Animal Nutriomics

cambridge.org/anr

Culturomics: A critical approach in studying the roles of human and animal microbiota

Samantha Howe, Ziyu Liu, Bin Zuo and Jiangchao Zhao

Department of Animal Science, Division of Agriculture, University of Arkansas, Fayetteville, AR, USA

Review

Cite this article: Howe S, Liu Z, Zuo B, Zhao J (2024) Culturomics: A critical approach in studying the roles of human and animal microbiota. *Animal Nutriomics* **1**, e6, 1–16. https://doi.org/10.1017/anr.2024.6

Received: 27 December 2023
Revised: 5 March 2024
Accepted: 7 March 2024

Keywords:
microbiota; culturomics; sequencing; animal health

Corresponding author: Jiangchao Zhao; Email: jzhao77@uark.edu

Abstract

The rise of sequencing technologies has greatly contributed to our knowledge of the microbiota and its role in animal health and production. However, many members of the microbiota have historically been considered "unculturable." Culturomics can be utilized to bring these fastidious microbes into cultivation and can be used in conjunction with culture-independent methods to study the microbiota in a more comprehensive manner. This review paper details culturomics' role in revolutionizing human, swine, and bovine microbiota research and how its use has greatly increased the bacterial repertoire. Additionally, it describes how culturomics can be applied to develop microbiota-derived therapeutics, such as next-generation probiotics, and to study the role of the microbiota. Finally, this review provides potential methods and considerations for designing future culturomics studies.

Introduction

There is a growing body of research on the isolation, culture, and identification of microbes because of their non-negligible role in human (Chen et al. 2021; Maruvada et al. 2017) and animal disease (Chai et al. 2022; Gresse et al. 2017; Howe et al. 2023; Welch et al. 2022), plant science (Mendes et al. 2013), and even forensics (Cho and Eom 2021). In 1980, only 1,761 bacterial species had been validated (Janda and Abbott 2007). This number has now increased to around 20,000 (Parte et al. 2020). Next-generation sequencing technologies have greatly increased our knowledge of and have been vital in establishing the bacterial repertoire (Deng et al. 2024; Lagier et al. 2015). However, a large part of the microbes identified by sequencing cannot be cultured *in vitro* and remain to be characterized (Lagier et al. 2015), which limits the exploration of microorganisms to a certain extent. Moreover, growing the microbiota in pure culture plays a crucial role in experimental models and therapeutic research applications. Therefore, the gradual development of culturomics, a high-throughput culture method, has contributed to solving these challenges in the field of microbiology in the 21st century. In this review paper, we first describe the development of culturomics and its progress in human, swine, and bovine cultured microbiota research. Then, we discuss how culturomics can be beneficial in developing host-derived microbiome-based therapeutics and can be applied to analyze the role of the microbiota. Lastly, we cover methods and considerations for the reader to use when integrating culturomics into their research.

Culturomics

Evolution and progression of bacterial culture

Microbiome research has evolved significantly over the past few decades. Historically, microorganisms were obtained and identified strictly through culture-based methods and phenotypic observations, such as gram stain and carbohydrate degradation (Lagier et al. 2015; Russell 1979; Salanitro et al. 1977). These methods were expensive and time-consuming. However, until the development of more advanced molecular techniques, they were considered the "gold standard." The development of molecular techniques, such as Sanger sequencing of the 16S rDNA gene and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) reduced the effort required to identify bacterial isolates and allowed for the easier identification of difficult-to-culture bacteria. The creation of next-generation sequencing replaced the use of conventional culture microbiology in many laboratories and has greatly increased our understanding of microbes and their communities. However, the importance of microbial culture is being re-recognized (Lagier et al. 2015). As our knowledge of microorganisms has increased, culture methods involving multiple technologies have emerged. Nowadays, culturomics is a high-throughput culture method involving numerous culture conditions, including but not limited to differing media compositions, pretreatments, oxygen levels, temperatures, etc., paired with techniques such as MALDI-TOF MS and full-length bacterial 16S rRNA gene sequencing to identify the isolates (Duquenoy et al. 2020; Lagier et al. 2012; Wang et al. 2021).

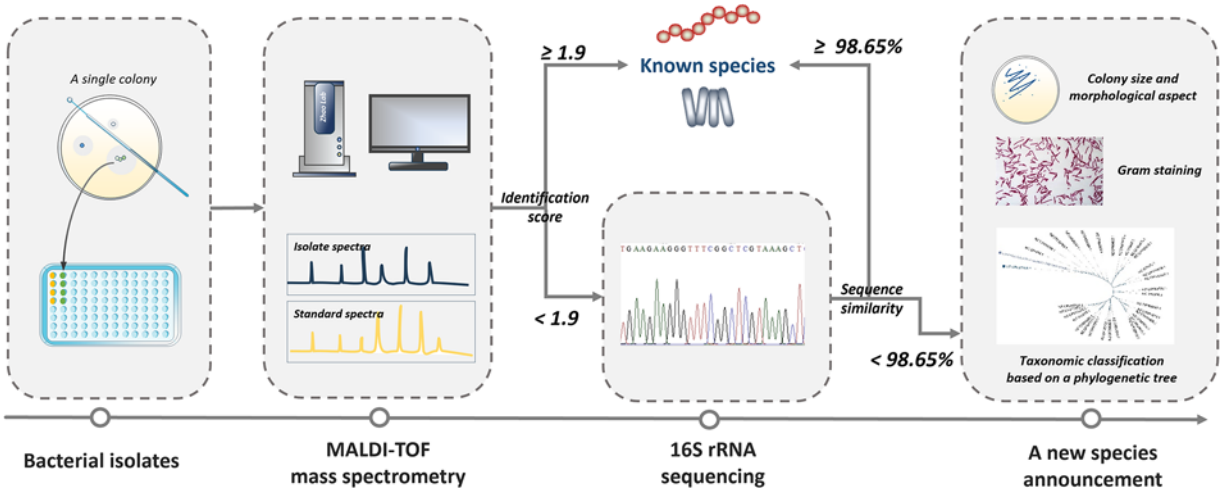

Figure 1. Identification of a new species in culturomics.

