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Droplet microfluidics: unveiling the hidden complexity of the human microbiome

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The human body harbors diverse microbial communities essential for maintaining health and influencing disease processes. Droplet microfluidics, a precise and high-throughput platform for manipulating microscale droplets, has become vital in advancing microbiome research. This review introduces the foundational principles of droplet microfluidics, its operational capabilities, and wide-ranging applications. We emphasize its role in enhancing single-cell sequencing technologies, particularly genome and RNA sequencing, transforming our understanding of microbial diversity, gene expression, and community dynamics. We explore its critical function in isolating and cultivating traditionally unculturable microbes and investigating microbial activity and interactions, facilitating deeper insight into community behavior and metabolic functions. Lastly, we highlight its broader applications in microbial analysis and its potential to revolutionize human health research by driving innovations in diagnostics, therapeutic development, and personalized medicine. This review provides a comprehensive overview of droplet microfluidics' impact on microbiome research, underscoring its potential to transform our understanding of microbial dynamics and their relevance to health and disease.

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1. Introduction

The human microbiome regulates various physiological functions, including digestion, metabolism, and immune response.[1,2] Approximately 10–100 trillion multi-kingdom microbes, primarily bacteria but also archaea, protozoa, fungi, and viruses, inhabit the human body.[3] These microbes contain 50 to 100 times more genetic information than the human host. Recent advances in multi-omics technologies, such as high-throughput sequencing, along with large-scale initiatives such as the integrative human microbiome project (iHMP) and the metagenomics of the human intestinal tract (MetaHIT), have significantly expanded our understanding of microbial composition and community structure at populational levels. Moreover, researchers have leveraged bioinformatics tools, including metagenomic analysis, metabolic network analysis, and machine learning, to explore associations between specific microbial taxa and host health outcomes. For instance, with the help of causality validation through microbial isolation, cultivation, and animal modeling, numerous studies have proved how probiotics can improve host immunity and regulate metabolic pathways while pathogenic microbes invade tissues and cause diseases.[4]

Despite significant advances, several challenges continue to impede microbiome research. One major obstacle is the interindividual variability in microbiome composition, which complicates efforts to generalize findings and predict clinical outcomes across diverse populations. Furthermore, microbial communities' spatiotemporal composition and gene expression are influenced by diet,[5] immune status,[6] and circadian rhythms.[7] Another challenge lies in the substantial genomic variation between strains within the same species,[8,9] which can significantly affect their metabolic potential, antibiotic resistance, and roles in host immunity and pathogenicity. Finally, although metagenomic studies have revealed the importance of unculturable microbes,[10] the lack of isolation and validation remains a critical barrier to fully understanding their functions. To address these issues, developing high-throughput cultivation methods and *in vitro* models that mimic the host environment is crucial for uncovering these microbes' specific effects and mechanisms.

Microfluidics, the applied manipulation of fluid at the microscale, offers distinct and complementary solutions to many challenges in microbiome research.[11] In particular, droplet microfluidics process picoliter to nanoliter aqueous droplets within an immiscible carrier fluid,[12] such as fluorinated oil, with each droplet as a miniaturized reaction chamber. This technology enables the isolation of individual microbial cells, providing a platform for high-throughput,

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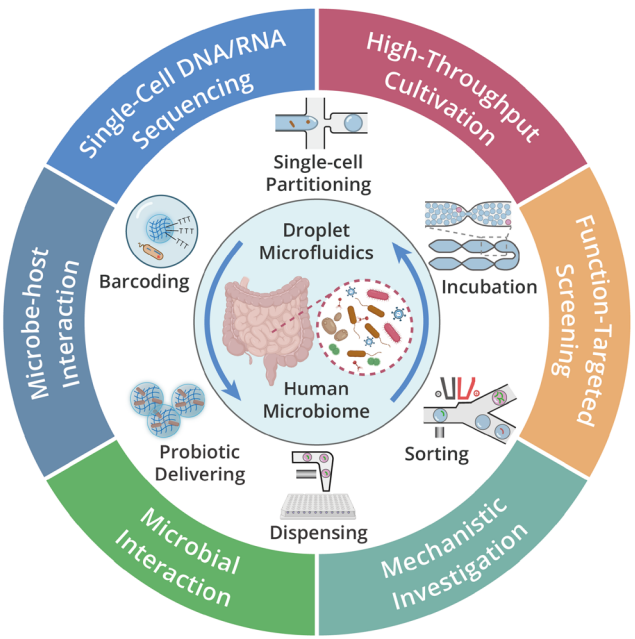
dynamic analysis of microbial physiology and genetic material. It is precious for addressing interindividual variability by allowing single-cell resolved analysis and capturing dynamic interactions between microbes and their environment. Moreover, droplet microfluidics facilitates the cultivation and functional screening of previously unculturable microbes, bridging the gap between correlation and causality in microbiome studies. Integrating droplet microfluidics with advanced sequencing and analytical tools offers unprecedented insights into microbial interactions.

This review will explore the diverse applications of droplet microfluidics in microbiome research, focusing on microbial single-cell sequencing, cultivation, functional screening, and interaction studies (Scheme 1). We will highlight how these technologies reshape our understanding of microbiome dynamics and their impact on host health. Special attention will be given to how droplet microfluidics is used to overcome challenges in isolating unculturable microbes, enabling high-throughput functional assays, and advancing microbial community synthesis and targeted delivery. This review aims to provide a comprehensive overview of how droplet microfluidics advances microbiome research and paves the way for new therapeutic approaches.

2. The complexity and diversity of human microbiota

The complexity and diversity of the human microbiota have been extensively reviewed.[13,14] Here, we provide a brief overview of the physiological and pathological aspects of the human microbiome. Microbial colonization occurs at birth, or even *in utero*, with over 50% of an infant's microbiota originating from maternal sources, including nasopharynx, saliva, skin, breast milk, feces, and the vaginal microbiota.[15] Consequently, the infant microbial population initially mirrors the maternal microbiota during the first weeks or months, gradually evolving to a more complex structure by 12 months and resembling the adult microbiome by age three.[16] Numerous factors influence this colonization process, including genetics, body environments, breastfeeding, diet, medication, and geographic location. These factors contribute to human microbiota's remarkable intra- and inter-individual diversity.

Recent studies have attempted to categorize the human microbiome into core and variable components to better understand the relationship between dysbiosis and health conditions.[17,18] Multi-kingdom microbiota has been applied as biomarkers for disease diagnosis and prognosis, such as colorectal cancer[19] and autism spectrum disorder.[20] The core microbiome is primarily defined by microbial composition,[21] though functional properties,[22] ecological parameters,[23] and temporal stability[24] also play significant roles. Here, Table 1 highlights the predominant microbes identified across various body sites through sequencing and their associations with dysbiosis-related diseases, emphasizing the critical role of the microbiome in human health.



Scheme 1 Applications of droplet microfluidics in human microbiome research, highlighting fundamental techniques and their diverse applications.

3. The versatile toolbox of droplet microfluidics

Droplet microfluidics is a specialized branch of microfluidics that involves manipulating tiny, uniform droplets within immiscible carrier fluids.[12,61,62] It emerged in the early 2000s and significantly advanced chemical and biological studies by enabling precise control of micro-reactions, facilitating high-throughput screening, and allowing single-cell analysis. The initial breakthrough came with the innovation of the T-junction device, which can produce monodispersed droplets through shear forces.[63,64] This innovation enlightened subsequent droplet generation techniques, which enable a broad range of applications (Fig. 1).

Droplet microfluidics offers several critical advantages, including precise control over reaction volumes, customizable mixing and reaction conditions,[64] stochastic confinement of single cells or molecules,[77] and minimal risk of cross-contamination.[78] Its high throughput enables the rapid generation of droplets, making it ideal for large-scale biological analyses. Its flexibility in handling individual droplets enables a wide range of functional operations, such as splitting, mixing, and targeted sorting.[62] These features make droplet microfluidics particularly valuable for single-cell analysis,[79,80] microbial cultivation,[81–84] nucleic acids amplification,[85,86] and enzymatic reaction or screening.[87,88] Given its versatility and scalability, understanding the various methods of droplet manipulation is essential for optimizing specific experimental outcomes. In the following sections, we will explore approaches for droplet generation and manipulation, highlighting their corresponding applications in biological research.

