This is a “preproof” accepted article for *Animal Nutriomics*.

This version may be subject to change during the production process.

10.1017/anr.2024.17

Title: Culturomics: Bringing Culture Back to the Forefront for Effective Gastrointestinal Bacterial Capture

Lingxi Chen[1,2#], Yujie An[1,2#], Kailang Huang[1,2], Junhong Wang[1,2], Yuanyuan Zhang[1,2] and Jiakun Wang[1,2,3*]

[1] MoE Key Laboratory of Molecular Animal Nutrition, Zhejiang University, Hangzhou, China
[2]Institute of Dairy Sciences, College of Animal Sciences, Zhejiang University, Hangzhou, China
[3]Key laboratory of Animal Feed and Nutrition of Zhejiang Province, Zhejiang University, Hangzhou, China
[#]Equal contributors

Competing interests: The author(s) declare none

Abstract

The gastrointestinal microbiota plays a crucial role in host nutrition and health. While culture-independent techniques have advanced microbial research, they often overlook low-abundance bacteria in the microbial community. Furthermore, empirical studies and mechanistic research rely on bacterial isolates obtained through culturing. The introduction of culturomics in 2012 significantly advanced culture-dependent techniques, contributing to microbial research. However, these methods remain labor-intensive, time-consuming, and costly, similar to traditional culture methods. This review provides an overview of the contributions of culturomics, summarizes procedures for optimizing sample and culture processes, and offers guidance on utilizing metagenomic data to enhance culturomics workflows. By collating and synthesizing these developments, this review aims to provide valuable insights into improving the practicality and productivity of culturomics for capturing gastrointestinal bacterial communities.

Keywords

Culturomics, Gastrointestinal bacteria, Workflow, Metagenomics, Uncultured bacteria.

Introduction

The gastrointestinal microbiome is as significant as a major organ; it plays a vital role in host health and nutritional status [1-4]. The gastrointestinal microbiome profoundly impacts the economic and ecological benefits of livestock, including ruminants, pigs, and poultry [5-7]. Over the past two decades, advances in culture-independent techniques, particularly metagenomics, which focuses on recovering genetic material from any sample, have revolutionized our understanding of microbial ecology [8-10].

However, 16S rRNA gene amplicon sequencing results can vary due to differences in primer selection, and classifying rare and novel bacteria remains challenging [11, 12]. Although metagenomics offers significant advantages and promising results, it often fails to provide reliable taxonomic characterization between strains of bacterial species [11]. Moreover, the diversification of bioinformatics software has introduced complexities and inconsistencies in sequencing analysis, potentially obscuring some taxa [13, 14]. The isolation of bacterial strains and subsequent validation represents the most compelling approach to demonstrating their roles in ecological niches and developing novel biotechnological applications. However, numerous gastrointestinal bacteria remain uncultured [15-17].

Lagier et al. [18], Dubourg et al. [19], and Ferrario et al. [20] reported that only 15% of the detected species overlapped between culture-independent and culture-dependent techniques, while Li et al. [21] reported an overlap of 8%. These findings support the essential role of culture in describing new prokaryotic species

and bridging metataxonomic gaps. With the advent of culturomics, culture-dependent techniques have regained prominence, complementing culture-independent approaches in gastrointestinal bacterial research and contributing to significant progress. The number of cultured bacterial species has increased rapidly, supplementing the repertoire of isolated gut bacteria [22-24]. Furthermore, culturomics enables the capture of bacteria of interest, including those with low abundance and specific taxa. For instance, Lagier et al. [25] isolated 174 species previously undescribed in the human gut. Further, Browne et al. [26] identified 90 species from the Human Microbiome Project's 'most wanted' list, and Liu et al. [27] successfully cultured 12 bacterial species harboring urease genes, contributing to a 34.38% increase in ureolytic species.

Similar to traditional culture methods, culturomics faces inefficiencies such as tedious and labor-intensive procedures, substantial hands-on time, and a low success rate of isolating targeted microorganisms. Therefore, this review aims to outline strategies for optimizing culturomics by improving sample conditioning and processing techniques, customizing media formulations, updating culture vectors, diversifying gelling agents, prolonging pre-incubation periods, implementing oligotrophic culturing methods, optimizing colony-picking procedures, and leveraging insights from metagenomics data. Our objective is to provide valuable insights into enhancing culturomics methods for more effective gastrointestinal bacterial capture.

The contribution of culturomics to scientific knowledge

'Culturomics', pioneered in 2012, is a comprehensive approach to expanding the bacterial repertoire by diversifying culture conditions, complemented by identification through matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and/or 16S rRNA gene sequencing [25]. There are two alternative strategies for culturomics: non-targeted and targeted [28]. Through these strategies,

culturomics can culture and identify species that were previously unculturable or unreported within specific niches and pave the way for practical applications (Figure 1).

Filling metataxonomic gaps

The term 'microbial dark matter' refers to uncultured microbes that can only be detected through sequencing. However, 16S rRNA gene sequencing may miss low-abundance microorganisms due to limited sequencing depth. Although metagenomic sequencing has improved with short-read methods, excelling in sequencing depth and diversity, and long-read methods, enhancing accuracy and 16S amplicon recovery, detecting low-abundance species remains challenging. Additionally, factors such as sampling, DNA extraction, and quality control can obscure certain taxa [13, 14, 29]. An increasing number of studies have indicated that some bacterial taxa, especially low-abundance bacteria, are overlooked in culture-independent sequencing. For instance, Zehavi et al. [30] reported that 60% (1,012 out of 1,698) of cultured

operational taxonomic units (OTUs) were absent in 16S rRNA gene sequencing results, representing rare taxa within the rumen. Whelan et al. [31] merged culture-dependent and culture-independent sequencing technologies to investigate cystic fibrosis lung bacteria, revealing that 63.34% of OTUs were exclusively detected with culture-dependent sequencing technology, with only 5.65% detected by culture-independent methods. Goldman et al. [22] enriched four types of vertebrate fecal samples with various culture media before 16S rRNA gene amplicon sequencing, finding 340 bacterial genera across culture-independent and culture-dependent samples, with 115 genera exclusively identified via culture-dependent sequencing, including 12 uncultured candidate genera. Li et al. [32] reported similar results in their study on desert soil microbiota using cultured-based sequencing, revealing that 2,799 amplicon sequencing variants (ASVs; 74.1%) were exclusively detected through culture-dependent sequencing, and only 83 ASVs (2.2%) overlapped between culture-dependent and culture-independent sequencing. Feehily et al. [33] confirmed the vertical transmission of *Bifidobacteria* using dual culture-based and metagenomic approaches, identifying 16 low-abundance strains solely through culture-dependent sequencing. Through five consecutive cultures of drinking water samples followed by Oxford Nanopore Technology sequencing, Fu et al. [34] demonstrated that culturing significantly improved the quality of metagenome-assembled genomes (MAGs). This approach yielded 86 high-quality MAGs, including 70 target pathogenic bacteria, whereas culture-independent methods produced only 12 MAGs.

With culturomics, the repertoire of isolated human bacterial species, particularly from the gastrointestinal tract, has rapidly expanded. Didier Raoult's team at King Abdulaziz University systematically tracked cultured human prokaryotic species, establishing and regularly updating the repertoire through comprehensive searches of culture databases and scientific literature. Their findings revealed that in 2015, only 2,172 prokaryotic species isolates were reported from humans [35]. This number increased to 2,776 in 2018 and further to 3,253 by 2020. Notably, 63% of the additional 477 species from 2018 to 2020 were isolated via culturomics [36, 37]. Another research group led by Liang Xiao at BGI-Shenzhen focused on human gut bacteria using culturomics. In 2019, they identified 338 species from 155 fecal samples using culture-dependent whole-genome sequencing and established the culturable genome reference (CGR), the largest collection of human gastrointestinal bacterial genomes, which included 149 candidate new species [24]. By 2023, they expanded the database to CGR2, comprising 527 species, including 179 unidentified ones, of which 126 were cultured for the first time [38]. Furthermore, they cultured 195 species from human oral samples, identifying 95 potential novel ones, and established the cultured oral bacteria genome reference (COGR) [39]. Despite these advances, the full spectrum of human bacteriota remains to be explored through culturomics.

Initial reports of culturomics primarily focused on human subjects and have progressively expanded to include various domestic animals (i.e., chicken in Ferrario

et al., 2017; cow in Zehavi et al., 2018; swine in Wang et al., 2021 [20, 30, 40]) and wildlife species (i.e., Egyptian mongoose in Pereira et al., 2020 [41]). Ferrario et al. [20] curated a collection of 420 unique bacterial strains, including isolates uncommon in the chicken gut. With systematic culturomics, Crhanova et al. [42] increased the bacterial culturability *in vitro* to over half of the chicken cecal bacteriota. Zehavi et al. [30] reported that 23% of rumen prokaryotic microorganisms were culturable. Wang et al. [40] comprehensively cultured swine gut bacteria and found that approximately 50% of total bacterial ASVs and 75% of genera and families, including several novel species, were culturable. Using 25 types of culture media in conjunction with metagenomics, Dong et al. [43] expanded the gut bacteriota repertoire of weanling piglets by identifying 333 new species.

In conclusion, the key benefit of culturomics is its ability to recover bacterial taxa that are overlooked in culture-independent sequencing, thereby enhancing the resolution of bacterial community profiles and improving our understanding of the composition of complex bacterial communities. In the context of bacterial research, the integration of high-throughput methods and culture is the most efficient approach to expanding the repertoire of gut bacterial isolates. Culturomics, in particular, can facilitate the identification of numerous candidate strains for prospective experiments.