Culturomics workflow

MALDI-TOF mass spectrometry is a critical method to identify bacterial species in culturomics research due to its advantages in efficiency, specificity, and low cost (Bilen 2020). The workflow of culturomics for unknown microbes includes the following key steps (Figure 1): bacterial isolation, MALDI-TOF mass spectrometry identification, 16S rRNA sequencing, and a new species announcement. Briefly, the colony from bacterial isolates should be first analyzed through MALDI-TOF MS several times. An identification score < 1.9 suggests that the isolate may be an unknown bacterial species (Seng et al. 2009). Then, the full-length 16S sequencing is performed. If the sequence similarity between the isolate and known species is $< 98.65\%$, there is a high probability that the isolate is a new species (Lagier et al. 2018). Finally, an article format describing the isolation process and main characteristics, including phenotypic data and taxonomic classification, is needed for the new species announcement (Fournier et al. 2017).

Microbiome sequencing and culturomics

Since the early 2000s, the development of high-throughput sequencing technologies has continued to transform many biological fields (Goodwin et al. 2016). Today, numerous sequencing "generations" and technologies are available (Satam et al. 2023). These technologies have revolutionized microbial ecology and microbiome/microbiota research, allowing researchers to explore microbial communities' diversity, structure, composition, and role in different environments (Chai et al. 2024; Deng et al. 2023; Wang et al. 2019). Additionally, the "sequencing era" has also been crucial for the development of microbe-based therapeutics (Sorbara and Pamer 2022) and bioremediation (Malla et al. 2018).

However, although they have many strengths, these technologies also have shortfalls (Satam et al. 2023). The use of these sequencing technologies found that, in the human gastrointestinal tract, approximately 80% of bacteria were unknown (Lagier et al. 2018). Many of these unknown bacteria cannot be classified to lower taxonomic levels and have been described as "microbial dark matter" (Rinke et al. 2013). Moreover, other weaknesses of microbiome sequencing include sequencing depth bias (Lagier et al. 2018; Lynch and Neufeld 2015), lack of causality from typical microbiome association studies (Chaudhari et al. 2021), and differences regarding laboratory and data analysis methods (Howe et al. 2023). Augmenting sequencing-based approaches by including culture-dependent analyses may help researchers overcome the shortfalls of culture-independent studies, as culture-dependent analyses are integral for identifying most causal relationships and mechanisms (Zhao and Zhao 2021), as illustrated by Fei et al. (2020), Pleguezuelos-Manzano et al. (2020), and Zagato et al. (2020). Nevertheless, many microbes are not easily cultured, with some estimates being that 99% of all bacterial and archaeal species are "unculturable" using conventional methods (Jiao et al. 2021). This creates a problematic conundrum because increasing the number of available reference genomes is necessary to decrease microbial dark matter, which cannot be accomplished without pure cultures. These pure cultures are also required to characterize novel microbes and genes (Liu et al. 2022).

Culture-dependent studies allow for the analysis of low-abundance microbes, providing information regarding "rare" bacteria often "missed" by culture-independent studies (Wang et al. 2021; Zehavi et al. 2018). This phenomenon has been coined the "rare biosphere," and the potential ecological importance of these taxa is becoming recognized (Lynch and Neufeld 2015). These "rare" microbes are likely essential in their respective environments. In humans, it has been observed that members of the rare biosphere may be involved in both health and disease (Hajishengallis et al. 2011; Jousset et al. 2017; van der Gast et al. 2011), and in plants, members of the rare biosphere likely protect the host by producing anti-pathogen compounds (Hol et al. 2015; Jousset et al. 2017). Clearly, studying the "rare biosphere" is highly important, and one method for this is culture. Lynch and Neufeld and Jousset et al. provide methods and considerations for studying these microbes (Jousset et al. 2017; Lynch and Neufeld 2015). Therefore, even in the sequencing age, culture-based analyses are highly important. Culturing the rare biosphere and previously uncultured members of the microbiota go hand in hand, and culturomics is a valuable tool for both.

The human culturomics

The changing bacterial repertoires identified in humans

Due to technological limitations in earlier years, only a small minority of the microbiota could be readily cultured *in vitro*

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

(Vartoukian et al. 2010), resulting in most bacteria being considered unculturable. However, the number of bacterial species isolated from humans has been rapidly increasing and updating with the development of culturomics. Researchers have tried to build a comprehensive compilation including all human-associated prokaryotic species described. The bacterial repertoire isolated from humans has been updated from 2,172 first reported in 2015 (Hugon et al. 2015), to 2,776 in 2018 (Bilen et al. 2018), and 3,253 in 2021 (Diakite et al. 2021). Among them, the proportion of novel isolated species from 2015 to the present is up to 26% ($N = 831$). Bacterial species in this repertoire were mainly classified into four different phyla, including the Firmicutes (37%; $N = 1200$), Proteobacteria (25; $N = 812$), Actinobacteria (25%; $N = 805$), and Bacteroidetes (8%; $N = 262$) (Diakite et al. 2021). Specifically, a total of 711 bacterial genera were listed in the human repertoire, mostly in *Mycobacterium, Clostridium, Bacillus, Corynebacterium*, and *Streptococcus* (Diakite et al. 2021). It is worth noting that the number of intestinal bacterial species accounted for the largest proportion of bacteria isolated from human anatomical sites, which includes isolates from the vagina, skin, urine, respiratory tract, and so on (Diakite et al. 2021).

Bacterial species isolated from different human body parts

In humans, bacterial species showed varied abundance in different sites of the body (Figure 2). Understanding and obtaining the gut microbiota is important for parsing the potential molecular mechanisms of human health and disease (Fan and Pedersen 2021). It has been predicted that there were 10^{11}–10^{12} bacteria/g of human feces (Van Houte 1966), which dramatically exceeds the number identified and cultured from the gut, suggesting that a considerable portion of microbes in the gut have not been isolated using current methods (Lagier et al. 2012). Lagier et al. isolated 1057 bacterial species from the human gut in different geographical regions, comprising 56% Firmicutes ($N = 600$), 17% Actinobacteria ($N = 181$), 16% Proteobacteria ($N = 173$), and 8% Bacteroidetes ($N = 88$) phyla (Lagier et al. 2016).

The respiratory tract spans from the nostrils to the lungs, which have a large surface area of approximately 70 m^2 and harbor diverse microbial communities (Man et al. 2017). Previous studies have shown that the microbiota in the respiratory tract could prevent pathogen colonization and maintain homeostasis (Pust et al. 2020; Shah et al. 2021). Fonkou et al. identified 756 different bacterial species totally from the respiratory tract, including the oral cavity and upper and lower respiratory tracts (Fonkou et al. 2018). Specifically, 355, 202, and 514 species were listed in the oral cavity, upper respiratory tract, and lower respiratory tract, respectively, with a proportion of 11.1% ($N = 84$) shared between all three sites. Actinobacteria (33.7%; $N = 255$), Proteobacteria (31.6%; $N = 239$), and Firmicutes (20.2%; $N = 153$) were the most represented phyla.