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Table 1 Predominant multi-kingdom microbiota across different body sites and key dysbiosis-associated species

Sites	Predominant multi-kingdom microbes				Dysbiosis-related diseases and core species	
	Bacteriome	Mycobiome[25,26]	Archaeome[27,28]	Virome[29]		
Gut[2,18,21,30]	Firmicutes Bacteroidetes	*Saccharomyces*, *Malassezia*, *Candida*	*Methanobrevibacter*	Bacteriophage	Inflammatory bowel diseases (IBD)[31–33]	*Klebsiella pneumoniae* *Clostridioides difficile*
	Proteobacteria				Insulin resistance[34]	*Candida albicans*
	Actinobacteria				Colorectal cancer[35]	Increased *Lachnospiraceae* (*family-level*) *Fusobacterium nucleatum*
Respiratory tract[36–39]	*Streptococcus*, *Veillonella*, *Prevotella*, *Haemophilus*	*Candida*, *Saccharomyces*, *Penicillium*	Nanoarchaeota	Bacteriophage, Anelloviridae, Redondoviridae	Chronic obstructive pulmonary disease[40]	*Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*
					Pulmonary fibrosis[41,42]	Increased *Streptococcus*, SARS-CoV-2
					Chronic pulmonary aspergillosis[43]	*Aspergillus* species (commonly *Aspergillus fumigatus*)
					Tuberculosis[44]	*Mycobacterium tuberculosis*
Oral cavity[39,45,46]	Firmicutes Actinobacteria Proteobacteria Fusobacteria Bacteroidetes Spirochaetes	*Candida*	*Methanobrevibacter*	Redondoviridae, Anelloviridae, Bacteriophages	Periodontitis[47] Dental caries[48] Apical periodontitis[49,50]	*Porphyromonas gingivalis* *Candida albicans* *Streptococcus mutans* *Enterococcus faecalis*
Skin[51,52]	Actinobacteria	*Malassezia*	Nitrososphaeria	*Polyomaviridae*, *Papillomaviridae*, *Circoviridae*	Acne[53]	*Cutibacterium acnes* (formerly *Propionibacterium acnes*)
	Firmicutes Bacteriodetes Proteobacteria				Atopic dermatitis[54]	*Staphylococcus aureus*
Vagina[55]	Firmicutes (majorly *Lactobacillus*)	*Candida*, uncultured *Saccharomycetale*	Methanobacteria	Bacteriophage	Bacterial vaginosis[56,57]	*Gardnerella vaginalis*, *Atopobium vaginae*, Prevotella spp., increased vaginal anaerobes decreased *Lactobacillus* spp.
					HIV infection[58]	Human immunodeficiency virus (HIV)
					HPV infection[59]	Human papillomavirus (HPV)
					Preterm birth[60]	*Sneathia amnii*, Lachnospiraceae, BVAB1, *Saccharibacteria* TM7-H1, decreased *Lactobacillus crispatus*

3.1 Droplet generation and incubation

Microfluidic droplet generation involves the continuous production of monodisperse droplets, typically ranging from picoliter to nanoliter volumes, by precisely shearing the dispersed phase with the continuous phase. Various methods have been developed for this process, including flow-focusing,[65] step emulsification,[66] capillary-based methods,[89] and centrifugal-based techniques (Fig. 1A–C).[90] Among these, flow-focusing and T-junction designs are the most widely used due to their simplicity, high throughput, and ability to generate droplets with sizes ranging from picoliters to microliters. Step emulsification, which uses parallel step structures, enables high-throughput droplet generation with minimal sensitivity to flow rate fluctuations. For instance, step emulsification chips can generate tens of thousands of droplets per second with sub-nanoliter volumes.[66] Interfacial emulsification and OsciDrop leverage inertia forces using periodical vibrating capillaries or pipette tips to generate size-tunable droplets.[91,92] Alternatively, SlipChip technology enables droplet generation by breaking a continuous stream into predefined volumes in microchambers following relative sliding of top and bottom plates, encapsulated by a continuous carrier fluid.[67,93]

Droplets can be incubated with well-controlled temperatures and atmospheres as uniform templates for spatially confined biochemical processes and advanced materials manufacturing.[94] For example, picoliter to nanoliter droplets can undergo hours of thermal cycling without coalescence, enabling droplet digital PCR.[92] On-chip incubation uses chambers integrated into the microfluidic device to provide precise environmental control,[95] such as regulating oxygen levels to support the growth of anaerobes.[96] In contrast, off-chip incubation is used for larger-scale or extended studies, where droplets are transferred to

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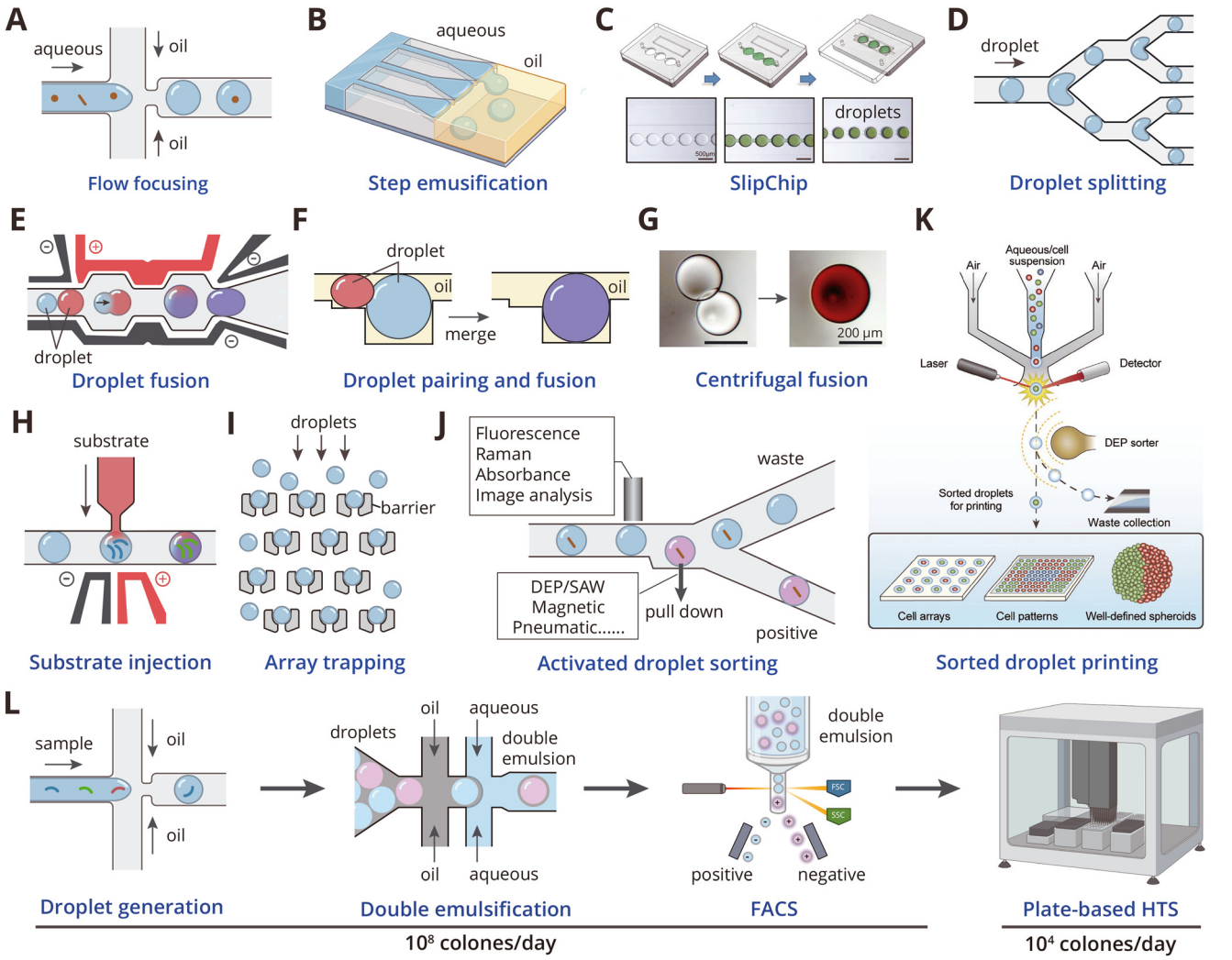

Fig. 1 Overview of droplet microfluidic techniques. (A–C) Droplet generation using flow-focusing,[65] step emulsification,[66] and SlipChip.[67] (D) Bifurcating droplet splitting.[68] (E) Droplet fusion through a chamber of high electric field.[69] (F) Droplet fusion within differential anchor array.[70] (G) Off-chip droplet fusion by centrifugation in a 96-well microtiter plate.[71] (H) Picoinjection.[72] (I) Geometry-based trapping arrays.[73] (J) Activated droplet sorting coupling different detection methods and external forces to select and collect target droplets.[74] (K) Sorting and printing single-cell droplets based on fluorescence.[75] (L) Integration of droplet microfluidics with flow cytometry and automated liquid handling for high-throughput screening.[76] The schematics in B and L were created with **https://www.BioRender.com**. Panel C adapted from ref. 67 with permission, copyright 2020, Elsevier.