Paving the way toward bacteriotherapy

Culturomics plays an important role in clinical microbiology with its ability to improve bacterial identification, facilitate mechanistic studies of host-microbial

interactions, and increase the isolation of potential pathogenic or therapeutic organisms. Using culturomics, Dubourg et al. [44] identified pathogenic bacteria such as *Clostridioides saudii*, *Clostridioides jeddahtimonense*, *Clostridioides culturomicsense*, *Butyricimonas phocaeensis*, and *Anaerosalibacter massiliensis*, which were among the first cultured from human fecal samples. Using a non-strictly defined culturomics approach, Khanna et al. [45] developed SER-109, an oral microbiome therapeutic comprised of 50 spore-forming bacterial species isolated from healthy fecal microbiota transplantation donors. SER-109 successfully prevented *Clostridium difficile* infection and demonstrated greater safety than fecal microbiota transplantation. This finding further supports the potential of designing and developing personalized formulations that include spore formers for the transfer of obligate anaerobes. Additionally, Ghimire et al. [46] discovered that 66 out of 102 bacterial species cultured from healthy human donors inhibited *C. difficile*. They observed the importance of species composition and interactions in achieving effective inhibition.

Based on culturomics and metagenomics investigations of gut bacteria in individuals with kwashiorkor and healthy children, Alou et al. [47] identified the 12 species detected only in healthy individuals as potential probiotics. Through investigations of intestinal bacteria in two patients and two healthy individuals using culturomics, Fellag et al. [48] found that four *Enterococcus mundtii* and *Enterococcus casseliflavus* strains could inhibit the growth of *Mycobacterium tuberculosis*, the causative agent of

pulmonary tuberculosis. Future research integrating culturomics, metagenomics, and machine learning technologies will improve the identification of probiotics and their contributions to bacteriotherapy.

Creating a new method for drug screening

Li et al. [49] integrated culturomics, bionics, and metabolomics to study probiotic-driven drug metabolism. They discovered that *Lacticaseibacillus casei* Zhang could metabolize racecadotril into its active ingredients in a system mimicking the human intestine *in vitro*, capturing individual responses to the drug. However, the drug metabolism pattern may differ between a single strain and bacterial communities, with the latter being more suitable for drug screening before clinical application, as it more closely resembles the bacterial status *in vivo* [50].

The non-targeted strategy of culturomics has the potential to maximally reflect the genuine status of the bacteriota from specific ecological niches, making it invaluable for drug screening and precision medicine [51, 52]. Javdan et al. [53] developed a BG medium by mixing modified Gifu anaerobic medium (mGAM) and Bryant and Burkey medium (BB) at a ratio of 3:7. Using this medium to screen 23 drugs in fecal sample cultures from 20 donors, they quantified the interpersonal differences in microbiome-derived metabolism. Building on the study by Javdan et al. [53], Tao et al. [54] optimized a new medium, termed GB, by mixing mGAM and BB at a ratio of 5:5 through multi-dimensional evaluation (Figure 2), which more accurately mimics the status of the human gut bacteriota than BG. Tao et al. [54] tested three representative

drugs using bacteriota from 10 donors and found that individual responses to different drugs, influenced by personalized gut bacteriota, could be captured by GB. This medium can effectively capture the human fecal bacteriota in a personalized manner, providing researchers with a valuable tool that can significantly advance personalized medicine.

Improving culturomics workflows for enhanced bacterial capture

Despite its significant contributions to bacterial culture over the past decade, culturomics remains a labor-intensive and time-consuming methodology with a low success rate for isolating low-abundance bacteria. Optimizing sample conditioning to preserve the viability of bacteria and increase the competitiveness of low-abundance bacteria is essential. Furthermore, many bacteria require highly specific *in vitro* growth conditions. In these instances, the culturomics-based method must be tailored to meet these unique requirements.

Sample conditioning

Goldman et al. [22] found that only 109 ASVs (5% of all detected ASVs) could be assigned to the species level and overlapped between culture-independent sequencing and culturomics. Most bacteria detected exclusively by culture-independent methods belonged to *Lachnospiraceae*, *Ruminococcaceae*, and *Erysipelotrichaceae*, consistent with results reported by Pfleiderer et al. [55] (Figure 3). These families predominantly comprise strictly anaerobic species, indicating that conditions of sample collection

play a critical role in culturomics. Pfleiderer et al. [55] recommended collecting fecal samples into containers with continuous gas generation and immediate transfer to an anaerobic chamber at 4 °C.

Bellali et al. [56] investigated human fecal samples using flow cytometry and the plating technique, revealing that culturability was 50% when the samples were exposed to oxygen for 120 min without antioxidants, but increased to 67% when protected by a protectant medium (patented N°1H53316 CAS 25 FR; Table 1). Furthermore, maintaining samples under anaerobic conditions during processing and limiting oxygen exposure to less than 2 min resulted in an 87% culturability rate, showing no significant differences between these two techniques. Given the anaerobic nature of the gastrointestinal tract and the difficulty in culturing strictly anaerobic species, it is essential to protect gastrointestinal samples from oxygen by shortening the exposure time to oxygen and using a protectant medium.

Sample processing

Sample dilution exhibits a strong effect on the richness of isolated bacteria. Zehavi et al. [30] diluted cows' rumen fluid from 10^{-1} to 10^{-6} and plated samples on medium 10 (M10) and M10 supplemented with rumen fluid, revealing that sample dilution had a significant impact on increasing the richness of isolated bacteria. Notably, dilution explained a higher variance (30%) in bacterial isolation compared to the type of medium used, illustrating that serial dilution can efficiently reduce the dependence of culturability on initial abundance (Figure 4). Increasing dilution led to changes in

bacterial composition: at the phylum level, the relative abundance of *Bacteroidetes* decreased, while *Firmicutes* increased. At the order level, there was a progressive increase in *Lactobacilli*, *Bacilli*, and *Enterobacteria*. Yadav et al. [57] established the 'two-dimensional cell separation' by combining serial dilution with gradient centrifugation, successfully isolating species at a rate more than three-fold higher compared to using dilution alone.

Passive filtration is another useful process for isolating motile and small-sized *Treponema* species. Belkacemi et al. [58] demonstrated the effectiveness of a device consisting of two chambers separated by a 0.2 µm filtration membrane, which enabled *Treponema* movement through the membrane from the upper compartment to the lower compartment, facilitating isolation. After serial dilution and secondary isolation on agar medium, Belkacemi et al. [58] successfully obtained pure cultures of 10 strains of *T. pectinovorum* and five strains of *T. denticola*.

Harsh treatment methods are effective for isolating bacterial spores due to their higher resistance compared to growing cells. Pre-treatment with heat, chemicals, or radiation has been shown to efficiently isolate spores [59]. For instance, Hong et al. [60] heated human fecal samples at 65 °C for 1 h, resulting in the isolation of 88 heat-resistant bacteria when combined with serial dilution. Golnari et al. [61] heat-shocked various samples, including fecal, soil, and dairy products, at 80 °C for 15 min and successfully isolated 36 strains of spore-producing bacteria. Browne et al. [26] treated human fecal samples with 70% ethanol at an equal volume for 4 h, revealing that

ethanol treatment profoundly changes the composition of culturable bacteria with an increase in the abundance of ethanol-resistant taxa. Afouda et al. [62] pretreated human stool samples with 50% ethanol (final concentration) for 1 h, demonstrating that ethanol treatment enhances the proportion of spores and complements direct culturing (Figure 5). Sample dilution, passive filtration, and harsh treatments effectively modify bacterial community compositions within the niches, increasing the relative abundance of specific taxa and enriching the diversity of cultured bacterial species.

Media customizing

Reducing the workload is essential to applying culturomics. In 2012, Lagier et al. explored 212 culture conditions with three stool samples, finding that only 70 were necessary to capture all isolated species [25]. Diakite et al. [63] further refined these conditions in 2020 to 16 highly efficient formulations (Table 2), achieving a 98% isolation rate of cultured human gut bacteriota. The effectiveness of yeast extract-casitone-fatty acids agar (YCFA) was previously reported by Browne et al. [26]. Lagier et al. [64] suggested three key procedures for maximizing bacterial isolation from the human gastrointestinal tract: (i) pre-incubation in a blood culture bottle (yielding 56% of the newly isolated species); (ii) the supplementation of filter-sterilized rumen fluid during pre-incubation (resulting in 40% of the newly isolated species); and (iii) the addition of 5% sheep blood (contributing to 25% of the newly isolated species).

The culture media used for the cultivation of gastrointestinal bacteria in pigs or chickens differ slightly from those used for humans (Table 3). The mGAM, *Bifidus* selective medium (BSM), Wilkins-Chalgren anaerobe broth (WCA), fastidious anaerobe agar (FAA), phenylethyl alcohol agar (PEA), modified peptone-yeast extract-glucose medium (MPYG; with or without sheep blood), and sheep blood-spore medium have been found suitable for the cultivation of gastrointestinal bacteria from pigs [43, 64, 66, 67]. Wang et al. [40] cultured the fecal samples from pigs at four growth stages using 53 different culture conditions, generating a heatmap to guide the isolation of specific bacterial taxa (Figure 6). Crhanova et al. [42] suggested that for the chicken cecal bacteria, nutrient broths with mono- or di-saccharides were suitable for selective culture of *Lactobacillaceae*. Supplementing with bile salts efficiently enriched *Veillonellaceae*, while YCFA was beneficial for increasing the abundance of *Desulfovibrionaceae.* Additionally, the addition of trimethoprim, colistin, nalidixic acid, and streptomycin selectively enriched *Erysipelotrichaceae*.

For the *Treponema* species, a T-Raoult medium [58], derived from oral treponeme enrichment broth (OTEB), OMIZ-Pat medium, new oral spirochaetae medium (NOS), and spirochaetae medium, have proven effective for culturing various *Treponema* species. Given the diverse niches and bacterial compositions within the gastrointestinal tracts of different hosts, culturomics can achieve greater efficiency by employing host-specific media. Future research integrating metagenomics and

metabolomics will provide valuable insights into capturing specialized bacteria within their respective hosts.