For decades, the urine of healthy individuals was usually considered to be sterile in clinical studies subjected to methodological biases (Wolfe et al. 2012). However, the ensuing evidence of detected bacteria in the urine revolutionized cognition and provided the urobiome more association with human health and disease (Hilt et al. 2014; Wolfe and Brubaker 2019). Morand et al. reviewed a total of 562 species from human repertoires in 2018, which shared 350 species in common with the gut microbiota (Morand et al. 2019). The four most represented phyla in the human urinary tract are Proteobacteria (35.5%; $N = 200$), Firmicutes (31.3%; $N = 176$), Actinobacteria (22.4%; $N = 126$), and Bacteroidetes (6.4%; $N = 36$). Importantly, most pathogenic bacteria constitute part of the microbiota composition in the human urinary tract and have the potential to lead to the occurrence of disease once the microbiota is disturbed. Dubourg et al. expanded this repertoire to 672 bacterial species by culturomics in 2020, among which 64.1% ($N = 431$) had been previously cultured from the human gut, hinting that a possible origin of the urine microbiota is derived from the gut (Dubourg et al. 2020). Strikingly, the authors also listed the 10 most prevalent bacteria from male and female urine specimens in clinical experiments, and the top 3 were *Escherichia coli, Klebsiella pneumoniae*, and *Enterococcus faecalis*.

Vaginal microbes, such as *Lactobacillus iners*, were reported to be involved in women's reproductive health and disease (Bloom et al. 2022). The imbalance of vaginal microbiota could result in bacterial vaginosis (Zhu et al. 2022). Diop et al. inventoried a repertoire of total 571 bacteria species from the vagina in 2019 (Diop et al. 2019), comprised of 39.1% Firmicutes ($N = 227$), 25.8% Proteobacteria ($N = 150$), 17.4% Actinobacteria ($N = 101$), and 12.7% Bacteroidetes ($N = 74$), respectively. Focused on Firmicutes phylum, a predominance of Lactobacillaceae family accounted for 17.2% ($N = 39$). However, only 49.1% ($N = 285$) of this repertoire were identified through culturomics (Diop et al. 2019). The more detailed characterization of the vaginal microbiota based on culturomics anticipates further reports and analyses in the future.

Overall, these pure isolations and cultures of bacterial species fill the gaps, at least partly, in identifying bacteria through culturomics, providing a basis for a more long-term and systematic study of human–microbial interactions.

The swine culturomics

Early culture-based study on pig gut microbiota

Since the 1950s, researchers have employed culture-based methods to study the intestinal microbiota of pigs (Fewins et al. 1957; Kenworthy and Crabb 1963). One of the early studies revealed that around 30% of swine fecal bacteria could be recovered using a rumen fluid medium; and the majority (90%) of these isolated bacteria were gram-positive (Salanitro et al. 1977), such as facultatively anaerobic *Streptococcus, Eubacterium* sp., *Clostridium* sp., and *Propionibacterium acnes*. Russell's study also reported that over 90% of the bacteria he isolated were gram-positive (Russell 1979). Furthermore, his research delved into the distribution of bacteria in different locations of the swine colon, noting a lower bacterial count in the intestinal wall tissue compared to the luminal content and surface layer. Similar results were found when bacterial populations in the pig cecum and colon were analyzed with different energy sources present in the media (Allison et al. 1979), which underscored the diversity and adaptability of microbial communities. Moreover, pig cecal content culture showed dominant *Bacteroides ruminicola* (35%) and *Selenomonas ruminantium* (21%) in the isolates (Robinson et al. 1981). As for colon microbiota, a study found that in healthy pigs, over 71% of the bacteria were gram-positive, including *Streptococcus* sp., *Lactobacillus acidophilus*, and *Bifidobacterium adolescentis* (Robinson et al. 1984). In contrast, pigs with dysentery showed a higher prevalence (88%) of gram-negative bacteria. Most early studies employed strictly anaerobic techniques with several roll tube media (Hungate and Macy 1973) and recovered mostly gram-positive bacteria.

