasses several aspects. Firstly, functional annotation involves comparing predicted gene sequences against known functional databases to determine the function of each encoded protein. Common gene prediction tools include Prodigal, GeneMark, and Glimmer. Common functional databases include KEGG, EggNOG, GO, COG, and Nr. Similarity search tools such as BLAST, DIAMOND, and HMMER can be used for functional annotation. Secondly, structural annotation involves identifying and annotating repetitive sequences and transposable elements to determine structural features such as introns, exons, and intergenic regions within sequencing fragments. Tools and methods like RepeatMasker and RepeatModeler can be used for structural annotation analysis. Lastly, species annotation involves identifying and annotating the sequences in the metagenome data to determine which microbial species they originate from. The tool Kraken is a fast metagenomic DNA sequence species annotation software based on k-mer exact alignment. It uses the most lowest common ancestor voting results for species annotation, enabling accurate and rapid annotation (Wood *et al.* 2014).

In addition, we can screen strains with potential probiotic effects by predicting the presence of gene clusters that may produce beneficial metabolites (such as organic acids, enzymes, exopolysaccharides, etc.) to provide more effective strategies for future disease prevention and treatment.

4. Perspective and outlook

Probiotics play an important role in maintaining intestinal and host health by regulating the structure and metabolic function of gut microbiota. We have realized the great potential of probiotics in gut microbiota balance and host health, and summarized relevant research techniques and methods. These techniques and methods are of great significance for mining new resources of probiotics and providing more effective strategies for the prevention and treatment of diseases in the future. By using advanced technologies and methods, we are able to gain insight into the composition and function of the gut microbiota. In recent years, with the continuous development of microbiome technology and molecular biology technology, the methods for identifying bacteria types and probiotic activity are constantly updated and improved. At present, the research mainly covers two aspects: on the one hand, it is to use molecular biology-based methods to accurately identify. On the other hand, it is to use metagenomic sequencing technology to conduct in-depth analysis of the microbiome.

Metagenomic sequencing has been applied more and more widely in the field of microbiology, especially in the study of the effects of probiotics on gut microbes. Compared to traditional 16S rRNA gene sequencing, cMAGs provide deeper and more comprehensive microbial genomic information, enabling more accurate identification and isolation of microbial species, and interpretation of microbial diversity and function. In metagenomic technology, our requirements for accuracy and completeness are also increasing. Assembling complete bacterial genomes from metagenomic data has long been an important basis for microbiome research. In the field of microbiology, the composition and identification of gut microbes have always been the focus of research. There are genome-level interactions between gut microbes and host organisms. Maintaining the balance of the gut microbiome is essential for maintaining good health. Adequate probiotics can interact with the gut microbiota and help the host maintain a healthy state of mind and body. By applying complete metagenomic techniques, we can gain a more comprehensive understanding of the interrelationships between probiotics and gut microbes. This technology is able to provide more in-depth and complete data so that we can delve deeper into the mechanisms of this interaction.

Although the gut microbiome has always been the focus of researchers, the redundancy of the large number of short sequences produced by NGS has made it difficult to classify them at the species and strain level. In addition, the reference catalogs used for 16S rRNA sequencing, 16S rDNA sequencing, and metabolome sequencing are also incomplete. These limitations have led to the idea of complete metagenomic assembly, advancing our comprehensive analysis of gut microbes (Bickhart *et al.* 2022). At present, the complete bacterial genome assembly based on second-generation metagenomic sequencing and assembly methods cannot be realized, which has the defects of chimerism and incomplete assembly. Even metagenomic binning has problems such as fragmented assembly and high contamination rates. However, with the continuous progress of sequencing technology, we are highly likely to achieve complete genome assembly through the third-generation long-read long-length sequencing, which significantly improves the assembly effect of metagenomes, and successfully solves the problems such as the small number of species identified by NGS metagenomes, the poor quality of genome assembly of dominant strains, and the incomplete analysis of core genome functional information. In particular, HiFi (especially the PacBio Revio TGS platform) combined with Hi-C sequencing technology can greatly improve the quality of metagenomic assembly. This approach addresses the limitations of previous studies based on short-read metagenomes, capturing a substantial number of previously overlooked genomes. However, it's worth noting that HiFi metagenomic sequencing is currently associated with higher costs and demands larger DNA samples (exceeding 1.5 micrograms) compared to NGS, which requires only 0.1 micrograms. Additionally, Hi-C technology, while powerful, has fewer applications in metagenomics and is less convenient for use in microbial communities. Therefore, there is a need for the development and adoption of efficient Hi-C technologies tailored for microbial metagenomics research. In addition, in the subsequent steps such as genome assembly and annotation, how to complete the work more accurately and quickly, and constantly improve the assembly quality while reducing the use of memory is also very important. For example, although Hifiasm-meta and metaFlye run at similar speeds, Hifiasm-meta uses more memory than metaFlye and HiCanu. However, Hifiasm-meta is superior to metaFlye and HiCanu in terms of assembly effect (Feng X *et al.* 2022). Given current metagenomic assembly software has some shortcomings, we not only need to continuously improve assembly algorithms and combine various strategies, but also utilize advanced sequencing technologies. Moreover, in probiotic research, it is essential to select or design appropriate research plans based on data types, as well as the characteristics of various sequencing technologies and assembly software. This will enable us to better obtain cMAGs from metagenomic assemblies, providing a more accurate and comprehensive data foundation for the genomic study of the interactions between probiotics and gut microbiota. For instance, as mentioned earlier, to further improve the quality of metagenomic assemblies, the *in situ* Hi-C technique can be utilized to assist assembly. Compared to traditional Hi-C techniques, it has advantages such as higher interaction frequency and lower background noise (Kong and Zhang 2019). Furthermore, based on the actual performance of servers or the characteristics of the data, we can choose the best-performing quality control and metagenomic assembly software for preliminary assembly, such as HiC-Pro and Hifiasm-meta. Subsequently, by combining Hi-C binning methods for chromosome-level optimization and reassembly of the preliminary results, we can obtain high-quality MAGs. Finally, appropriate software like CTDB and antiSMASH can be chosen for taxonomic annotation and functional annotation, to acquire more accurate and comprehensive metagenomic

information. This information can also predict biosynthetic gene clusters, helping us screen strains with probiotic effects.

In addition, the rapid development of machine learning and artificial intelligence technologies in recent years has brought new opportunities to the field of probiotic research. By training models on large datasets, enabling accurate prediction or data analysis, we hope to apply these technologies in probiotic research. However, how to build efficient models and choose appropriate training datasets also poses a significant challenge, requiring researchers to conduct continuous experiments and exploration.

Acknowledgements

This work was supported by the Guangdong Basic and Applied Basic Research Foundation, China (2022A1515010766), the Shenzhen Science and Technology Program, China (RCBS20210609104512021 and KCXFZ20201221173205015); the National Natural Science Foundation of China (32202653); the Project Funded by the China Postdoctoral Science Foundation (BX2021367 and 2021M703543).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used ChatGPT to assist in improving the clarity and readability of the language. All content generated was carefully reviewed and revised by the authors as necessary, and the authors take full responsibility for the final content of the article.

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