Vector updating

Conventional tools for bacterial culturing include plates, tubes, and bottles. Given the demanding nature of culturomics, there is a strong demand for new vectors with higher throughput. Cheng et al. [69] successfully used 96-well plates to culture 104 bacterial isolates and construct a synthetic community. These plates are capable of harvesting numerous bacterial isolates from a complex microbial community. Zhang et al. [70] established a protocol for bacterial isolation using 96-well plates with limited dilution and bacterial identification through the 'Culturome' bioinformatic pipeline (https://github.com/YongxinLiu/Culturome), achieving high-throughput cultivation and identification of root-associated bacteria within 8-9 weeks. This approach using 96-well plates could be adapted for isolating gastrointestinal bacteria, significantly reducing labor and costs. In another study, Liu et al. [27] used 96-well plates as incubation vectors in the isolation of ureolytic bacteria from cattle rumen, yielding a total of 976 single-strain colonies, comprising 404 unique strains and 52 clusters with < 98% 16S rRNA gene identity. One strain from each cluster underwent whole-genome sequencing, revealing 28 strains carrying urease genes classified into 12 species. This increased the number of phenotypically confirmed ureolytic species by 45.83%. Due to its convenience, cost-effectiveness, and high throughput, the

96-well cell culture plates prove to be a promising tool for advancing culturomics research.

Gelling agents diversifying

Agar is the most commonly used gelling agent in bacterial culture media due to its advantageous material qualities [71]. However, the traditional preparation process of agar medium has a significant drawback: autoclaving agar together with phosphate produces hydrogen peroxide, which can inhibit the growth of some taxa. To mitigate this issue, it has been suggested that phosphate and agar should be autoclaved separately and mixed just before solidification [72]. Further, Tanaka et al. [73] reported that using a soft agar medium with 0.4% agar, which supported faster molecule diffusion, is more effective in isolating single colonies compared to the conventional 1.5% agar concentration. Nyonyo et al. [74] found that modified basal liquid medium (MBM) supplemented with 1.8% agar captures a greater diversity of isolates from cow rumen content compared to MBM adding gellan gum (0.8% Phytagel or 1% Gelrite). However, isolates from certain genera could only be cultured using gellan gum. Building on this, Nyonyo et al. [75] added azo-carboxymethylcellulose to MBM containing either agar or gellan gum to facilitate the isolation of ruminal cellulolytic bacteria, revealing that gellan gum performed better in supporting the growth of these bacteria. Nishioka et al. [76] achieved a similar result, where several recalcitrant bacteria from *Dendrobium* plants were only isolated using a medium containing gellan gum. These findings illustrate the

complementarity between agar and gellan gum and emphasize the importance of diversifying gelling agents. Other gelling agents that could be explored in culturomics include gelatin, xanthan gum, guar gum, isubgol, and carrageenan [71]. Diversifying gelling agents and their concentrations may enhance the success rate of gastrointestinal bacterial isolation.

Pre-incubation prolonging and oligotrophic culturing

Given the variable growth rates of bacteria in laboratory culture environments, it is important to account for slow-growing taxa in culturomics research. Microorganisms are generally classified as eutrophic and oligotrophic based on their nutritional requirements [77]. However, this classification is often applied to bacteria from extreme environments, such as deserts and oceans [78-80], indicating that a large proportion of oligotrophic bacteria in the gastrointestinal tract may be overlooked. Traditional culture media are typically nutrient-rich, which can overshadow the presence of oligotrophic bacteria, rendering some bacteria 'unculturable'. Pulschen et al. [81] successfully cultured several previously uncultured bacteria from Antarctic soils using oligotrophic conditions and prolonged incubation periods. Gray et al. [82] studied a non-sporulating *Bacillus subtilis* strain from the soil that could be cultured in extremely diluted media, growing very slowly under deep starvation conditions while remaining metabolically active. They termed this slow growth the 'oligotrophic growth state'. These findings highlight the different growth patterns of bacteria in varying media and underscore the importance of oligotrophic culture. Considering the

accumulation of metabolism waste, Chang et al. [83] examined the effect of regularly supplementing the culture with fresh media in a 30-day continuous enrichment period, isolating a total of 106 bacterial species from human fecal samples, including three novel species and six not previously isolated from the human intestine, thereby increasing the isolation rate by 22%. Huang et al. [84] subsequently conducted a trial of oligotrophic culture, demonstrating that 10-fold diluted media yielded a higher number of bacterial species than undiluted media, and 24 species account for 34.7% of the total isolated species which are unique to the oligotrophic media. This highlights the importance of employing oligotrophic media to suppress the dominance of eutrophic bacteria in laboratory cultures. Future studies should aim to isolate more gastrointestinal bacteria using oligotrophic media and a prolonged culture period. Comprehensive genomic analyses of these oligotrophic bacteria have the potential to further enhance our understanding and discovery of 'microbial dark matter' in the gastrointestinal tract.

Colony picking

The morphological characteristics of colonies, including size, color, smoothness and so on, should be considered when picking colonies from a solid plate. There is no standardized method, instead, researchers rely on subjective judgment based on their experience. This inevitably results in some bacteria being lost due to researchers' biases. To mitigate this, colonies are sometimes picked in large numbers, leading to an increased workload in culturomics. Chang et al. [83] compared 'experienced'

selective colony picking with picking all the colonies from the plate and found that the former decreased the rate of bacterial acquisition by only 8.5%, significantly reducing labor. Huang et al. [23] developed CAMII, a platform for automated microbiome imaging and isolation. This platform can analyze the size, density, color, circularity, convexity, and inertia of the colonies on the agar plate using a dual-illumination imaging system. To select valuable colonies, it employs a robotic colony picker for high-throughput microbial isolation and can learn from colony morphology data, guiding more standardized colony picking. This method can be combined with sequencing results to correlate taxonomic and morphological data. Additionally, potential interactions between bacteria can be observed by monitoring the growth status of adjacent colonies on agar plates. With the assistance of computers and robots, colonies can be comprehensively picked, reducing human labor.

Efforts to optimize sample handling and culture procedures in culturomics are summarized in Figure 7. Key steps include handling samples quickly and using antioxidants to protect them from oxygen, employing sample dilution or combining it with centrifugation for better isolation, implementing passive filtration for *Treponema*, and adopting harsh treatments for spore-forming bacteria before the culture procedure to ensure efficient culturing. During the culture process, several methods can enhance the efficiency of culturomics while reducing time, labor, and cost. These include customizing media for samples from diverse hosts or specific bacteria, updating vectors by using 96-well plates to improve throughput, diversifying gelling agents by

varying types and concentrations, prolonging pre-incubation, employing oligotrophic culturing, and optimizing colony picking.

Using metagenomic data to bring uncultured bacteria into cultivation

Advancements in culturing techniques, such as microcapsules [85], micro-Petri dishes, million-well growth chips [86], and diffusion chambers for environmental samples [87], have enhanced microbial culturability. However, the inability to replicate natural habitats *in vitro* means that some bacteria remain uncultured. Metagenome assembly facilitates the extraction of bacterial genome sequences from metagenomic data. Currently, numerous software tools are available for assembling MAGs. The combination of short-read and long-read sequencing can enhance MAG quality [88]. single amplified genomes (SAGs) can be obtained by single-cell sorting from complex microbiota. The interpretation of genomic information of target bacteria can guide various stages of the culturing process, including pre-culture sorting, media and condition optimizing, and post-culture analysis. By integrating metagenomic data, culturomics can overcome traditional limitations and reduce the associated workload (Figure 8).

Pre-culture screening

Traditional culturing methods typically employ a ‘culture-first’ strategy, which significantly increases the workload and often results in the loss of target bacteria during non-*in situ* culture processes. In contrast, the ‘screen-first’ strategy requires the

identification of target bacteria from complex communities, followed by cell sorting to isolate them precisely. Metagenomics offers comprehensive genomic data about bacteriota, enabling the design of specific probes and primers, predicting bacterial extracellular membrane structures, and reconstructing metabolic pathways. The integrated data facilitates the rapid identification of target bacteria and enables the efficient implementation of the 'screen-first' strategy, thus substantially reducing the overall workload.

Metagenomic data can be utilized to reconstruct the metabolic pathways of target bacteria, while single-cell Raman spectroscopy (SCRS) combined with stable isotope probes allows for distinguishing metabolic phenotypes of cells based on Raman spectral shifts caused by substrate uptake. For instance, heavy water is often used to incubate target bacteria and subsequent metabolic activity is detected through Raman spectral shifts in the carbon-deuterium (C-D) vibration band at 2000–2300 cm^{-1} [89]. Following identification using SCRS, the next challenge shifts to isolating single cells from samples. Raman-activated cell sorting (RACS) integrates SCRS technology with single-cell sorting to achieve targeted bacterial with specific metabolic functions. Previously, RACS was combined with Raman-activated cell ejection (RACE) to obtain single cells. However, the small size and light weight of bacteria resulted in low ejection success rates and limited high-throughput cell sorting capability. To address these limitations, Wang et al. [90] introduced the integrated Raman-activated droplet sorting (RADS) microfluidic system, which is capable of high-throughput

sorting at rates up to 260 cells/min. Xu et al. [91] developed the Raman-activated gravity-driven single-cell encapsulation (RAGE) system, which performs SCRS in the liquid phase within a biologically active environment before packaging the target bacteria into picoliter droplets, obtaining 'one-cell-one-tube' samples of target bacteria. This system significantly improves genome coverage from less than 10% to over 92% of the sorted single-cell genome. Subsequently, Jing et al. [92] developed single-cell Raman-activated sorting equipped with a RAGE chip to pre-screen phosphate-solubilizing microbes (PSM) in wastewater, followed by culturing the bacteria. This led to the isolation of four strains of strong organic-PSM and one low abundance organic-PSM strain, *Cutibacterium* spp., which had not been previously reported for phosphate-solubilizing activity. Notably, the phosphate-solubilizing activity of these strains in pure culture was significantly lower than *in situ*, underscoring the importance of pre-screening in the natural environment.