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

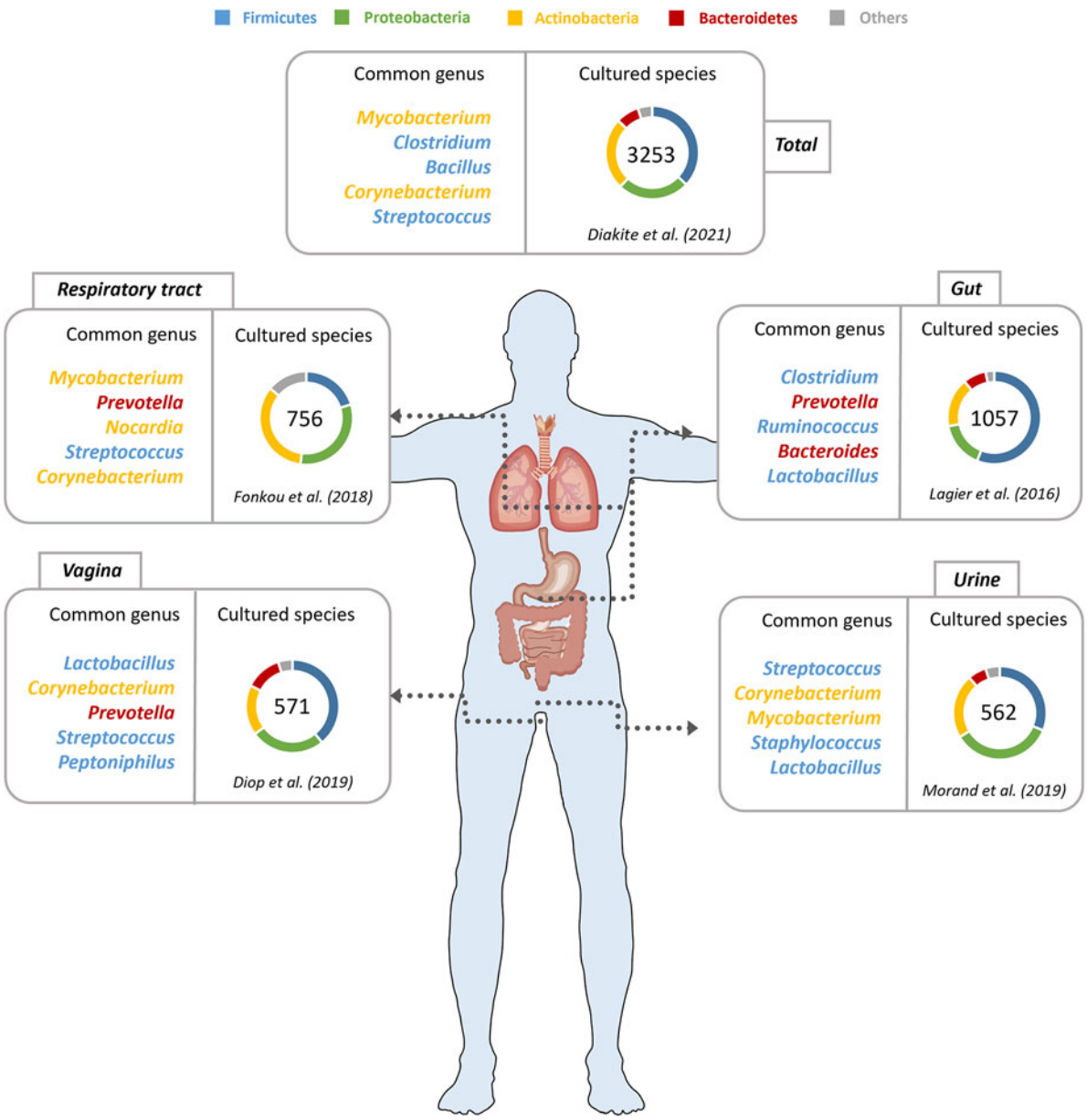

Figure 2. Cultured species repertoires in humans from different body sites.

These techniques and results laid the foundation for the future culture-dependent and -independent studies of the swine microbiome.

Culturomics on pig gut microbiota

The growing knowledge of swine microbiome and culture technology allows researchers to isolate and study various bacteria from pig intestines, focusing on their types, functions, and diversity. By analyzing these bacteria, scientists learn about interactions between microbes and hosts, disease development, and pigs' overall health (Ma et al. 2024). Wang et al., using 53 different cultivation methods, demonstrated an increase in microbial diversity across different growth stages in pigs (Wang et al. 2021). The study found that culture-dependent methods revealed higher bacterial diversity than previously known, with significant amounts of bacterial amplicon sequence variants (ASVs). It also developed reference culture maps for specific bacterial taxa, highlighting the influence of various factors like oxygen, medium, and pig age on microbiota cultivation. The findings are crucial for understanding specific bacterial roles in swine production and health, aiding in the isolation of beneficial bacteria for potential use as probiotics. One of the more recent studies identified 267 bacterial colonies, with 42 species classified into 23 genera in culturomics study of weaning pig gut microbiome (Lee et al. 2022). *Lactobacillus* species, particularly *L. curvatus, L. sakei*, and *L. mucosae*, were predominant among the cultured species. Additionally, the establishment of PiBAC, a comprehensive collection of about 1100 bacterial pure cultures from 31 different media of pig gut microbiome, marks a notable advancement in this field (Wylensek et al. 2020). This assemblage, encompassing 117 strains that correspond to 110 species across 40 families and 9 phyla, was curated to ensure

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

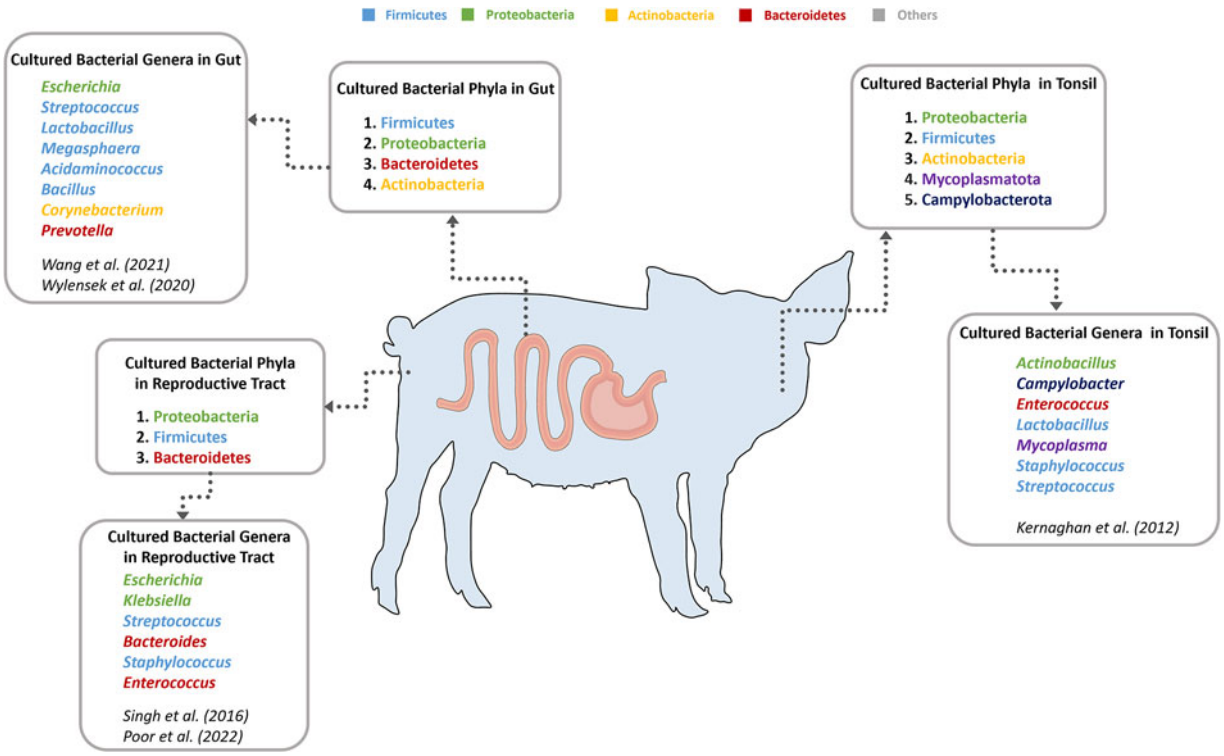

Figure 3. Most commonly cultured bacteria from swine.

extensive species-level representation. The majority of these species are commonly found in the dominant communities within the pig gut microbiota (Figure 3). Remarkably, 38 of these strains are categorized as novel taxa, highlighting a substantial broadening in our understanding of the pig gut microbiome's diversity. These findings are pivotal in elucidating both the functional roles and taxonomic diversity of these bacteria.