Eppendorf tubes or sealed environments to maintain stable conditions over time.[97–99] Additionally, droplet microfluidics facilitates the creation of hydrogel microspheres from biocompatible polymers like agarose, alginate, or Matrigel, which encapsulate bacterial or mammalian cells for long-term growth with continuous media exchange.[100] Droplets can also be tailored to study microscale events such as gene transfer, metabolic exchange, and biomolecular self-assembly.[101]

3.2 Droplet splitting, fusion, and picoinjection

Droplet splitting, fusion, and picoinjection are essential methods in droplet microfluidics that enable complex biological and chemical processes. Droplet splitting allows droplets to be divided symmetrically or asymmetrically into two or more smaller droplets (Fig. 1D). Symmetrical splitting is typically achieved by directing the droplet through bifurcating channels, where equal hydrodynamic resistance ensures the droplets divide evenly into two equal droplets.[68]

Droplet fusion involves merging two or more droplets to combine their contents, which is often needed in cascaded reactions where the mixing of reagents is necessary. In typical fusion devices, droplets of varying sizes are paired and compressed in a fusion chamber, where a high electric field facilitates their merging (Fig. 1E).[69] While this method is highly efficient for contamination-sensitive and high-throughput applications, its success can be limited by imperfect matching droplet pairs. A more recent technique, the differential anchor array, selectively traps and immobilizes with different trapping strengths, allowing precise droplet pairing and fusion in a 2D

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array (Fig. 1F).[70,102] Meanwhile, for on-demand droplet fusion off the chip, droplets can be dispensed in round-bottomed microtiter plates with carrier oil containing surfactant at the critical micelle concentration (CMC), followed by centrifugation, resulting in high fusion rates for single-cell assays (Fig. 1G).[71]

Picoinjection allows the precise introduction of picoliter volumes of reagents into individual droplets, also facilitated by an electric field, while maintaining droplet stability and integrity (Fig. 1H).[72] A modified approach, stepinjection, utilizes a stepped injection channel design to balance injection success rates while minimizing contamination risks.[103] Electrode-free picoinjection can be achieved by using dissolved electrolytes in the injection fluid as a conductor to apply the electric field.[104] Alternatively, electricity-free picoinjection uses precise pressure control within the microfluidic channels to inject reagents without needing an electric field.[105]

3.3 Droplet trapping and array assembly

Droplets' trapping and array assembly are essential for prolonged observation, dynamic analysis, and rare event recovery. Automated detection systems can facilitate continuous and dynamic characterization by assigning positional information to each droplet fixed to a planar array. One commonly used approach involves geometry-based trapping designs, which utilize an array of microfabricated trap structures for high-throughput droplet capture (Fig. 1I).[73] Hydrodynamic trapping chips guide droplets sequentially into chambers using fluid dynamics to enhance capture efficiency.[106] Co-trapping of multiple droplets is another widely adopted strategy, essential for advanced applications requiring droplet interactions. For instance, the CARMEN (combinatorial arrayed reactions for multiplexed evaluation of nucleic acids)[107] platform combines sample droplets with detection-mix droplets in a microwell array, enabling the random yet systematic pairing of diverse samples with amplification assays targeting various pathogens. This innovative approach achieves highly multiplexed target detection across numerous samples, significantly enhancing throughput and versatility in nucleic acid diagnostics.

Additionally, droplets can be assembled autonomously into planar monolayer droplet arrays (PMDA) within microfluidic chambers, creating large-scale arrays suitable for high-throughput imaging and analysis.[108] Furthermore, in open reservoirs with flat bottoms, PMDAs can form through gravity-driven sedimentation and self-assembly, offering a streamlined method for organizing droplets on a large scale.[91,92,109]

3.4 Droplet sorting

Efficient sorting of droplets based on their properties is essential when processing large numbers of droplets. Sorting methods can be classified into passive and active techniques.[74] Passive sorting leverages hydrodynamic properties to separate droplets based on factors like size or density.[110,111] Passive sorting is cost-effective and scalable, ideal for high-throughput screening without external controls. This approach is particularly valuable in microbiome research, where bacterial growth within droplets can cause a size reduction,[110] allowing the identification of droplets containing actively growing bacterial cells. In contrast, active sorting applies external forces such as dielectrophoresis,[112] acoustic waves,[113] magnetic forces,[114] and pneumatic valves,[115,116] to manipulate droplets on demand, overcoming flow resistance and directing selected droplets to collection channels. These platforms enable real-time sorting based on characteristics such as fluorescence intensity,[117] absorbance,[118,119] and real-time pattern recognition using high-speed imaging (Fig. 1J).[120] Active droplet sorting has been widely used in applications such as antibody screening,[121] drug-resistant strain screening,[120] and bioactive molecules discovery.[122]

3.5 Integrated droplet microfluidics and downstream off-chip platforms

Integrating multiple droplet-based functional units reduces manual steps and enhances the efficiency of biochemical processes. Systems that combine droplet generation, incubation, and sorting within a single device have shown considerable progress in high-throughput applications. For example, one study reported a 30-fold increase in enzyme activity using a droplet microfluidic system that integrated single-cell lysis, enzymatic reactions, and fluorescence-activated droplet sorting (FADS).[123] In another case, a microfluidic system combined droplet splitting and sorting modules for label-free screening using electrospray ionization mass spectrometry (ESI-MS). The droplets were split, with one portion analyzed by ESI-MS and the other sorted based on the results. This mass-activated droplet sorting (MADS) system achieved a throughput of 15 000 samples in 6 hours with an accuracy of 98% for *in vitro*-expressed transaminase.[124]

Additionally, integrating droplet microfluidics with other techniques can further expand its capabilities. For instance, combining droplet and digital microfluidics (DMF) enables automated sample preparation and high-throughput analyses.[95,125] One integrated system performed nucleic acid extraction and reagent mixing in the DMF module, followed by droplet generation and collection in parallel chambers for multiplex digital loop-mediated isothermal amplification (dLAMP).[125] This system demonstrated high accuracy (91.1%), speed, and a broad detection range for pathogen detection in urine samples. These innovations underscore the potential of integrated droplet microfluidic systems in advancing high-throughput biochemical analysis.

The potential of droplet microfluidics extends beyond miniaturized systems when integrated with established microtiter plate-based screening and liquid handling platforms, enabling a seamless transition from on-chip experiments to traditional laboratory workflows.[126] For instance, the Cyto-Mine platform automates the encapsulation, screening, sorting, isolation, and dispensing of high-secreting clones into microtiter plate wells, achieving high precision and efficiency

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for monoclonality assurance.[127] Similarly, a high-definition single-cell printing (HD-SCP) device was developed, featuring a miniaturized microfluidic sorter that selects single cells from a mixture based on fluorescence for printing with an accuracy of ~10 μm and a speed of ~100 Hz (Fig. 1K).[75] This method has potential applications in printing bacterial cells encapsulated in droplets or hydrogel microspheres into microtiter plates, offering promising advancements for microbial studies and high-throughput screening. An alternative approach is transforming water-in-oil droplets into double emulsions, which can then be sorted using widely available flow cytometers (Fig. 1L).[76] This method enables high-throughput screening of large droplet libraries, producing double emulsions at 6–12 kHz rates and sorting approximately 10^8 droplets in a day. Recently,