Cross et al. [93] used the SAG data from the human oral bacterium *Saccharibacterium* TM7a to identify and retrograde its extramembrane proteins. These proteins were then screened as candidate antigens for antibody development. After incubation with fluorescent-labeled antibodies, the target bacteria were sorted using flow cytometry, and living cells were enriched and cultured. A modified high-speed flow cytometry system has proven valuable in gut bacteriota research as it enables the maintenance of anaerobic conditions during cell sorting [94].

Batani et al. [95] modified the standard fluorescence *in situ* hybridization (FISH)

technique to enhance bacterial survival during the process a method they termed ‘live FISH’. This modified approach involved designing FISH probes based on bacterial 16S rRNA gene sequences. These probes were used in conjunction with fluorescence-activated cell sorting (FACS) to obtain target live bacteria for subsequent enrichment cultures.

Bacteriophages are highly host-specific viruses, making their native or derived molecules excellent candidates for specific bacterial binding probes. Advances in metagenomics have enabled the deduction of phage sequences and their host interactions from genomic data. Hosokawa et al. [96] developed a probe for the specific detection of *Streptococcus* using SAGs from oral uncultured bacteria. They identified a cell-wall binding domain (CBD) phage endolysin as the basis for the probe and combined it with magnetic separation flow cytometry to specifically enrich *Streptococcus* populations. In conclusion, pre-culture screening can be divided into two key steps: screening and sorting. Screening identifies target bacteria from complex microbial samples while sorting techniques isolate these target bacteria for subsequent enrichment cultures. Prioritizing pre-culture screening is essential for more efficient isolation of gastrointestinal bacteria. Furthermore, many techniques from environmental microbiology, when used in conjunction with anaerobic environments, can facilitate the effective isolation of gastrointestinal bacteria.

Media composition optimizing

The analysis of MAG or SAG sequences can provide important insights for

successfully culturing target bacteria, guiding the optimization of medium composition, and significantly enhancing isolation success rates. Over the past 20 years, advancements in metagenomics have led to numerous successful applications of using metagenomics data for optimizing selective media [65, 97-99]. In these cases, target bacterial genomes were analyzed to (i) identify functional or resistance genes; (ii) reconstruct metabolic pathways by screening for complete gene clusters; and (iii) detect genetic defects, such as the absence of essential functional genes.

Using MAG or SAG data, functional genes of target bacteria can be inferred to aid in configuring selective media that screens out non-target bacteria lacking these genes. For instance, Tyson et al. [98] analyzed sequencing data from a low-diversity subsurface acid mine drainage (AMD) biofilm and identified a single *nif* operon in a previously uncultured *Leptospirillum* Group III genomic fragment. This operon was presumed to be unique to nitrogen-fixing bacteria in this bacteriota. Based on this finding, a nitrogen-free medium was designed, leading to the successful isolation of *Leptospirillum* Group III. Additionally, incorporating antibiotics into the culture medium is another effective strategy to inhibit non-target bacterial growth. The comprehensive antibiotic research database (CARD) facilitates this by predicting resistance genes present in target bacteria based on genomic sequences. CARD is continuously updated, with its current version 3.2.4 encompassing 6627 ontology terms, 5010 reference sequences, 1933 mutations, 3004 publications, and 5057 detection models [100, 101]. In addition to identifying individual functional genes,

MAG and SAG sequences can be used to reconstruct metabolic pathways, aiding in selecting appropriate substrates for the medium. The antibiotics and secondary metabolite analysis shell (antiSMASH), a powerful tool for mining bacterial secondary metabolites based on genomic data, allows for the analysis of biosynthetic gene clusters in bacterial genomes [102, 103]. For instance, Viacava et al. [99] performed metagenomic and metatranscriptomics analyses on enriched soil bacteria, annotating 17 key enzymes of MAGs and their involved metabolic pathways. This data was instrumental in designing a selective medium tailored for isolating the anaerobic arsenic-methylating soil bacterium *Paraclostridium* sp. strain EML.

It is also possible to derive information about genomic deficiencies to optimize medium composition, enabling the growth of target bacteria under non-*in situ* culture conditions. *Tropheryma whipplei*, the pathogen of Whipple's disease, is particularly challenging to cultivate under laboratory conditions and has been isolated only in association with eukaryotic cells. Renesto et al. [97] predicted the metabolic pathways of *T. whipplei* from its genome sequence, revealing the absence of all genes required for synthesizing nine amino acids. This genomic deficiency indicated that *T. whipplei* relies on exogenous sources for these amino acids. Consequently, by supplementing the culture medium with these nine amino acids, the researchers successfully isolated a new strain of *T. whipplei.*

Culture conditions optimizing

Genomic data offers valuable insights into bacterial adaptations to the environment.

Weimann et al. [104] developed Traitar, a fully automated software package designed to predict phenotypic traits from genomic sequences. This tool can predict various microbial characteristics, including substrate utilization for carbon and energy sources, morphology, antibiotic susceptibility, proteolysis, enzymatic activities, oxygen requirements, and optimal growth temperature. Traitar's accuracy was confirmed with data from 572 species spanning eight phyla. Sauer et al. [105] expanded on the principles of reverse ecology through big data analysis, demonstrating that genomic data can predict not only the optimal growth temperature of bacteria but also other quantifiable environmental parameters. These parameters include pH, salinity, osmolality, and oxygen concentration. By uncovering these characteristics from genomic data, their approach can facilitate culturomics studies. Genomic data contains a vast amount of information that can guide the refinement of culture conditions, suggesting that future software could be developed to predict comprehensive, quantifiable culture conditions based on MAG or SAG sequences guiding culturomics studies.

Post-culture screening

The use of extreme dilution combined with 96-well plates has proven to be an effective method for isolating a single bacterium per well, ensuring that each well contains no more than one bacterium due to the high dilution factor. Following high-throughput culture in 96-well plates, Liu et al. [27] used *ureC*-specific primers for post-culture screening, which efficiently and conveniently identified candidate

urea-degrading bacteria. Functional genes identified from MAG or SAG data can be used to design specific primers to guide the isolation process. Additionally, pre-culture screening methods, including fluorescently labeled antibodies, can also be applied after the culture to further refine isolation and identification.

Furthermore, different genera of colonies exhibit distinct morphological characteristics. For example, *Dorea*, *Bacteroides*, and *Collinsella* form larger colonies on agar with dense hyphae but vary in colony diameters, whereas *Faecalibacterium* produces smaller, darker colonies and is less culturable. Therefore, the colony characteristics of the target bacteria on the plate can be predicted based on taxonomic information from target MAGs or SAGs, especially at the genus level. Huang et al. [23] developed the automated CAMII system, which collects microbial morphological data, learns from it, and integrates morphological and genomic data to guide preliminary screening, thus reducing the workload when picking bacteria. Although such advanced systems are not yet widely available in traditional laboratories, further research should focus on designing specific primers or probes and predicting morphological characteristics from genome sequences to guide post-culture screening.

Conclusion

With the advancement of culture-independent techniques, our understanding of gastrointestinal bacteriota has significantly deepened. Sequencing has revealed greater diversity in bacterial species and functions, highlighting the importance of culturing bacterial isolates for further research. Thus, culture-dependent techniques, particularly

culturomics, have regained prominence in studying bacteriota from specific niches. Despite numerous efforts to optimize culturomics, further efficiency improvements are needed to meet the demands of higher-throughput bacterial separation. The combination of culturomics and metagenomics represents a promising trend due to their complementary roles in bacterial study. To support high-throughput bacterial culture, advanced equipment and techniques such as RACS, live-FISH, and fluorescently labeled antibodies should be further developed and tailored for gastrointestinal bacterial isolation. As culture technology evolves and microbial information databases improve, culturomics has the potential to become a more efficient technique with a reduced workload, lower costs, and a higher success rate for capturing gastrointestinal bacteria effectively.

Reference

[1] Brody H. (2020) The gut microbiome. NATURE, 577(7792), S5-S5.

[2] Gavin P.G.G., Kim K.W., Craig M.E.E. et al (2022) Multi-omic interactions in the gut of children at the onset of islet autoimmunity. MICROBIOME, 10(1).

[3] Ghosh T.S., Shanahan F., O'Toole P.W. (2022) The gut microbiome as a modulator of healthy ageing. NATURE REVIEWS GASTROENTEROLOGY & HEPATOLOGY, 19(9), 565-584.

[4] Kuziel G.A., Rakoff-Nahoum S. (2022) The gut microbiome. CURRENT BIOLOGY, 32(6), R257-R264.

[5] Bindari Y.R., Gerber P.F. (2022) Centennial review: Factors affecting the chicken gastrointestinal microbial composition and their association with gut health and productive performance. POULTRY SCIENCE, 101(1).

[6] Maltecca C., Bergamaschi M., Tiezzi F. (2020) The interaction between microbiome and pig efficiency: A review. JOURNAL OF ANIMAL BREEDING AND GENETICS, 137(1), 4-13.

[7] Matthews C., Crispie F., Lewis E. et al (2019) The rumen microbiome: A crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. GUT MICROBES, 10(2), 115-132.

[8] Gueimonde M., Collado M.C. (2012) Metagenomics and probiotics. CLINICAL MICROBIOLOGY AND INFECTION, 18, 32-34.

[9] Pavlopoulos G.A., Baltoumas F.A., Liu S. et al (2023) Unraveling the functional dark matter through global metagenomics. NATURE, 622(7983), 594-602.