Culture-based study on reproductive tract microbiome

Earlier studies on the porcine vaginal microbiota identified a dominant presence of species such as *Streptococcus* sp., *E. coli, Staphylococcus* sp., *Corynebacterium* sp., *Micrococcus* sp., and *Actinobacillus* sp., by cultivation from 142 isolates (Bara et al. 1993; Larsen 1993). In a study of large black sows, the 115 isolates from vaginal swabs revealed a broader diversity, encompassing 30 species across 16 different genera (Singh and Ebibeni 2016). In this study, *Aeromonas* was the most frequently isolated at 39.2%, followed by *Enterococcus, Klebsiella, Escherichia*, and *Citrobacter*. Bacterial culture and identification by MALDI-TOF MS were employed to reveal a rich population of bacterial species in the vaginal canal of sows (Poor et al. 2022), showing that healthy sows had a higher frequency of *Enterococcus faecalis, Streptococcus hyovaginalis* and *Acinetobacter lwolffii* than the purulent vulvar discharge sows (Figure 3). This suggested a complex and dynamic bacterial ecosystem within the porcine vaginal environment, with certain species potentially playing key roles in maintaining reproductive health or contributing to disease states.

Culture-based study on pig tonsil microbiome

The presence of bacteria in the tonsil of the soft palate of swine has also been well-documented (Kernaghan et al. 2012), with reports up until 2012 indicating that more than 70 different bacterial species across 14 families have been cultured from this region (Figure 3). These studies have largely concentrated on identifying primary pathogens of swine and zoonotic agents. In instances involving organisms like *Mycoplasma hyopneumoniae* (Marois et al. 2007) or *Salmonella enterica* (Lomonaco et al. 2009), it is quite evident that these entities possess pathogenic capabilities. Conversely, bacteria such as *Actinobacillus minor* (Chiers et al. 2001) and *Actinobacillus porcitonsillarum* (Tonpitak et al. 2007) were likely to be harmless commensals. This diversity in the microbial population of the swine tonsils highlighted the complexity of the microbiome in these animals, encompassing a range of organisms with varying impacts on swine health.

Culturomics in pig microbiome research is an evolving field that combines traditional culture-dependent methods with modern molecular techniques. In addition, studies have shown the importance of integrating both culture-dependent and -independent methods to gain a more coherent picture of the pig gut microbiome. One study found that using a single culture medium with selective screens identified 46 distinct bacterial species, demonstrating an effective way to increase species diversity (Fenske et al. 2020). However, these cultured species did not fully represent the most abundant taxa in the microbiome, as revealed by metagenomic analysis. This discrepancy underscored the importance of combining both approaches for a more comprehensive understanding of the pig gut microbiome. This has led to significant advancements in understanding the diversity and function of microbial communities of swine microbiome.

The bovine culturomics

According to the United States Department of Agriculture National Agriculture Statistics Service, in 2022, there were 39.6 million

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

breeding cows (dairy and beef) and 27 million calves (dairy and beef) (Service UNAS 2022a, 2022b) and is the most economically important agricultural sector (Knight 2022). Therefore, due to its impact on cattle health and production, the bovine microbiota is of great importance. However, compared to traditional microbiome studies, very few have focused on culturing the microbiota, as most culture-based analyses focus on isolating opportunistic pathogens (Chai et al. 2022). Although no "tried and true" methods exist for culturomics studies (i.e., how many and which culture conditions should be included), it should be noted that many of the studies described below are not "true" culturomics studies because of the low number of media compositions and culture conditions they utilize. Regardless, due to a lack of true culturomics studies used to study the bovine microbiota, they will be described to provide readers with the current culture-based methods used to study the bovine microbiota and a starting point when designing their bovine culturomics studies.

Rumen

The bovine rumen microbiome has been heavily researched due to its relationship with feed efficiency and methane production (Newbold and Ramos-Morales 2020; O'Hara et al. 2020). Both the Global Rumen Census (Henderson et al. 2015) and Hungate 1000 (Creevey et al. 2014; Seshadri et al. 2018) projects have vastly increased our knowledge of the rumen microbiota. While the Global Rumen Census described the rumen microbiota of 32 species from 35 countries, it utilized only culture-independent methods (Henderson et al. 2015), while the goal of the Hungate 1000 project is to create a reference database of genome sequences and cultured rumen microbes. A meta-analysis was conducted to identify many target organisms for the Hungate 1000 project (Creevey et al. 2014). As of 2018, the Hungate collection was comprised of 21 archaea and 480 bacterial genomes, of which 410 are from cultured microbes and 91 are from previously available reference genomes. However, this collection has been estimated to only consist of approximately 75% of taxa in the rumen at the genera level, indicating a need for additional efforts to bring the remaining 25% into cultivation. Regardless, the Hungate collection is an extremely valuable resource for rumen microbiota research (Seshadri et al. 2018).

Early pre-Hungate1000, efforts focused on increasing the number of culturable rumen microbiota organisms include (Kenters et al. 2011; Nyonyo et al. 2013). Kenters et al. utilized a novel culture medium with a salt composition similar to the rumen fluid and dilution method. While this study utilized sheep rumen samples, not bovine, the authors isolated 60 new isolates, 19 of which likely belonged to previously undescribed genera (Kenters et al. 2011). Furthermore, Nyonyo et al. examined the inclusion of gelling agents in the culture medium and found that of the 69 unclassified isolates cultured, 24.4% were cultured on basal media (BM) supplemented with 1.8% agar (agar BM: A-BM), 56.6% were isolated on a modified basal medium (MBM) (0.1% $MgCl_2$ instead of KH_2PO_4) supplemented with 1.8% agar (agar MBM: A-MBM), 34.5% were isolated on the modified basal media supplemented with 0.8% Phytagel™ (Phytagel MBM: P-MBM), and 13.8% were isolated on the modified basal media modified supplemented with 1% Gelrite® (Gelrite MBM: G-MBM). The authors noted that previously uncultured bacteria were isolated from all media, but A-MBM, G-MBM, and P-MBM media increased the previously uncultured to total bacteria ratio (Nyonyo et al. 2013). Although not focused on rumen samples, Ziemer observed that including cellulose and xylan–pectin in an 8-week fermentation of bovine feces resulted in the isolation of many previously uncultured microbes (Ziemer 2014). Together, these studies indicate the importance of media composition in isolating fastidious microbes.