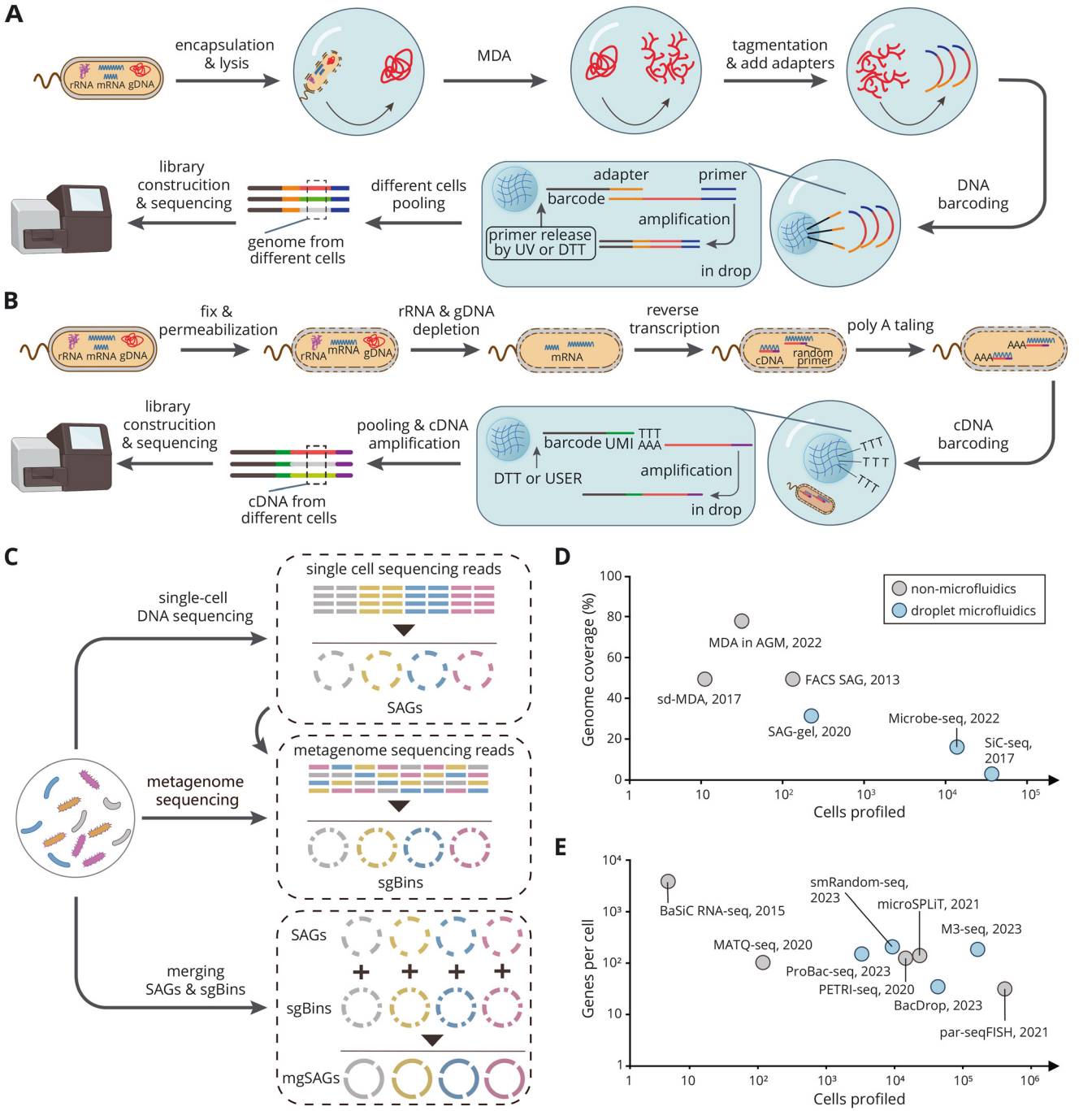

Fig. 2 Advances in droplet-based microbial single-cell sequencing. (A) Workflow of microbe-seq for microbial single-cell DNA sequencing.[129] (B) Workflow of BacDrop for microbial scRNA-seq.[130] (C) Integrating single-cell genome sequencing and metagenomics to improve genome completeness.[131] (D) Analysis throughput (cell numbers per run) and genome coverage in representative publications for microbial single-cell genome sequencing. (E) Analysis throughput and transcriptome coverage (genes per cell) in representative publications for microbial scRNA-seq.

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a high-capacity approach developed with an optical-based microbubble technique has been applied to retrieve target droplets from a static droplet array (SDA).[259] Unlike sorting at a fixed time point based on droplet content, this method utilizes a large-scale microchamber array to track real-time processes in each droplet simultaneously, allowing sorting based on reaction kinetics. This system showed nearly 100% success in releasing targeted droplets, proving its utility in high-throughput assays such as single-cell analysis and phenotype-based sorting.

4. Microbial single-cell sequencing

Microbial sequencing offers a culture-independent means of exploring microbial diversity and its functional roles in human health. However, traditional metagenomic methods always provide aggregate data from microbial populations, potentially masking key differences within subpopulations and overlooking rare microbes. Single-cell sequencing has emerged as a transformative approach for more detailed microbiome analysis and enabling the resolution of individual microbial cells (Fig. 2).

4.1 General methods in single-cell sequencing

Single cells contain genomic DNA in the femtogram range, making it challenging to extract enough material for direct sequencing. This problem can be solved by introducing whole-genome amplification (WGA) following single-cell lysis, which generates microgram quantities of DNA suitable for library preparation and sequencing. WGA techniques must carefully balance three key factors—amplification uniformity, genome coverage, and fidelity—to ensure reliable results, prompting the development of a range of innovative methods tailored to address these challenges. Early WGA techniques, such as degenerate oligonucleotide primer (DOP)-PCR[132] combined with random primers, utilized polymerase chain reaction to amplify genomic DNA. Multiple displacement amplification (MDA)[133] later emerged as a prominent method due to its use of Phi29 DNA polymerase with strand-displacement activity, enabling high-yield DNA amplification with improved coverage and accuracy. However, these exponential amplification-based methods can exacerbate amplification bias and amplification errors. To address these limitations, linear or semi-linear amplification approaches, like multiple annealing and looping-based amplification cycles (MALBAC)[128] and linear amplification *via* transposon insertion (LIANTI),[134] have improved amplification strategies to minimize bias and ensure uniform genome representation. Notably, LIANTI employs a transposon-based linear amplification principle, enabling precise detection of copy number variations and single nucleotide variations in human cells. However, its complex protocol has limited broader application on platforms like microfluidic systems. Recently, a derivative of MDA called primary template-directed amplification (PTA) has been introduced.[135] By incorporating exonuclease-resistant terminators, PTA confines amplification primarily to the original template, achieving quasi-linear amplification. This approach not only maintains high amplification performance but also keeps simple experimental workflows. More detailed information about these aforementioned WGA techniques can be found in the review published by Wen *et al.*[136]

In single-cell RNA sequencing (scRNA-seq), the small amount of RNA in individual cells requires specialized techniques for capture and amplification. The process starts with single-cell lysis and RNA extraction, followed by reverse transcription into complementary DNA (cDNA). Techniques like Smart-seq,[137,138] which use oligo-dT priming and template switching, generate full-length cDNA for amplification. Alternately, CEL-Seq[139] utilizes oligo-dT primers integrated with T7 promoter elements and barcoding sequences, realizing linear amplification of cDNA through *in vitro* transcription and ensuring precise identification of transcripts originating from individual cells. Notably, the barcoding strategy[140] allows sample pooling during library preparation, significantly improving throughput and reducing workload. These foundational techniques have laid the groundwork for advanced droplet-based single-cell sequencing platforms.

4.2 Advancements from traditional to droplet-based microbial single-cell sequencing

Early techniques for sequencing individual microbial cells relied on characteristics such as morphology, fluorescence labeling, or Raman spectroscopy, employing methods like flow cytometry,[141] optical tweezers,[142,143] and laser capture microdissection.[144,145] These isolated cells were subjected to lysis, amplification, and library construction. Although these approaches had limited throughput, they successfully revealed genomic information from previously elusive microbial populations. More recently, high-throughput methods like PETRI-seq[146] and microSPLiT[147] have been developed, utilizing combinatorial barcoding to label permeabilized microbial cells in microtiter plates, enabling large-scale transcriptome sequencing. However, the multi-round barcoding process in these methods often results in significant data loss, making them less suitable for precious samples with limited cell numbers.

In contrast, droplet microfluidics has emerged as a transformative platform for single-cell multi-omics by transferring molecular reactions into individual droplets, offering high-throughput capabilities and efficient sample utilization. By enrichment of target microbial single cells through FADS, microbial genomes of rare species can be recovered with high quality.[148] Encapsulating microbial single cells within gel beads enables in-bead cell lysis and whole-genome amplification in bulk systems, dramatically increasing operational throughput. Following fluorescence-activated droplet sorting (FACS) of gel beads, a large number of high-quality single-amplified genomes (SAGs) can be collected in plates for further research or storage.[149] Furthermore, another similar study demonstrated that gel

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bead could achieve an average genome completeness significantly higher than that obtained using conventional microliter-scale amplification methods.[150]

Droplet microfluidics also enables the co-encapsulation of individual cells and barcoding beads within droplets, achieving in-droplet labeling to distinguish genetic material from different cells. Techniques such as Indrop[151] and Drop-seq[152] have leveraged this approach to achieve high-throughput single-cell transcriptomic sequencing in eukaryotic cells, laying the foundation for subsequent studies in microbial omics. Recently, several in-droplet cell labeling methods tailored for microbiological applications have been developed, employing diverse methodologies. To provide a clearer understanding of the available droplet-based platforms, we outline a general workflow for both genomic and transcriptomic analyses of prokaryotic cells, offering insights into their potential and applicability in microbial research. (Fig. 2A and B).

4.3 Droplet-based microbial single-cell genome sequencing

Microbial single-cell genomic sequencing has historically lagged behind eukaryotic applications. Droplet microfluidics has revolutionized whole-genome amplification (WGA) by introducing emulsion WGA (eWGA), which partitions single-cell genomic DNA into numerous picoliter droplets, ensuring uniform amplification gains across DNA fragments.[153] This advancement enables high-fidelity analysis of low-input samples, such as single cells, for both eukaryotic and prokaryotic organisms.[153,154] Recent innovations, such as SiC-seq and Microbe-seq, have further advanced the field by significantly increasing the throughput for microbial single-cell analysis.[129,155] SiC-seq performs direct single-cell tagmentation and barcoding within droplets,[155] while Microbe-seq integrates all processing steps into a droplet-based workflow, using MDA before genome labeling.[129] This approach significantly enhances nucleic acid capture from single cells and improves genome coverage during sequencing (Fig. 2A). Microbe-seq has been used to reconstruct horizontal gene transfer networks in the gut microbiome and to identify host–phage interactions, such as the association between crAssphage and *Bacteroides vulgatus*.

Despite these advancements, SAGs often suffer from incomplete and uneven coverage due to artifacts introduced by MDA, prompting the development of co-assembly methods to generate more complete genome drafts such as ccSAG.[156] This tool could effectively eliminate chimeric and co-assemble multiple and closely related SAG into a nearly complete genome with overall quality equivalent to those assembled from bulk DNA. Additionally, an approach integrating environmental metagenomic data has also been developed to overcome this bottleneck, which helps fill gaps within SAGs, significantly improving the completeness of genomes, such as those of SAR324 bacterioplankton and *Thaumarchaeota*.[157] The bioinformatic tool SMAGLinker was designed to integrate SAG and metagenomic data to reconstruct single-cell genome-guided bins (sgBins), resulting in metagenome-guided SAGs (mgSAGs) with improved genome completeness compared to original SAGs (Fig. 2C).[131]

Recent single-cell technologies like DoTA-Seq focus on targeted gene profiling rather than whole-genome sequencing.[158] This approach reduces experimental costs and time while enhancing data accuracy, making it ideal for detecting subtle expression changes in microbial communities and studying the role of low-abundance microbes in human disease. Additionally, SIP-seq has been developed to isolate and sequence HIV proviruses and their chromosomal integration sites at the single-cell level.[159] This method employs FACS to select infected host cells, followed by two rounds of amplification—initially using MDA to amplify both viral and host DNA and then TaqMan PCR to detect HIV sequences. SIP-seq effectively eliminates interference from the host genome, allowing for precise identification of HIV provirus integration sites and offering insights into the clonal architecture and persistence of the HIV-1 reservoir.