[10] Tas N., de Jong A.E.E., Li Y. et al (2021) Metagenomic tools in microbial ecology research. CURRENT OPINION IN BIOTECHNOLOGY, 67, 184-191.

[11] Lynch M.D.J., Neufeld J.D. (2015) Ecology and exploration of the rare biosphere. NATURE REVIEWS MICROBIOLOGY, 13(4), 217-229.

[12] Lagier J.C., Dubourg G., Million M. et al (2018) Culturing the human microbiota and culturomics. NATURE REVIEWS MICROBIOLOGY, 16(9), 540-550.

[13] Liu Y.X., Qin Y., Chen T. et al (2021) A practical guide to amplicon and metagenomic analysis of microbiome data. PROTEIN & CELL, 12(5), 315-330.

[14] Bharti R., Grimm D.G. (2021) Current challenges and best-practice protocols for microbiome analysis. BRIEFINGS IN BIOINFORMATICS, 22(1), 178-193.

[15] Almeida A., Mitchell A.L., Boland M. et al (2019) A new genomic blueprint of the human gut microbiota. NATURE, 568(7753), 499-+.

[16] Lagkouvardos I., Overmann J., Clavel T. (2017) Cultured microbes represent a substantial fraction of the human and mouse gut microbiota. GUT MICROBES, 8(5), 493-503.

[17] Yadav A., Ahlawat S., Sharma K.K. (2023) Culturing the unculturables: Strategies, challenges, and opportunities for gut microbiome study. JOURNAL OF APPLIED MICROBIOLOGY, 134(12).

[18] Lagier J.C., Million M., Hugon P. et al (2012) Human gut microbiota: Repertoire and variations. FRONTIERS IN CELLULAR AND INFECTION MICROBIOLOGY, 2.

[19] Dubourg G., Lagier J.C., Armougom F. et al (2013) The gut microbiota of a patient with resistant tuberculosis is more comprehensively studied by culturomics than by metagenomics. EUROPEAN JOURNAL OF CLINICAL MICROBIOLOGY & INFECTIOUS DISEASES, 32(5), 637-645.

[20] Ferrario C., Alessandri G., Mancabelli L. et al (2017) Untangling the cecal microbiota of feral chickens by culturomic and metagenomic analyses. ENVIRONMENTAL MICROBIOLOGY, 19(11), 4771-4783.

[21] Li C., Luan Z., Zhao Y. et al (2022) Deep insights into the gut microbial community of extreme longevity in south chinese centenarians by ultra-deep metagenomics and large-scale culturomics. NPJ BIOFILMS AND MICROBIOMES, 8(1).

[22] Goldman S.L., Sanders J.G., Yan W. et al (2022) Culture-enriched community profiling improves resolution of the vertebrate gut microbiota. MOLECULAR ECOLOGY RESOURCES, 22(1), 122-136.

[23] Huang Y., Sheth R.U., Zhao S. et al (2023) High-throughput microbial culturomics using automation and machine learning. NATURE BIOTECHNOLOGY, 41(10), 1424-+.

[24] Zou Y., Xue W., Luo G. et al (2019) 1,520 reference genomes from cultivated human gut bacteria enable functional microbiome analyses. NATURE BIOTECHNOLOGY, 37(2), 179-+.

[25] Lagier J.C., Armougom F., Million M. et al (2012) Microbial culturomics: Paradigm shift in the human gut microbiome study. CLINICAL MICROBIOLOGY AND INFECTION, 18(12), 1185-1193.

[26] Browne H.P., Forster S.C., Anonye B.O. et al (2016) Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. NATURE, 533(7604), 543-+.

[27] Liu S., Yu Z., Zhong H. et al (2023) Functional gene-guided enrichment plus *in situ* microsphere cultivation enables isolation of new crucial ureolytic bacteria from the rumen of cattle. MICROBIOME, 11(1).

[28] Wan X., Yang Q., Wang X. et al (2023) Isolation and cultivation of human gut microorganisms: A review. MICROORGANISMS, 11(4).

[29] Orellana L.H., Krueger K., Sidhu C. et al (2023) Comparing genomes recovered from time-series metagenomes using long- and short-read sequencing technologies. MICROBIOME, 11(1).

[30] Zehavi T., Probst M., Mizrahi I. (2018) Insights into culturomics of the rumen microbiome. FRONTIERS IN MICROBIOLOGY, 9.

[31] Whelan F.J., Waddell B., Syed S.A. et al (2020) Culture-enriched metagenomic sequencing enables in-depth profiling of the cystic fibrosis lung microbiota. NATURE MICROBIOLOGY, 5(2), 379-+.

[32] Li S., Lian W.H., Han J.R. et al (2023) Capturing the microbial dark matter in desert soils using culturomics-based metagenomics and high-resolution analysis. NPJ BIOFILMS AND MICROBIOMES, 9(1).

[33] Feehily C., O'Neill I.J., Walsh C.J. et al (2023) Detailed mapping of *Bifidobacterium* strain transmission from mother to infant via a dual culture-based and metagenomic approach. NATURE COMMUNICATIONS, 14(1).

[34] Fu S., Zhang Y., Wang R. et al (2023) A novel culture-enriched metagenomic sequencing strategy effectively guarantee the microbial safety of drinking water by uncovering the low abundance pathogens. JOURNAL OF ENVIRONMENTAL MANAGEMENT, 345.

[35] Hugon P., Dufour J.C., Colson P. et al (2015) A comprehensive repertoire of prokaryotic species identified in human beings. LANCET INFECTIOUS DISEASES, 15(10), 1211-1219.

[36] Bilen M., Dufour J.C., Lagier J.C. et al (2018) The contribution of culturomics to the repertoire of isolated human bacterial and archaeal species. MICROBIOME, 6.

[37] Diakite A., Dubourg G., Raoult D. (2021) Updating the repertoire of cultured bacteria from the human being. MICROBIAL PATHOGENESIS, 150.

[38] Lin X., Hu T., Chen J. et al (2023) The genomic landscape of reference genomes of cultivated human gut bacteria. NATURE COMMUNICATIONS, 14(1).

[39] Li W.X., Liang H.W., Lin X.Q. et al (2023) A catalog of bacterial reference genomes from cultivated human oral bacteria. NPJ BIOFILMS AND MICROBIOMES, 9(1).

[40] Wang X., Howe S., Wei X. et al (2021) Comprehensive cultivation of the swine gut microbiome reveals high bacterial diversity and guides bacterial isolation in pigs. MSYSTEMS, 6(4).

[41] Pereira A.C., Bandeira V., Fonseca C. et al (2020) Crosstalk between culturomics and microbial profiling of egyptian mongoose (*Herpestes ichneumo*) gut microbiome. MICROORGANISMS, 8(6).

[42] Crhanova M., Karasova D., Juricova H. et al (2019) Systematic culturomics shows that half of chicken caecal microbiota members can be grown *in vitro*

except for two lineages of *Clostridiale* and a single lineage of *Bacteroidete*. MICROORGANISMS, 7(11).

[43] Dong B., Lin X., Jing X. et al (2022) A bacterial genome and culture collection of gut microbial in weanling piglet. MICROBIOLOGY SPECTRUM, 10(1).

[44] Dubourg G., Baron S., Cadoret F. et al (2018) From culturomics to clinical microbiology and forward. EMERGING INFECTIOUS DISEASES, 24(9), 1683-1690.

[45] Khanna S., Pardi D.S., Kelly C.R. et al (2016) A novel microbiome therapeutic increases gut microbial diversity and prevents recurrent clostridium difficile infection. JOURNAL OF INFECTIOUS DISEASES, 214(2), 173-181.

[46] Ghimire S., Roy C., Wongkuna S. et al (2020) Identification of *Clostridioides difficulty*-inhibiting gut commensals using culturomics, phenotyping, and combinatorial community assembly. MSYSTEMS, 5(1).

[47] Alou M.T., Million M., Traore S.I. et al (2017) Gut bacteria missing in severe acute malnutrition, can we identify potential probiotics by culturomics? FRONTIERS IN MICROBIOLOGY, 8.

[48] Fellag M., Gouba N., Bedotto M. et al (2020) Culturomics discloses anti-tubercular enterococci exclusive of pulmonary tuberculosis: A preliminary report. MICROORGANISMS, 8(10).

[49] Li B., Kwok L.Y., Wang D. et al (2023) Integrating metabolomics, bionics, and culturomics to study probiotics-driven drug metabolism. FRONTIERS IN PHARMACOLOGY, 14.

[50] Shetty S.A., Smidt H., de Vos W.M. (2019) Reconstructing functional networks in the human intestinal tract using synthetic microbiomes. CURRENT OPINION IN BIOTECHNOLOGY, 58, 146-154.

[51] Li H., He J., Jia W. (2016) The influence of gut microbiota on drug metabolismand toxicity. EXPERT OPINION ON DRUG METABOLISM & TOXICOLOGY, 12(1), 31-40.

[52] Weersma R.K., Zhernakova A., Fu J.Y. (2020) Interaction between drugs and the gut microbiome. GUT, 69(8), 1510-1519.

[53] Javdan B., Lopez J.G., Chankhamjon P. et al (2020) Personalized mapping of drug metabolism by the human gut microbiome. CELL, 181(7), 1661-+.

[54] Tao X., Huang W., Pan L. et al (2023) Optimizing *ex vivo* culture conditions to study human gut microbiome. ISME COMMUNICATIONS, 3(1).

[55] Pfleiderer A., Lagier J.C., Armougom F. et al (2013) Culturomics identified 11 new bacterial species from a single anorexia nervosa stool sample. EUROPEAN JOURNAL OF CLINICAL MICROBIOLOGY & INFECTIOUS DISEASES, 32(11), 1471-1481.

[56] Bellali S., Lagier J.C., Raoult D. et al (2019) Among live and dead bacteria, the optimization of sample collection and processing remains essential in recovering gut microbiota components. FRONTIERS IN MICROBIOLOGY, 10.