This is further emphasized by Zehavi et al., who cultured rumen samples on both defined (M10 media) and undefined (M10 + sterile rumen fluid) media with numerous sample dilutions and then sequenced the original rumen sample to estimate the percent of cultivable rumen operational taxonomic units (OTUs) (Zehavi et al. 2018). Overall, they observed that only 23% of the rumen microbiota detected using culture-independent methods was culturable using these two media compositions, with only 3.6% of cultured OTUs overlapping with the Hungate1000 database. After ruling out possible contamination, the authors concluded that they had confirmed the existence of a "rare rumen biosphere," as they cultured many isolates not detected using culture-independent methods, indicating the importance of conducting culture-dependent analyses in conjunction with conventional culture-independent studies. The authors also concluded that culture repetition and sample dilution increased the microbial diversity captured by culture. Although they only utilized two culture media, Zehavi et al. clearly indicate the importance of culture media composition and dilutions in isolating rumen microbes and illustrate the clear existence of a "rare rumen biosphere" (Zehavi et al. 2018).

As culturomics and culture-dependent analyses have grown in popularity, and the rare biosphere has been increasingly recognized, new methods have been developed to aid in isolating lower abundance microbes. Liu et al. utilized three methods to isolate ureolytic microbes from the bovine rumen, providing a valuable methodology and workflow that can be applied to other low-abundance rumen microbes (Liu et al. 2023). Briefly, the authors first utilized urease gene (*ureC*) guided enrichment. The *ureC*-positive cultures were then serially diluted and embedded into agarose microspheres, which were incubated in a dialysis bag within an *in situ* rumen environment consisting of non-sterile rumen fluid, media, and the cattle ration. At differing time points, up to 72 hours, the microspheres were crushed to obtain the isolated microbe, and full-length 16S sequencing was performed on the isolates. Using these methods, they obtained 976 total isolates comprised of 404 unique isolates. Of these, 28 isolates belonging to 12 bacterial species contained the *ureC* gene, including *Aliarcobacter butzleri, Citrobacter koseri, C. farmeri, C. amalonaticus, Clostridium butyricum, Corynebacterium vitaeruminis, Enterobacter hormaechei, E. cloacae, Klebsiella pneumoniae, Paraclostridium bifermentans, Pseudomonas stutzeri*, and *Proteus penneri* (Liu et al. 2023).

It is clear that more wide-scale culturomics studies are needed to bring the previously uncultured microbes into cultivation and further characterize the rare rumen biosphere. While many of these studies only utilized a few different media compositions, they indicate the need for multiple media compositions, as is observed in human culturomics studies (Diakite et al. 2020). Including numerous culture conditions may result in an increased number of isolated fastidious microbes. Moreover, Zehavi et al. , Kenters et al. , and Ziemer illustrate the importance of media composition being similar to that of the niche of interest (Kenters et al. 2011; Zehavi et al. 2018; Ziemer 2014). Regardless, these provide useful media compositions for future culturomics analyses focused on isolating microbes from the rumen and a methodology for incorporating new methods, such as *in situ* cultivation and gene-guided enrichment, into a culturomics workflow (Liu et al. 2023).

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

Reproductive

While the uterus has been historically considered sterile, it is now well-accepted that a uterine microbiome does exist. The reproductive (vaginal and uterine) microbiome is considered low biomass, and numerous environmental factors have been observed to contribute to reproductive microbiome variation. Additionally, while uterine microbiota diversity has been linked to health, dysbiosis has been associated with metritis. It has been speculated that host-derived probiotics may be developed to maintain microbiota stability and protect against disease (Çömlekcioğlu et al. 2024).

Similarly, most culture-dependent analyses on the reproductive microbiota have focused on comparing health statuses (Kronfeld et al. 2022; Paiano et al. 2022; Wagener et al. 2015). Wagener et al. cultured 2052 isolates from the uterus of postpartum dairy calves from 76 genera. However, 24.2% and 13.2% of the isolates were from the genera *Staphylococcus* and *Trueperella*, respectively. However, only two media types (sheep blood and MacConkey) were used (Wagener et al. 2015). Kronfeld et al. cultured bacteria from the vagina and uterus of healthy postpartum cattle and those with puerperal disorders utilizing five culture conditions consisting of aerobic, anaerobic, and microaerophilic conditions. In total, from vaginal samples, they isolated 561 bacteria from 46 genera, and from uterine samples, they isolated 409 bacteria from 42 genera. In both locations, regardless of health status, *Streptococcus* was the most isolated genera (Kronfeld et al. 2022). Paiano et al. isolated 127 bacterial species from the uteri of healthy cattle and those with clinical and subclinical endometritis utilizing four aerobic culture conditions. In healthy cattle, 97 bacterial species were isolated, while in cattle with clinical and subclinical endometritis, 53 and 21 bacterial species were isolated, respectively. The opportunistic pathogens *Bacillus cereus, Escherichia coli*, and *Aerococcus viridans* were the most isolated bacterial species and were isolated from all health statuses. Interestingly, bacteria were unable to be cultured from 35.4%, 40%, and 18.1% of samples from healthy, subclinical, and clinical cattle, respectively (Paiano et al. 2022). However, this is likely due to the low number of culture conditions used. Wagener et al. (2015), Kronfeld et al. (2022), and Paiano et al. (2022) indicate that there is increased research interest in utilizing culture-based methods to isolate both commensal microbes and opportunistic pathogens from the bovine reproductive tract and illustrate the diversity of the cultivable bovine reproductive microbiome.

Unlike the aforementioned studies, Webb et al. utilized both culture-dependent and -independent analyses to study the uterine and vaginal microbiota and its relationship with beef cattle fertility (Webb et al. 2023). They utilized five culture conditions consisting of both aerobic and anaerobic isolation, resulting in 512 isolates from 52 genera from the vaginal samples and 221 isolates from 29 genera from the uterine samples. *Streptococcus* was the most isolated genera regardless of location. Although they combined both culture-dependent and -independent analyses, the authors did not comment on the percentage of the vaginal and uterine microbiota that was culturable utilizing their conditions (Webb et al. 2023).