4.4 Droplet-based microbial scRNA-seq

Transcriptomic sequencing is crucial in uncovering microbial diversity, heterogeneity, dynamic functions, and interactions in various environments.[160] In microbial scRNA-seq, specific challenges arise compared to genome sequencing. Bacterial ribosomal RNA (rRNA) makes up over 80% of total RNA, while mRNA constitutes only a small fraction. Additionally, bacterial mRNA lacks the 5′ cap and PolyA tail found in eukaryotic mRNA, contributing to its instability and difficulty in capture.

Two key strategies have been developed to capture mRNA to address the challenges of prokaryotic scRNA-seq. The first approach uses random primers, which target a wide range of mRNA sequences. Technologies like scDual-Seq replaced poly(A)-based primers with random hexamers to capture eukaryotic RNA and synthesize the second strand using CEL-Seq2 barcoded primers.[161] Recent technologies like BacDrop[130] and smRandom-seq[162] have further enhanced throughput, enabling the analysis of thousands of microbes per run. BacDrop depletes rRNA using a duplex-specific nuclease, followed by a two-step barcoding strategy (Fig. 2B).[130] smRandom-seq uses the CRISPR-Cas9 system to selectively remove rRNA and applies short random primers for reverse transcription.[162] The second approach employs gene-specific primers to improve the capture efficiency of specific mRNA sequences. For example, ProBac-seq was developed, utilizing large oligonucleotide libraries with sequences complementary to specific microbial genomes, pre-adenylated with PolyA tails, for targeted and efficient mRNA reverse transcription.[163] This method has revealed previously unreported transcriptional heterogeneity in *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) and has provided insights into toxin expression regulation in *Clostridium perfringens*.

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Dual scRNA-seq technologies have been developed to simultaneously monitor the expression profiles of eukaryotic cells and microbes at the single-cell level. For example, smRandom-seq has been used to study the interaction between *Acinetobacter baumannii* (*A. baumannii*) and macrophages, revealing a relationship between ferroptosis and *A. baumannii* infection.[164] An unbiased dual-scRNA-seq method was applied to detect cells parasitized by *Leishmania donovani*, highlighting its ability to identify infected cells that were previously undetectable, such as hematopoietic stem cells in the bone marrow.[165] Dual RNA-seq has also been employed to explore the tropism and receptor specificity of pandemic viruses.[166] This approach provides valuable insights into microbial interactions within host cells and supports the development of targeted therapies for microbe-related diseases.

A comparative overview of major single-cell sequencing parameters for microbial genome sequencing (Fig. 2D) and scRNA-seq technologies (Fig. 2E). Emerging single-cell sequencing techniques are primarily based on droplet microfluidics platforms. While these platforms often yield lower per-cell data than traditional well-plate methods, they offer a significant advantage in throughput. Recent advances in droplet microfluidics have continuously improved genome coverage in DNA sequencing and increased the number of genes detected per cell in RNA sequencing. These advancements underscore the tremendous potential of this technology for a wide range of future applications. However, subtle variations in the microbial species and sequencing depths used across these methods imply that the parameters are more suited for general reference rather than direct comparison.

5. Droplet-based microbial isolation and cultivation

Microbial isolation aims to cultivate pure cultures to study microbial functions and their roles in human health. While culture-based methods have long been essential for classifying microbes by their physiological traits, modern culture-free approaches, such as metagenomics, have significantly expanded our understanding of microbial diversity by directly analyzing genetic material from natural environments. These methods have revealed numerous previously unknown microbes in the human gut,[167] including archaea and viruses. However, despite their advantages, culture-independent methods face challenges like experimental and computational biases, emphasizing the continued need for pure cultures in functional studies and therapeutic applications. The culturomics approach has emerged as a powerful post-omics tool, leveraging advanced techniques to cultivate previously uncultivable microbial "dark matter".[168] Culturomics integrates high-throughput culturing methods with state-of-the-art sequencing and mass spectrometry to identify novel microbial species, significantly increasing the number of known bacteria from 2776 in 2018 to 3253 in 2020, with 301 new species attributed to this method.[169] However, conventional colony-picking methods for isolating species still present drawbacks, such as fast-growing species dominating plates and inhibiting the growth of slower-growing microbes and the potential for mixed colonies, requiring time-consuming re-streaking.

Droplet microfluidics provides a highly efficient method for isolating complex microbial communities into individual cultures, offering scalability to cover diverse and low-abundance species while ensuring high purity and precision (Fig. 3). One example is the DropSpots device, which immobilizes thousands of picoliter-scale droplets, each containing a single microbial cell, for long-term cultivation and recovery (Fig. 3A).[170] Another system, the large-scale droplet array in a microcage array, generates up to 1 000 000 droplets on a chip within seconds, facilitating microbial growth and enabling scalable downstream applications, such as screening esterase-expressing bacteria (Fig. 3B).[171] The microfluidic streak plate (MSP) system arranges single-cell droplets in a spiral pattern for extended cultivation and recovery, significantly improving the growth of rare and slow-growing species (Fig. 3C).[97,172] Droplet-based cultivation has also been employed to enhance the growth of strains typically found in low-abundance (<1%) in metagenomic analyses of human stool samples.[120] Additionally, converting droplets into double emulsions allows them to be sorted *via* flow cytometry,[173] facilitating nutrient privatization and enriching slow-growing species like *Negativicutes* and *Methanobacteria* from human stool samples.

To investigate microbial interdependencies, such as cross-feeding and symbiotic relationships necessary for survival and proliferation, we can adjust the initial cell number based on the Poisson distribution of cells in droplets, facilitating the co-cultivation of symbiotic microbes.[96,174] For example, this approach enabled the cultivation of previously uncharacterized gut commensals, such as a novel genus from the *Neisseriaceae* family, after successful proof-of-concept studies with synthetic auxotrophic *E. coli* strains (Fig. 3D). Another study used a high-throughput picoliter droplet system combined with microbial-derived necromass supplements to enrich candidate phyla radiation (CPR) bacteria from groundwater, highlighting its effectiveness for cultivating microbes with specialized parasitic or symbiotic relationships (Fig. 3E).[179]

To test bacterial growth under varying nutrient conditions, multilayer droplet microfluidic systems have been developed to generate concentration gradients and conduct high-throughput screenings (Fig. 3F).[175] This method enhances the optimization of microbial culture environments, facilitating the evaluation of microbial responses to diverse conditions. Additionally, single-cell dispensers offer a precise alternative for testing cultivation conditions, with commercially available instruments enabling accurate droplet generation and cell isolation (Fig. 3G).[176] Notably, a study demonstrated that combining bioorthogonal non-canonical amino acid tagging (BONCAT) with FACS effectively identifies translationally active microbes under various substrate and physicochemical conditions.[180] This approach, paired with