[57] Yadav K.K., Nimonkar Y., Poddar B.J. et al (2022) Two-dimensional cell separation: A high-throughput approach to enhance the culturability of bacterial cells from environmental samples. MICROBIOLOGY SPECTRUM, 10(3).

[58] Belkacemi S., Khalil J.B., Ominami Y. et al (2019) Passive filtration, rapid scanning electron microscopy, and matrix-assisted laser desorption ionization-time of flight mass spectrometry for *Treponema* culture and identification from the oral cavity. JOURNAL OF CLINICAL MICROBIOLOGY, 57(10).

[59] Nicholson W.L., Munakata N., Horneck G. et al (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, 64(3), 548-+.

[60] Hong H.A., Khaneja R., Tam N.M.K. et al (2009) *Bacillus subtilis* isolated from the human gastrointestinal tract. RESEARCH IN MICROBIOLOGY, 160(2), 134-143.

[61] Golnari M., Bahrami N., Milanian Z. et al (2024) Isolation and characterization of novel bacillus strains with superior probiotic potential: Comparative analysis and safety evaluation. SCIENTIFIC REPORTS, 14(1), 1457-1457.

[62] Afouda P., Hocquart M., Pham T.P.T. et al (2020) Alcohol pretreatment of stools effect on culturomics. SCIENTIFIC REPORTS, 10(1).

[63] Diakite A., Dubourg G., Dione N. et al (2020) Optimization and standardization of the culturomics technique for human microbiome exploration. SCIENTIFIC REPORTS, 10(1).

[64] Lagier J.C., Khelaifia S., Alou M.T. et al (2016) Culture of previously uncultured members of the human gut microbiota by culturomics. NATURE MICROBIOLOGY, 1(12).

[65] Rettedal E.A., Gumpert H., Sommer M.O.A. (2014) Cultivation-based multiplex phenotyping of human gut microbiota allows targeted recovery of previously uncultured bacteria. NATURE COMMUNICATIONS, 5.

[66] Wylensek D., Hitch T.C.A., Riedel T. et al (2020) A collection of bacterial isolates from the pig intestine reveals functional and taxonomic diversity. NATURE COMMUNICATIONS, 11(1).

[67] Mun D., Kim H., Shin M. et al (2021) Decoding the intestinal microbiota repertoire of sow and weaned pigs using culturomic and metagenomic approaches. JOURNAL OF ANIMAL SCIENCE AND TECHNOLOGY, 63(6), 1423-1432.

[68]Mitsumori, Makoto (2018). Isolation of cellulolytic bacteria from the rumen. Humana Press.

[69] Cheng A.G., Ho P.Y., Aranda-Diaz A. et al (2022) Design, construction, and *in vivo* augmentation of a complex gut microbiome. CELL, 185(19), 3617-+.

[70] Zhang J., Liu Y.X., Guo X. et al (2021) High-throughput cultivation and identification of bacteria from the plant root microbiota. NATURE PROTOCOLS, 16(2).

[71] Das N., Tripathi N., Basu S. et al (2015) Progress in the development of gelling agents for improved culturability of microorganisms. FRONTIERS IN MICROBIOLOGY, 6.

[72] Tanaka T., Kawasaki K., Daimon S. et al (2014) A hidden pitfall in the preparation of agar media undermines microorganism cultivability. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 80(24), 7659-7666.

[73] Tanaka Y., Benno Y. (2015) Application of a single-colony coculture technique to the isolation of hitherto unculturable gut bacteria. MICROBIOLOGY AND IMMUNOLOGY, 59(2), 63-70.

[74] Nyonyo T., Shinkai T., Tajima A. et al (2013) Effect of media composition, including gelling agents, on isolation of previously uncultured rumen bacteria. LETTERS IN APPLIED MICROBIOLOGY, 56(1), 63-70.

[75] Nyonyo T., Shinkai T., Mitsumori M. (2014) Improved culturability of cellulolytic rumen bacteria and phylogenetic diversity of culturable cellulolytic and xylanolytic bacteria newly isolated from the bovine rumen. FEMS MICROBIOLOGY ECOLOGY, 88(3), 528-537.

[76] Nishioka T., Tamaki H. (2022) Improved cultivation and isolation of diverse endophytic bacteria inhabiting *Dendrobium* roots by using simply modified agar media. MICROBIOLOGY SPECTRUM, 10(6).

[77] Kuznetsov S.I., Dubinina G.A., Lapteva N.A. (1979) Biology of oligotrophic bacteria. ANNUAL REVIEW OF MICROBIOLOGY, 33, 377-387.

[78] Giraud M., Garçon V., de la Broise D. et al (2019) Potential effects of deep seawater discharge by an ocean thermal energy conversion plant on the marine microorganisms in oligotrophic waters. SCIENCE OF THE TOTAL ENVIRONMENT, 693.

[79] Norris N., Levine N.M., Fernández V.I. et al (2021) Mechanistic model of nutrient uptake explains dichotomy between marine oligotrophic and copiotrophic bacteria. PLOS COMPUTATIONAL BIOLOGY, 17(5).

[80] Tapia-Torres Y., Elser J.J., Souza V. et al (2015) Ecoenzymatic stoichiometry at the extremes: How microbes cope in an ultra-oligotrophic desert soil. SOIL BIOLOGY & BIOCHEMISTRY, 87, 34-42.

[81] Pulschen A.A., Bendia A.G., Fricker A.D. et al (2017) Isolation of uncultured bacteria from antarctica using long incubation periods and low nutritional media. FRONTIERS IN MICROBIOLOGY, 8.

[82] Gray D.A., Dugar G., Gamba P. et al (2019) Extreme slow growth as alternative strategy to survive deep starvation in bacteria. NATURE COMMUNICATIONS, 10.

[83] Chang Y., Hou F., Pan Z. et al (2019) Optimization of culturomics strategy in human fecal samples. FRONTIERS IN MICROBIOLOGY, 10.

[84] Huang Z., Han N., Chang Y. et al (2023) Optimization of oligotrophic culturomics for isolation of human gut microbiota. ACTA MICROBIOLOGICA SINICA, 63(09), 3641-3652.

[85] Zengler K., Walcher M., Clark G. et al (2005) High-throughput cultivation of microorganisms using microcapsules. METHODS IN ENZYMOLOGY, 379, 124-+.

[86] Ingham C.J., Sprenkels A., Bomer J. et al (2007) The micro-petri dish, a million-well growth chip for the culture and high-throughput screening of microorganisms. PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 104(46), 18217-18222.

[87] Bollmann A., Lewis K., Epstein S.S. (2007) Incubation of environmental samples in a diffusion chamber increases the diversity of recovered isolates. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 73(20), 6386-6390.

[88] Zhang Z., Yang C., Veldsman W.P. et al (2023) Benchmarking genome assembly methods on metagenomic sequencing data. BRIEFINGS IN BIOINFORMATICS, 24(2).

[89] Berry D., Mader E., Lee T.K. et al (2015) Tracking heavy water (D_2O) incorporation for identifying and sorting active microbial cells. PROCEEDINGS

OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 112(2), E194-E203.

[90] Wang X., Ren L., Su Y. et al (2017) Raman-activated droplet sorting (RADS) for label-free high-throughput screening of microalgal single-cells. ANALYTICAL CHEMISTRY, 89(22), 12569-12577.

[91] Xu T., Gong Y., Su X. et al (2020) Phenome-genome profiling of single bacterial cell by raman-activated gravity-driven encapsulation and sequencing. SMALL, 16(30).

[92] Jing X., Gong Y., Pan H. et al (2022) Single-cell raman-activated sorting and cultivation (SCRACS-culture) for assessing and mining *in situ* phosphate-solubilizing microbes from nature. ISME COMMUNICATIONS, 2(1).

[93] Cross K.L., Campbell J.H., Balachandran M. et al (2019) Targeted isolation and cultivation of uncultivated bacteria by reverse genomics. NATURE BIOTECHNOLOGY, 37(11), 1314-+.

[94] Thompson A.W., Crow M.J., Wadey B. et al (2015) A method to analyze, sort, and retain viability of obligate anaerobic microorganisms from complex microbial communities. JOURNAL OF MICROBIOLOGICAL METHODS, 117, 74-77.

[95] Batani G., Bayer K., Boege J. et al (2019) Fluorescence *in situ* hybridization (FISH) and cell sorting of living bacteria. SCIENTIFIC REPORTS, 9.

[96] Hosokawa M., Iwai N., Arikawa K. et al (2023) Target enrichment of uncultured human oral bacteria with phage-derived molecules found by single-cell genomics. JOURNAL OF BIOSCIENCE AND BIOENGINEERING, 136(1), 58-66.

[97] Renesto P., Crapoulet N., Ogata H. et al (2003) Genome-based design of a cell-free culture medium for *Tropheryma whipplei*. LANCET, 362(9382), 447-449.

[98] Tyson G.W., Lo I., Baker B.J. et al (2005) Genome-directed isolation of the key nitrogen fixer *Leptospirillum ferrodiazotrophum* sp nov from an acidophilic

microbial community. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 71(10), 6319-6324.

[99] Viacava K., Qiao J., Janowczyk A. et al (2022) Meta-omics-aided isolation of an elusive anaerobic arsenic-methylating soil bacterium. ISME JOURNAL, 16(7), 1740-1749.

[100] Alcock B.P., Huynh W., Chalil R. et al (2023) CARD 2023: Expanded curation, support for machine learning, and resistome prediction at the comprehensive antibiotic resistance database. NUCLEIC ACIDS RESEARCH, 51(D1), D690-D699.

[101] McArthur A.G., Waglechner N., Nizam F. et al (2013) The comprehensive antibiotic resistance database. ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, 57(7), 3348-3357.