Therefore, additional culturomics studies paired with culture-independent analyses to describe the cultivability of the bovine reproductive tract, isolate key players in the bovine reproductive microbiota, and screen isolates for potential function are needed. Regardless, these studies provide valuable information such as media compositions, culture conditions, and currently isolated bacteria, which can provide researchers with a framework for designing future more in-depth culturomics studies to study the bovine reproductive tract.

Other niches of interest

Two other disease areas that could greatly benefit from increased culturomics analyses include mastitis and bovine respiratory disease (BRD). Angelopoulou et al. conducted culturomics analysis on milk samples from cattle with mastitis from five different culture conditions (Angelopoulou et al. 2019). They observed a large difference between the culture-independent and -dependent results. Using 16S amplicon sequencing, 36 genera were present, of which only 8 (*Bacillus, Carnobacterium, Enterococcus, Escherichia/Shigella, Pseudomonas, Staphylococcus, Streptococcus*, and *Trueperella*) were cultured. In addition, four genera (*Barnesiella, Kocuria, Microbacterium*, and *Raoultella*) were cultured but not detected using 16S amplicon sequencing. While it is possible that these bacteria are the result of contamination, they likely represent the rare biosphere discussed previously. Therefore, the results of Angelopoulou et al. indicate a need for additional culturomics studies focusing on the udder and milk microbiota (Angelopoulou et al. 2019).

It is well accepted that the structure, diversity, and composition of the respiratory microbiome of healthy cattle and those with BRD differs (Chai et al. 2022; Howe et al. 2023). However, no true culturomics studies and very few culture-dependent analyses focusing strictly on commensal microbes have been conducted. Holman et al. isolated commensal bacteria from the nasopharyngeal tract at feedlot entry and after 60 days within the feedlot (Holman et al. 2015). They utilized three culture media (brain heart infusion (BHI), de Man, Rogosa, Sharpe (MRS), and 5% sheep blood agar) and 16S rRNA sequencing for identification paired with 454 pyrosequencing. Using these conditions, they isolated 605 isolates from 32 genera, including *Moraxella, Pasteurella, Mannheimia, Corynebacterium*, and *Acinetobacter*, most often isolated from the BHI and blood agar, and *Bacillus, Staphylococcus, Lactobacillus, Aerococcus*, and *Streptococcus* from MRS. The authors also noted differences between the culture-dependent and -independent analyses as *Aerococcus, Dietzia, Proteus, Rothia*, and *Micrococcus* were cultured but not present in the culture-independent data (Holman et al. 2015). Additionally, Amat et al. isolated many commensal bacteria from the bovine nasopharyngeal tract (Amat et al. 2019a), focusing specifically on lactic acid-producing bacteria previously identified as potentially health-associated members of the bovine nasopharyngeal microbiota (Amat et al. 2019b). They isolated 300 colonies from 14 genera; however, they utilized only two culture conditions (MRS and Rogosa agar), both of which were focused on isolating lactic acid-producing bacteria. The most commonly isolated genera were *Bacillus, Staphylococcus, Streptococcus*, and *Lactobacillus*. Moreover, although they were focused on isolating lactic acid-producing bacteria, 69% of isolates were not (Amat et al. 2019a). These studies illustrate the need for a true culturomics study focused on the bovine respiratory microbiota.

Culture-dependent analyses, similar to culturomics, have been valuable tools in studying the bovine microbiota, especially the rumen microbiota (Kenters et al. 2011; Liu et al. 2023; Nyonyo et al. 2013; Seshadri et al. 2018; Zehavi et al. 2018; Ziemer 2014); however, as a whole, high-throughput culture has not been as utilized to study the bovine microbiota as it has been in humans (Bilen et al. 2018; Diakite et al. 2020; Lagier et al. 2012, 2016). While it is clear that a rumen and mammary/milk rare biosphere exists (Angelopoulou et al. 2019; Zehavi et al. 2018), the true cultivability of the reproductive and respiratory tract microbiotas remains unexplored. Regardless, many microbes, both commensal

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

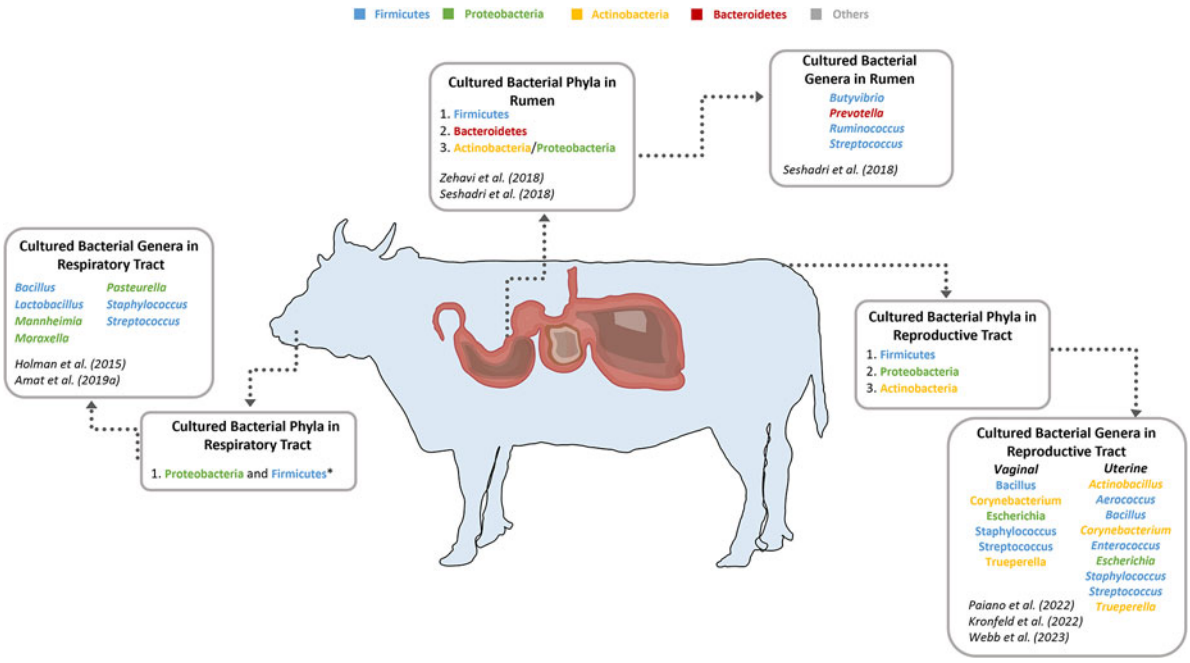

Figure 4. Most commonly cultured bacteria from bovine. * indicates an inability to differentiate due to commonly used selective agar.

and opportunistic pathogens, have been isolated from the bovine microbiota (Figure 4).