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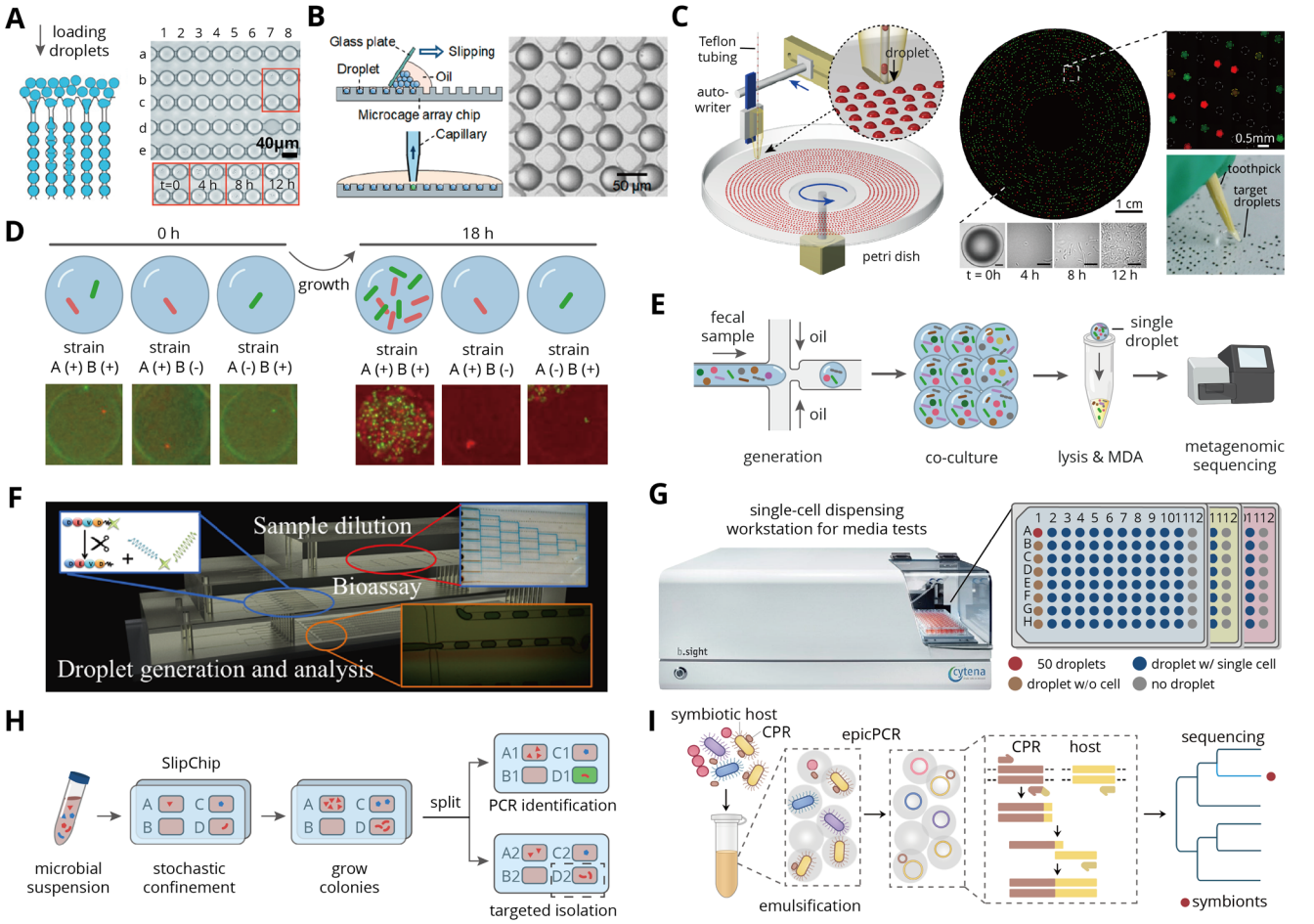

Fig. 3 Strategies for isolating and cultivating difficult-to-culture microbes using droplet microfluidics. (A) DropSpots device for single-cell cultivation in a droplet array.[170] (B) Microcage array chip for real-time microbial growth observation and droplet recovery.[171] (C) Microfluidic streak plate for microbial cultivation.[172] (D and E) Droplet co-encapsulation with essential symbiotic partners promotes microbial proliferation, as validated by imaging (D)[174] and sequencing (E).[96] (F) A 3D microfluidic chip integrates gradient dilution with droplet generation to adjust culture conditions.[175] (G) Single-cell droplet dispensing systems for media optimization.[176] (H) SlipChip for gene-targeted cultivation of microbes from the human gut.[177] (I) epicPCR unveils episymbiotic relationships within microbial communities.[178] Panel A adapted from ref. 170 with permission, copyright 2009, Royal Society of Chemistry. Panel B preprinted from ref. 171 with permission, copyright 2019, American Chemical Society. Panel C adapted from ref. 172 with permission, copyright 2016, American Society for Microbiology. Panel F reprinted from ref. 175 with permission, copyright 2015, American Chemical Society.

16S rRNA sequencing for taxonomic identification, allows researchers to experimentally test genomic predictions. This method could also inform the development of targeted cultivation media for uncultured microbes, including archaea and bacteria, under conditions closely resembling their natural environments.

In addition to non-targeted isolation and cultivation methods, droplet microfluidics can be combined with techniques like Raman-activated cell sorting (RACS),[181] optical tweezers,[142] and fluorescence *in situ* hybridization (FISH)[182] for precise capture and retrieval of target microbes based on phenotypes, genotypes, and surface proteins. For instance, a platform called Raman-activated gravity-driven single-cell encapsulation and sequencing (RAGE-Seq) was developed to identify antibiotic-resistant bacteria in urogenital tract infections.[183] This platform uses single-cell Raman spectroscopy to phenotypically screen cells and then encapsulates the target cells in picoliter droplets for genomic sequencing. Raman spectroscopy detects molecular fingerprints unique to each species or cell based on the vibrational information of cellular components like DNA, proteins, and lipids, providing non-destructive, label-free sorting capabilities. Surface-enhanced Raman scattering (SERS) and stimulated Raman scattering (SRS) further enhance sensitivity and speed, enabling the detection of low-abundance molecules and rapid analysis for high-throughput applications.[184,185]

Another technique, the SlipChip device, employs two complementary plates with microchambers that split microcolonies into two daughter droplets for gene-targeted microbial isolation from a complex community (Fig. 3H). One half undergoes analysis *via* FISH or PCR, while the other is retained for cultivation, allowing successful isolation of elusive strains such as *Oscillibacter* from the human microbiome.[177] Furthermore, the epicPCR (emulsion, paired isolation and concatenation PCR) technique,[186,187] combined with droplet

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microfluidics,[188] enables the isolation of episymbiotic bacteria like *Candidatus Saccharibacteria* (named TM7i) with its host (*Leucobacter aridocollis*) by linking phylogenetic markers to functional genes, facilitating the discovery of symbiotic relationships in microbial communities (Fig. 3I).[178]

Recent innovations in targeted microbial isolation also include a reverse genomics approach, where membrane protein-encoding genes are used to screen surface epitopes, and corresponding antibodies are created to capture previously uncultured *Saccharibacteria* along with their *Actinobacteria* hosts.[189] Similarly, genomic information from the cell wall binding domain (CBD) of *Streptococcus* phages has been used to generate fluorescent CBDs to detect and enrich specific *Streptococcus* species from saliva.[190] A digital microfluidics platform integrating optical tweezers and fluorescence-activated cell sorting was developed to capture *Salmonella Typhimurium* using magnetic beads coated with fluorescent-labeled antibiotics in droplets.[191] This approach highlights the potential of combining reverse genomics with droplet microfluidics and other advanced techniques for isolating and cultivating previously unculturable microbes.

6. Applications of droplet microfluidics for assessing microbial activity and interactions

Droplet-based microfluidics provides a versatile platform for high-throughput and high-content microscale analysis, enabling a wide range of studies related to microbial activities and interactions. Recent studies have leveraged this technology to observe microbial growth, screen bioactive compounds, evaluate antimicrobial susceptibility, and investigate microbial cell–cell interactions.

6.1 Observing microbial growth and collective behavior

A microchemostat is a microscale version of a traditional chemostat designed to maintain a continuous and controlled environment for microbial growth. In a microchemostat, nutrients are continuously supplied, and waste products are removed, allowing microbes to grow under steady-state conditions. This setup is ideal for studying microbial behaviors over time, as it ensures consistent environmental conditions while enabling precise control over variables such as nutrient concentration, oxygen levels, and microbial density.

Integrated droplet microfluidic systems can manipulate each droplet as an individual microchemostat, providing a controlled environment for studying microbial growth dynamics at both individual and population levels.[192] One study, for example, tracked the growth trajectory of hundreds of *E. coli* cells within droplets and demonstrated that the division times of individual cells were independent and uninherited, consistent with the Bellman–Harris model.[193] Another study encapsulated the bacterial cell-division protein FtsZ into droplets, revealing self-organization behavior within confined environments, which provided new insights into the mechanisms of bacterial cell division.[194] Also, microchemostats in droplet form have been used to study more complex microbial activities. For instance, a droplet-based study explored how droplet size and surface hydrophobicity influenced bacterial plasmid transfer rates in microscopic surface wetness (MSW) environments (Fig. 4A).[101] The findings indicated that larger droplets with lower liquid–solid interface ratios had higher cell densities and increased plasmid transfer rates, while surface hydrophobicity further enhanced transfer rates by reducing droplet spreading and promoting higher cell densities.

Droplet-based microchemostats are useful for studying isolated microbial growth and investigating collective microbial behaviors, such as quorum sensing and biofilm formation. For example, individual *Staphylococcus aureus* (*S. aureus*) cell in droplets was found to secrete autoinducer that initiated quorum sensing, as the confined droplet environment concentrated quorum-sensing factors, thereby activating regulatory genes (Fig. 4B).[195] A dynamic sessile-droplet system was developed to cultivate and analyze *P. aeruginosa* biofilms on hydrophilic micropatterns under shear force stimulation, providing a robust platform for studying biofilm development and testing antimicrobial susceptibility (Fig. 4C).[196]

6.2 Screening microbial bioactive molecules

Microbial genomes are incredibly diverse, offering various functional capabilities that allow microbes to catalyze numerous biochemical processes.[202,203] This diversity makes microbes valuable for discovering novel enzymes, metabolites, and bioactive compounds. Droplet-based microfluidics, particularly when combined with FADS or emulsified into double emulsions for use with traditional FACS, has emerged as a powerful tool for high-throughput screening of bioactive molecules.