[102] Blin K., Shaw S., Augustijn H.E. et al (2023) antiSMASH 7.0: New and improved predictions for detection, regulation, chemical structures and visualisation. NUCLEIC ACIDS RESEARCH, 51(W1), W46-W50.

[103] Medema M.H., Blin K., Cimermancic P. et al (2011) antiSMASH: Rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. NUCLEIC ACIDS RESEARCH, 39, W339-W346.

[104] Weimann A., Mooren K., Frank J. et al (2016) From genomes to phenotypes: Traitar, the microbial trait analyzer. MSYSTEMS, 1(6).

[105] Sauer D.B., Wang D.N. (2019) Predicting the optimal growth temperatures of prokaryotes using only genome derived features. BIOINFORMATICS, 35(18), 3224-3231.

Figure legends:

Figure 1. The contribution of culturomics (Created with BioRender.com).

Figure 1

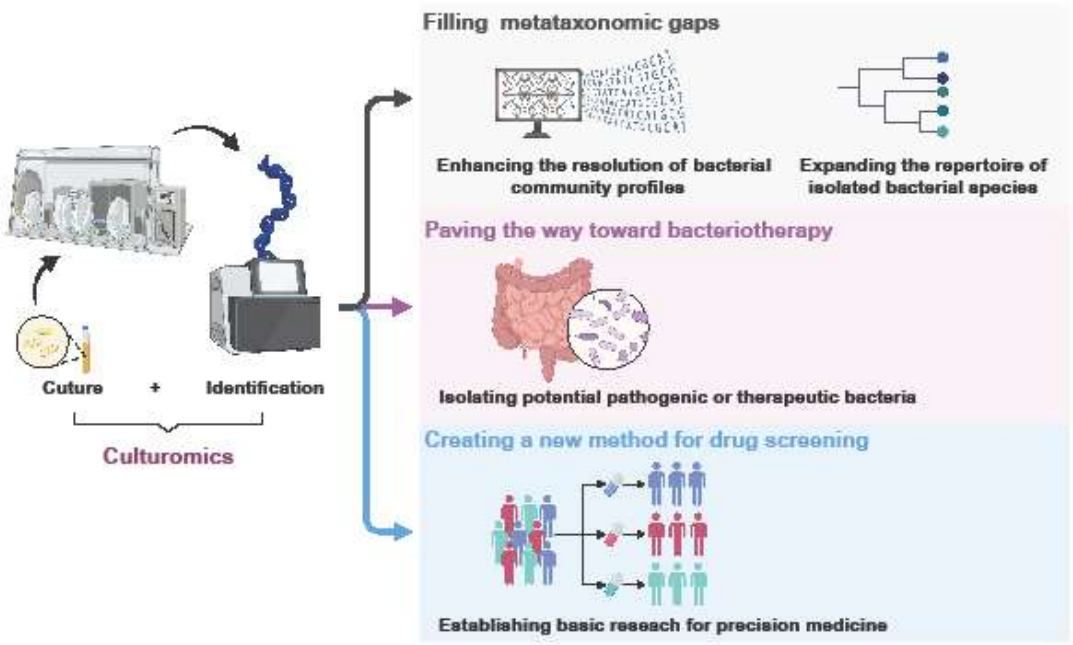

Figure 2. Multi-dimensional evaluation of medium [54].

Figure 2

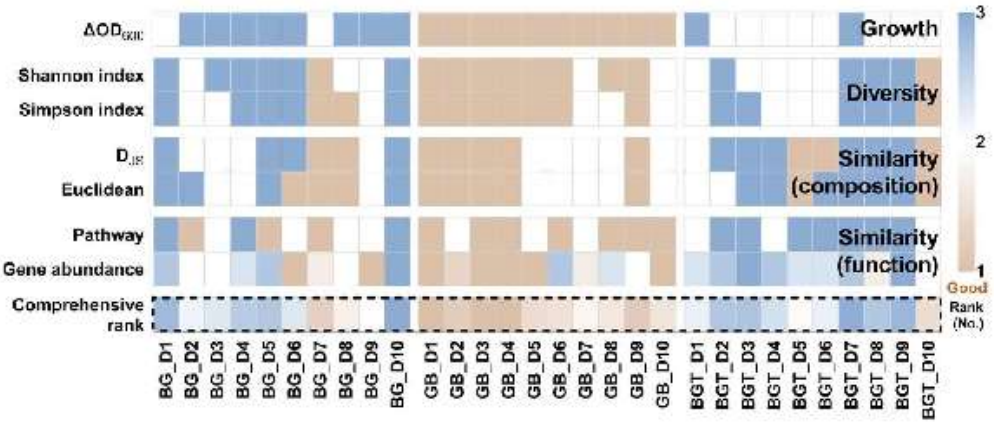

Figure 3. Comparison of the culture-independent sequencing and culture results. (A) The difference in 16S rRNA gene sequences was detected between fecal samples and plate contents [22]. (B) The difference between pyrosequencing and culture results [55].

Figure 3

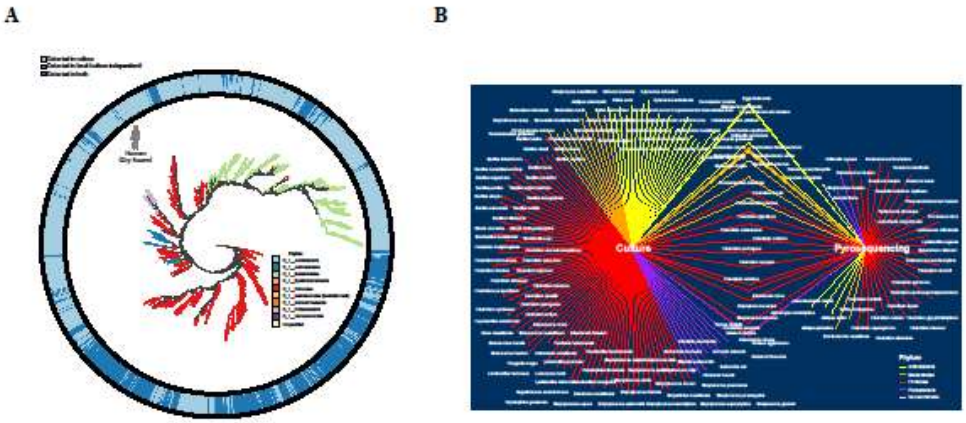

Figure 4. Experimental factors affecting cultivability [30]. (A) The proportion of the cultivable prokaryotic microorganisms in each rumen dilution. (B) The impact of abundance on cultivability.

Figure 4

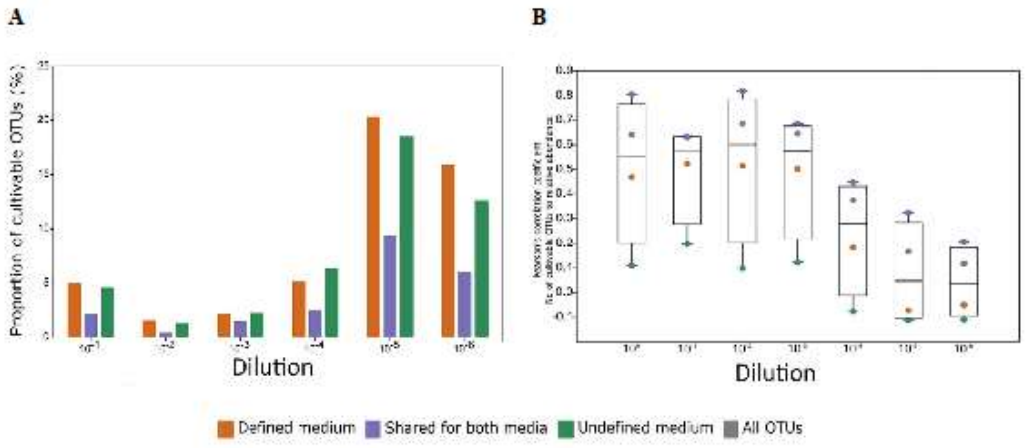

Figure 5. The impact of ethanol treatment [62].

Figure 5

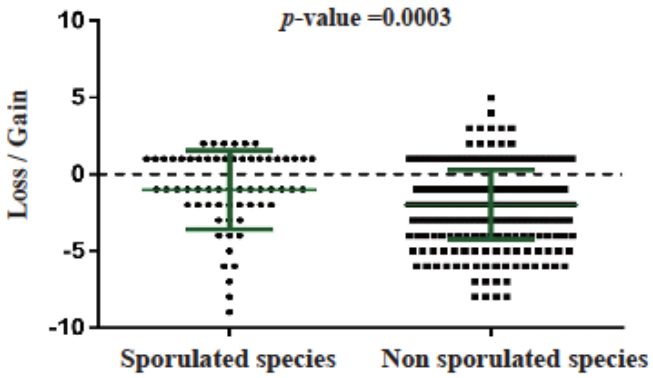

Figure 6. The enriched specific ASVs of interest in pigs with the 53 culture conditions [40].