Droplet microfluidics facilitates the detection of secreted products of microbial cells within picoliter droplets, preserving the critical genotype–phenotype linkage essential for screening in metabolic engineering.[88,122] For instance, a transcription-based *E. coli* biosensor, responsive to *p*-coumaric acid *via* the *B. subtilis* transcriptional repressor PadR, was used to sort yeast cells encapsulated in droplets based on their extracellular *p*-coumaric acid production, indicated by a PadR-regulated fluorescent signal.[204] RNA aptamer technology has been used to reflect target molecule concentrations, such as tyrosine, into fluorescence signals, enabling ultra-high-throughput screening of millions of yeast mutants. This method's adaptability for various target molecules through aptamer sequence modification enhances its versatility for bioactive molecule screening.[205] FADS has also been applied to detect antibiotic-producing microbes within microbial communities, identifying various antimicrobial compounds like surfactants and cytotoxic lipopeptides.[206] Additionally, a solid-state fermentation medium with 2% colloidal chitin was employed to fortify droplet structural integrity, preventing hyphae penetration

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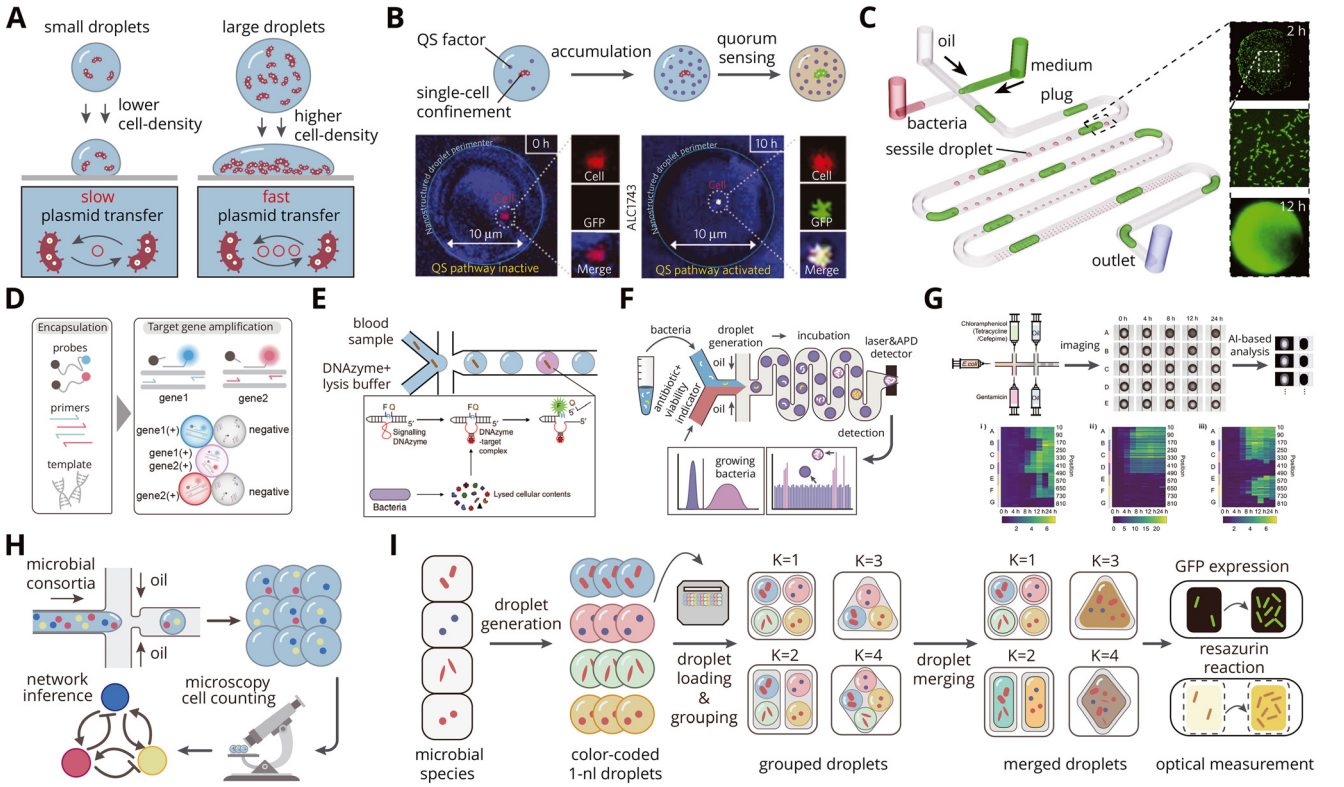

Fig. 4 Droplet microfluidics for assessing microbial activity and interactions. (A) Droplets with smaller sizes promote plasmid transfer in the microbial population.[101] (B) Single-cell quorum sensing activated in droplets.[195] (C) Microbial biofilm formation in sessile droplets for long-term studies.[196] (D) Multiplex ddPCR for amplification-based microbial identification.[197] (E) Amplification-free microbial identification using DNAzyme-based sensors.[198] (F) Microbial antibiotic susceptibility testing conducted *via* the DropFAST platform.[199] (G) AI-driven analysis platform for determining optimal antibiotic combinations based on high-throughput imaging and droplet content analysis.[200] (H) MINI-Drop method co-encapsulates multiple microbial species to investigate their interactions.[201] (I) The kChip for high-throughput and random fusion of droplets containing different microbial species. It allows analysis of growth dynamics and screening of synthetic microbial communities.[108] Panel B reprinted from ref. 195 with permission, copyright 2009, WILEY VCH. Panel C reprinted from ref. 196 with permission, copyright 2009, WILEY VCH. Panel G reprinted from ref. 200 with permission, copyright 2023, Royal Society of Chemistry.

and supporting long-term fungal cultivation, facilitating FADS-based screening of clones with high cell-wall-degrading enzymatic activity.[207] Furthermore, water–oil–water double emulsions were used in FACS to screen riboflavin-secreting mutant cells, achieving riboflavin production rates 56 times higher than the parent strain.[208]

Integrating microfluidic systems with advanced mass spectrometric techniques, such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), has revolutionized the analysis of microbial metabolites.[209,210] These methods offer high sensitivity and resolution for detecting metabolites at the single-cell level. For example, one study combined droplet microfluidics with ESI-MS to detect secondary metabolites like streptomycin from actinomycetales strains,[211,212] providing a robust tool for discovering novel antibiotics. Further innovations, such as using glass chips for droplet generation and fluorinated capillaries to eliminate surfactant interference in ESI-MS detection,[212,213] have facilitated biocatalysis detection at single-cell resolution. These droplet-MS detection techniques eliminate the need for labeling and streamline the discovery of functional microbial metabolites.

6.3 Droplet-based pathogen diagnostics and antimicrobial susceptibility testing

Antimicrobial resistance (AMR) poses a global health threat, with estimates suggesting it could result in 10 million deaths annually by 2050.[214] Delays in cultivation, pathogen identification, and antimicrobial susceptibility testing (AST) contribute to the overuse and misuse of antibiotics, exacerbating the AMR crisis. Droplet microfluidics offers a promising solution by facilitating faster pathogen cultivation, identification, and AST, thereby enabling precise antibiotic therapy and reducing the risks of treatment delays and inappropriate antibiotic use.

Droplet-based pathogen identification methods offer rapid detection, high sensitivity, and efficient processing of complex clinical samples. By compartmentalizing samples into thousands of droplets, droplet digital PCR (ddPCR) enables highly sensitive and multiplexed detection of methicillin-resistant *Staphylococcus aureus* (MRSA), even at low copy numbers, which is crucial for diagnosing early-stage infections (Fig. 4D). Compared to qPCR, ddPCR demonstrated significantly superior sensitivity (100% *vs.* 38.89%) and comparable or higher specificity (100% *vs.* 97.67%)

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in detecting MRSA.[197] Furthermore, in another comparative study, ddPCR exhibited greater precision and robustness than qPCR in quantifying *Cryptosporidium* oocysts from fecal samples, particularly under challenging conditions such as low DNA concentrations and the presence of PCR inhibitors.[215] Additionally, ddPCR played a critical role during the COVID-19 pandemic, assisting in the detection of low-copy-number viruses, viral load quantification, and monitoring viral concentrations in the environment.[216]

Amplification-free approaches, such as DNAzyme-based sensors, have also been integrated into droplets for rapid and specific bacterial detection directly from clinical blood samples (Fig. 4E).[198] Similarly, a droplet microfluidics system has been developed for the rapid diagnosis of urinary tract infections (UTIs) by using fluorogenic peptide nucleic acid (PNA) probes targeting 16S rRNA, allowing for amplification-free detection.[217] In antimicrobial susceptibility testing, ddPCR can assess susceptibility after 15 minutes of antibiotic exposure by measuring DNA replication through single-molecule counting, offering a rapid, phenotype-based approach to UTI testing.[218] Similarly, droplet digital loop-mediated isothermal amplification (ddLAMP) has been applied to determine *E. coli* susceptibility from clinical urine samples in less than 30 minutes, achieving accuracy comparable to standard methods.[219]

Droplet-based microfluidics provides additional benefits in AST by enabling faster detection, enhanced sensitivity, and high-throughput screening of antibiotic combinations.[220] For example, a rapid single-cell biosensing platform, dropFAST, was developed to encapsulate individual bacterial cells with a fluorescent growth indicator and antibiotics into picoliter droplets (Fig. 4F). After a short on-chip incubation, in-line fluorescence detection yields AST results within 1 hour.[199] This single-cell analysis enhances the accuracy of minimum inhibitory concentration (MIC) determinations by eliminating population-level interactions and nutrient competition, providing insights into antibiotic resistance mechanisms in clonally identical bacterial populations. A cascade filtration-droplet digital chromogenic assay system was also developed to identify carbapenem-resistant strains in urine samples without traditional isolation, achieving rapid identification by chromogenic reaction within 3.5 hours.[221] Furthermore, machine learning-based morphological recognition has been integrated with droplet microfluidics to automate the classification of bacterial growth within nanoliter droplets,[222,223] enabling the determination of colistin susceptibility profiles within 3 hours in a label-free manner and demonstrated similar accuracy to the traditional broth dilution method. Additionally, droplet microfluidics facilitates the assessment of antibiotic combinations, helping to optimize therapy, minimize side effects, and slow the evolution of multi-antibiotic-resistant bacterial strains (Fig. 4G).[200]

6.4 Investigating microbial interactions

In natural environments, microbes rarely exist in isolation, instead engaging in complex interactions with other species. This is equally true within the human body, where microbial interactions can significantly influence health and disease. These interactions generally fall into three categories: positive, negative, or neutral, based on phenotypic differences such as growth response or metabolic activity. However, despite advancements in bioinformatics tools like CoNet,[224] MetagenoNets,[225] and NetCoMi,[226] there remains a notable gap between *in silico* predictions and experimental outcomes in microbial interactions.