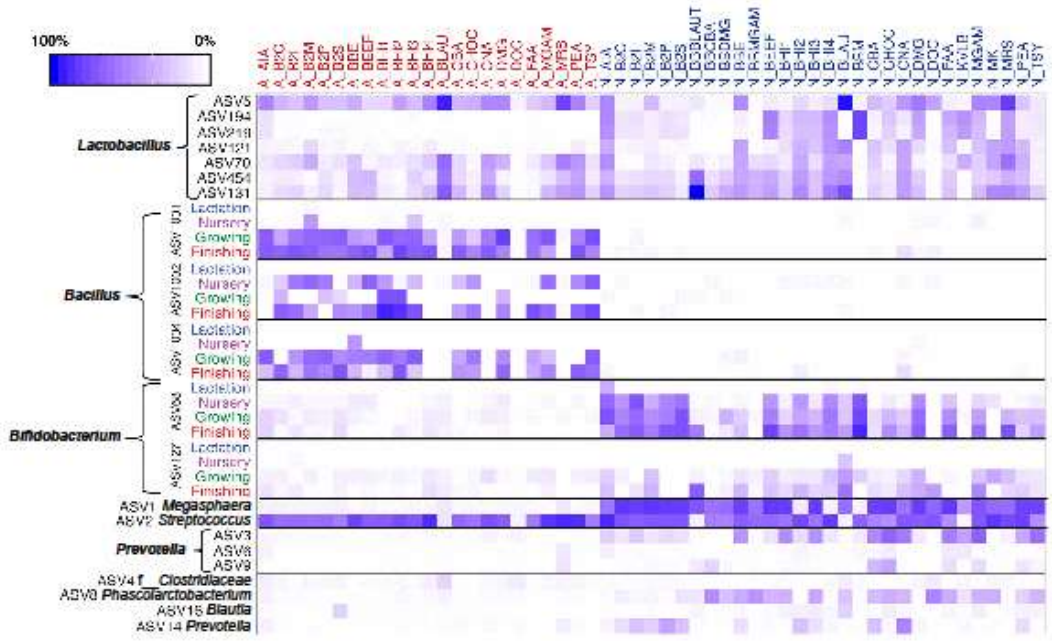

Figure 7. Improving the culturomics workflows (Created with BioRender.com).

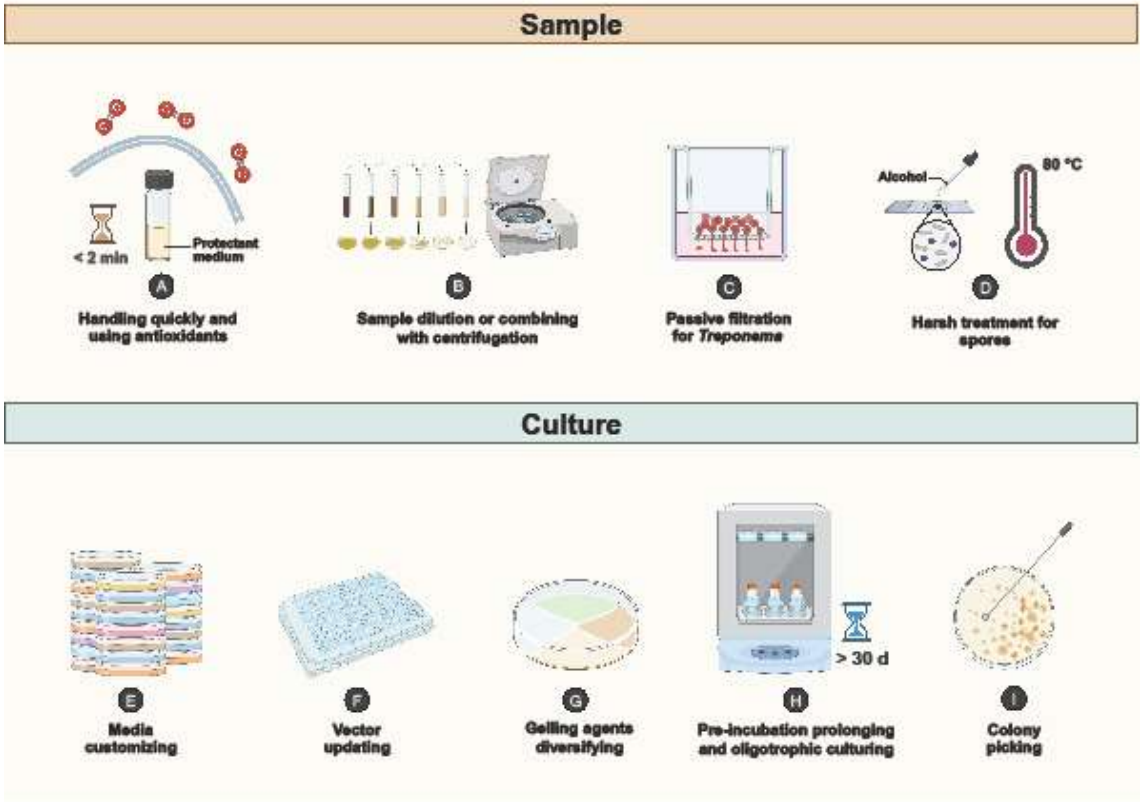

Figure 8. Novel technology used in metagenomic-directed cultureomics (Created with BioRender.com).

Figure 8

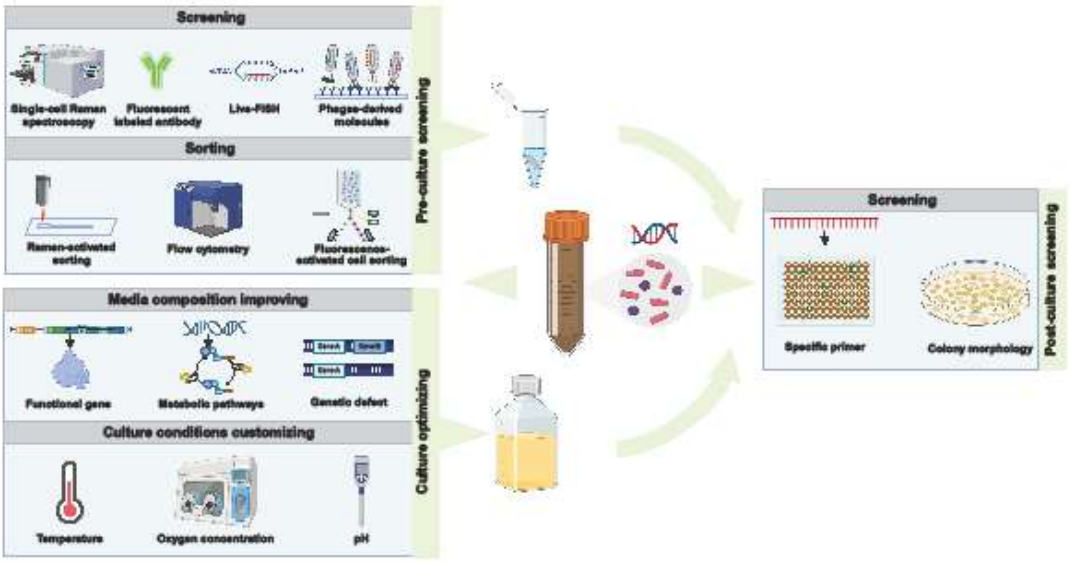

Table 1. Composition (g/L) of the protectant medium patented under the following number (N°1H53316 CAS 25 FR) by Bellali et al. [56].

Composition (pH 7.3±0.2)	Content
PBS	1 L
Skimmed milk	100 g
Sucrose	100 g
Trehalose	50 g
$CaCl_2$	0.1 g
$MgCl_2$	0.1 g
KOH	0.3/0.6 g
Ascorbic acid	1 g
Uric acid	0.4 g
Glutathione	0.1 g

Table 2. List of the 16 best culture conditions recommended for culturomics of human gut bacteriota by Diakite et al. [63].

Culture conditions	Number of species added and not previously isolated	Number of species isolated
Blood culture bottle with rumen fluid and sheep blood in anaerobic condition at 37 °C	306	306
R-medium with lamb serum with rumen fluid and sheep blood in anaerobic condition at 37 °C	64	172
Blood culture bottle with rumen fluid and sheep blood in anaerobic condition at 37 °C after pre-treatment of stool sample with alcohol	29	133
YCFA broth in anaerobic condition at 37 °C	21	152
Blood culture bottle with stool filtered at 0,45 µm in anaerobic condition at 37 °C	17	144
Blood culture bottle with 5 ml sheep blood in anaerobic condition at 37 °C	13	166
Blood culture bottle after thermic shock at 80 °C during 20 min in anaerobic condition at 37 °C	8	141
Marine broth in anaerobic condition at 37 °C	5	139
Blood culture bottle with stool filtered at 0.45 µm in aerobic condition at 37°C	5	35
Blood culture bottle with stool filtered at 5 µm in anaerobic condition at 37°C	4	126
Blood culture bottle with rumen fluid in anaerobic condition at 37°C after pre-treatment of stool sample with alcohol	4	64
R-medium with rumen fluid and sheep blood in anaerobic condition at 37°C	3	127
CNA agar medium in anaerobic condition at 37°C	3	50
5% sheep blood broth in anaerobic condition at 37°C	2	167
Schaedler broth in anaerobic condition at 37°C	2	123
Christensenella broth medium in anaerobic condition at 37°C	1	116

Table 3. The media are suitable for bacterial samples from diverse hosts.

The sample origin	Medium	Reference
Human	the 16 culture conditions listed in Table 2;	[63]
	Gifu anaerobic medium (GAM);	[65]
	mGAM;	[65]
		[53]
	diluting modified Gifu anaerobe medium (DMGAM);	[65]
	YCFA;	[26]
	MPYG;	[24]
	3mGAM:7BB (BG);	[53]
	5mGAM:5BB (GB);	[54]
	chocolate agar (CA);	[22]
	Columbia blood agar (CBA).	[22]
Pig	Columbia agar with sheep blood PLUS (Blood agar);	[66]
	BSM;	[66]
	WCA;	[66]
	mGAM;	[66]
	phenylethyl alcohol agar (PEA);	[67]
	fastidious anaerobe agar (FAA);	[67]
	MPYG;	[43]
	MPYG with sheep blood;	[43]
	spore medium with sheep blood.	[43]
Chicken	WCA;	[42]
	WCA with mucin;	[42]
	YCFA;	[42]
	YCFA with mucin.	[42]
Ruminant	M10;	[30]
	M10 with rumen fluid;	[30]
	rumen fluid-glucose-cellobiose agar (RGCA).	[68]