Droplet-based microfluidics has appeared as an effective tool for studying microbial interactions, offering greater precision and efficiency than traditional culture methods.[227] For instance, programmable droplet microfluidic devices have been designed to enable sequential merging of picoliter-volume droplets containing bacterial cells, allowing researchers to model complex microbial communities and study their stability and coexistence over time.[228] These systems help study symbiotic relationships, such as cross-feeding auxotrophs. One example involved co-culturing an RFP-labeled *E. coli* methionine auxotroph with a GFP-labeled *B. subtilis* tryptophan auxotroph, enabling the detailed study of positive microbial interactions (Fig. 4H).[201] Negative interactions have also been explored using droplet-based methods, such as a GFP-labeled *E. coli* strain producing a quorum-sensing molecule that inhibited another RFP-labeled *E. coli* strain. Further innovations include platforms like kChip, which can rapidly screen ~100 000 synthetic microbial consortia for growth promoters or inhibitors, providing valuable insights into species interactions, even under varying carbon sources (Fig. 4I).[108]

Microfluidic devices are not limited to studying interactions within microbial species but are also applied to interkingdom interactions, such as those between bacteria and fungi or phages and bacteria. For example, time-lapse fluorescence microscopy has been combined with droplet microfluidics to observe the growth and lysis dynamics of *E. coli* when challenged with phages.[229] In another study, a micrometabolomics device was used to demonstrate that *Aspergillus fumigatus* growth was inhibited in the presence of *P. aeruginosa*, while *Aspergillus flavus* exhibited enhanced chlamydospore formation when co-cultured with *Ralstonia solanacearum*.[230] These applications highlight the versatility of droplet microfluidics in uncovering a broad range of microbial interactions.

7. Application of droplet-microfluidics for investigating the role of microbes on human health

Research on microbe–host interactions has gained significant attention for its role in understanding human health. These interactions are crucial for the colonization and evolution of microbial communities and for regulating host physiology and immune responses. Traditionally, animal models, such as germ-free and pseudo germ-free mice, have been

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instrumental in studying these interactions. For example, germ-free mice have been used to demonstrate that certain microbial species can improve intestinal barrier function, modulate immune responses, or trigger diseases like intestinal inflammation and gastric cancer.[231,232]

Building on these insights, microbial therapies to deliver or cut specific microbes for disease treatment have garnered increasing interest. Droplet microfluidics, with its advantages in precise size control, high-throughput production, and efficient multilayer design, has been applied to encapsulate and target microbes and their byproducts. For instance, *Bacteroides fragilis*, enriched in breast cancer tissues, was utilized to create mineralized bacterial outer membrane vesicles using a microfluidic platform, enhancing antitumor immune responses when delivered to tumor tissues.[233] Similarly, microfluidic systems have been developed to co-encapsulate probiotics and postbiotics in double hydrogel droplets, targeting delivery to the colon for treating colitis by reducing inflammation and promoting intestinal barrier repair.[234] Furthermore, advanced colloidosome platforms with multiple protective layers were designed to enhance probiotic survival during gastrointestinal transit, allowing for targeted release in the colon with significant therapeutic effects on colitis.[235,236]

In addition to animal models, *in vitro* models play a crucial role in studying microbe–host interactions. Traditional two-dimensional (2D) cell cultures often fail to accurately mimic physiological conditions. Advances in tissue engineering have led to the development of three-dimensional (3D) culture systems, such as spheroids and organoids, which better replicate tissue microenvironments. Droplet-based microfluidics has created controlled environments for 3D cell growth and interactions through scaffold-free or scaffold-based methods. For example, scaffold-free droplet microfluidics has been used to generate multicellular spheroids for drug testing,[237] while scaffold-based methods incorporate extracellular matrix materials to study cell–matrix interactions and cellular differentiation.[238]

Despite these advances, droplet microfluidic devices have been less commonly applied to microbe–host interaction studies than human organ-on-chips systems.[239] This is primarily due to the latter's ability to more accurately replicate the complex physiological environments of human organs, including tissue architectures, fluid dynamics, and multi-organ interactions, such as the gut–liver axis.[240–244] Organ-on-a-chip systems also offer a continuous supply of nutrients and oxygen, which is crucial for long-term culture and monitoring of interactions. While droplet microfluidics has not yet reached this level of control, some analogous strategies have been developed. For instance, droplet pairing and fusion allow fresh medium to be introduced, mimicking fed-batch culture modes to some extent. Additionally, replacing the oil phase with culture media facilitates constant nutrient refreshment for immobilized cells within hydrogels, akin to continuous culture modes seen in bioreactors.[100,245] Such advancements highlight the potential for droplet microfluidics to evolve toward more robust and versatile platforms for modeling microbe–host interactions in the future.

8. Conclusion and outlook

Advances in multidisciplinary fields, particularly meta-omics technologies, have transformed human microbiome research from focusing solely on microbial composition to uncovering functional relationships between the microbiome and host health. Emerging evidence highlights that microbiota-derived bioactive compounds, including antigens, enzymes, small molecules, and extracellular vesicles, play essential roles in regulating both local and systemic physiological pathways. Despite these advances, the precise mechanisms by which individual microbial taxa influence host gene expressions and pathways remain largely unknown.

Droplet microfluidics has emerged as a transformative tool in microbiome research, enabling high-throughput isolation, cultivation, and analysis of individual microbial cells within picoliter to nanoliter droplets. This approach benefits both culture-dependent and culture-independent studies by enabling precise manipulation of microenvironments or reactions. Despite these advancements, several challenges remain. Variability in single-cell sequencing across different microbial species highlights the need for standardized preprocessing methods that can be applied to a broader range of microbes. Additionally, sequencing coverage and the amount of usable data are still limited, calling for improvements in molecular techniques, such as enhanced nucleic acid capture and amplification. Single-cell proteomics and metabolomics in microbes are still underdeveloped, and the integration of droplet-based multi-omics technologies to explore causal relationships between microbes and host phenotypes is in its preliminary stages. Further innovations in droplet-based spatial omics are also needed to better elucidate microbial spatial organization and interactions with their environments.

Despite its potential, droplet microfluidics also faces several limitations compared to traditional methods. Microbial cultivation within droplets can sometimes fail to replicate the complex interactions present in bulk cultures, potentially affecting the growth of cross-feeding species. Additionally, operational complexity and the lack of standardized interfaces hinder its accessibility for researchers with limited expertise in microfluidics. Droplet stability issues further complicate multistep, intricate reactions. Furthermore, the limited integration of droplet microfluidics with high-throughput automated platforms restricts its full potential for broader applications. These challenges underscore the need for automation and standardization of microfluidic device production and operation to enable scalable applications.

Recent advances in artificial intelligence (AI) offer promising solutions to these challenges. AI has demonstrated its ability to enhance device design,[246,247] sorting,[248] and functional analysis in droplet microfluidics.[249,250] AI-driven

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tools, including machine learning, are being used to optimize microbial media formulations,[251–253] perform image-based microbial identification,[254] and analyze multi-omics data.[255] Looking ahead, the convergence of large-scale experimental data generated through droplet microfluidics and AI-driven analytics holds the potential to revolutionize human microbiome research. By enabling high-resolution, large-scale investigations, these technologies can uncover novel therapeutic strategies that harness the microbiome's potential for improving human health.[256–258] This approach paves the way for transitioning from understanding microbial diversity to unlocking microbial functionality, driving advancements in precision medicine and the development of innovative treatments for microbe-associated diseases.

Data availability

No new data were generated or analyzed in this study. Data sharing is not applicable to this article as it is a review of existing literature.

Author contributions

Y. X. and Z. W. contributed equally to drafting and visualization of the original manuscript. C. L. contributed to visualization. S. T. was involved in investigation and editing. W. D. provided supervision, contributed to conceptualization, editing, and visualization.

Conflicts of interest

The authors declared no conflict of interest.

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