While most culture-based studies have focused on the rumen, increasing the number of "culturable" rumen microbes, many members of the rumen microbiota still have not been brought into cultivation (Zehavi et al. 2018). Likely, future culturomics studies utilizing a wide array of culture conditions, some of which should be highly similar to the niche of interest (Kenters et al. 2011; Zehavi et al. 2018; Ziemer 2014), will increase the number of cultured microbes, providing additional insight into these microbial communities and their overall role in health and productivity. Therefore, there is a great need for large-scale bovine culturomics studies to increase the cultivability of the bovine microbiota and further evaluate the existence and role of low-abundance microbes. Doing so will aid the development of host-derived bacterial therapeutics to increase cattle health and productivity.

Applications of culturomics in probiotic development

While probiotics are typically toted as the new "cure-all," their true effectiveness is debated, especially regarding their ability to colonize the host (Suez et al. 2020). However, developing next-generation probiotics (NGPs) may help overcome this problem. NGPs are commensal members of the host microbiome identified to likely confer health benefits to the host (O'Toole et al. 2017). Since they are host-derived, niche-specific microbes, there is a higher likelihood that they will colonize the administration site, presumably resulting in longer-lasting effects compared to conventional probiotics (Chuang et al. 2022). Many known health-associated human gastrointestinal microbiota members are currently being investigated as potential NGPs (El Hage et al. 2017). Tools such as machine learning algorithms like RandomForest and many differential abundance algorithms can identify potential NGPs from microbiome data, as they are often utilized to determine ASVs or OTUs associated with a specific disease state (Howe et al. 2023). It is currently recommended that multiple algorithms are utilized to determine differentially abundant taxa (Nearing et al. 2022). These potential NGPs must then be obtained in pure culture to confirm their health-promoting effects. As a result, culturomics is a highly valuable addition to the NGP development pipeline, as it can increase the number of available potential NGPs that can undergo further screening (Chang et al. 2019). The current research regarding NGP development is covered below and can be loosely split into two groups: studies utilizing culturomics and studies intensively screening isolates of interest.

Studies utilizing culturomics/high-throughput culture methods

Duquenoy et al. determined bacteria significantly enriched in the ceca of both low- and high-performing chicks and utilized culturomics to isolate these microbes (Duquenoy et al. 2020). Their anaerobic culture conditions included three base media (modified Gifu Anaerobic Medium (mGAM), Luria–Bertani Miller medium, and *Lactobacillus* medium) supplemented with antibiotics, rumen fluid, or sodium taurocholate. Samples also underwent pretreatment with heat and ethanol to isolate spore-forming bacteria. These culture conditions allowed them to isolate 541 colonies, although only 358 could be sub-isolated. These colonies were identified utilizing MALDI-TOF MS and full-length 16S sequencing. Using the full-length 16S sequencing, the authors were able to match the isolates to the corresponding OTU, as they used the QIIME pipeline for their culture-independent analysis. Based on the 16S sequences, the authors concluded that they likely isolated a novel bacterial species from the chicken ceca. Furthermore, the isolates were then screened for anti-*Campylobacter jejuni* activity. In total, nine isolates were observed to have moderate to strong anti-*C. jejuni* activity. These isolates belonged to the following genera: *Enterococcus, Lactobacillus, Bacillus*, and *Escherichia* (Duquenoy et al. 2020). Utilizing 16S sequencing to identify the isolated colonies alone or in conjunction with MALDI-TOF MS does increase the workload; however, it allows researchers to be confident they isolated the OTU or ASV of interest and can allow

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

the identification of potentially novel isolates, as illustrated by Duquenoy et al. (2020).

Tidjani Alou et al. utilized both 16S amplicon sequencing and culturomics to study the gut microbiota of humans with kwashiorkor, also known as severe acute malnutrition (Tidjani Alou et al. 2017). They utilized 18 culture conditions consisting of different preincubations and pretreatments, including filtration and thermic shock, as well as differing incubation temperatures and oxygen conditions, to isolate 12,000 colonies. The isolates were then identified using MALDI-TOF MS, and the ones that could not be identified using MS underwent 16S sequencing. In the kwashiorkor samples, the authors isolated nine novel bacterial species and nine novel bacterial genera. In the control samples, the authors isolated 26 new bacterial species, 8 new genera, and 1 new bacterial family. Furthermore, Tidjani Alou et al. isolated the 12 likely potential probiotics, as they were present only in the control samples, and bacteria of their classification are known to have probiotic characteristics. These potential probiotics included *Alistipes indistinctus, Anerostipes caccae, Bacillus lichenformis, B. subtilis, Bacteroides salyersiae, Bifidobacterium adolescentis, Intestinimonas butyriciproducens, Lactobacillus parabuchneri, L. perolens, L. vaccinostercus, Terrisporobacter glycolicus*, and *Weisella confusa* (Tidjani Alou et al. 2017). However, to our knowledge, they performed no further mechanism screening confirming the isolates' probiotic capabilities.

Li et al. utilized metagenomics and culturomics to examine the gut microbiota of centenarians (Li et al. 2022). Based on their metagenomic sequencing data, they identified taxa that differed based on age and identified taxa associated with increased longevity. The authors then utilized 98 sample pretreatments and 23 media compositions to isolate these taxa, resulting in over 8000 isolates, which mostly belonged to 203 known bacterial species; however, novel members of the gastrointestinal tract were also isolated. Under anaerobic conditions, the authors cultured 41 "undefined" isolates that were likely novel. Under microaerobic conditions, one undefined, likely novel isolate was cultured. In addition, the authors noted that 1430 species were identified in their study. However, only 116 species were identified utilizing both culture-independent and -dependent methods, and 140 species were identified using only culturomics (Li et al. 2022). This indicates the importance of utilizing culturomics with culture-independent methods, as it provides deeper insight into the microbial community and further emphasizes the possible existence of a rare biosphere in the human gastrointestinal tract, proving the importance of integrating culturomics with culture-independent studies when studying the microbiota. Furthermore, Li et al. cultured an increased number of bacterial species from the *Enterococcus* and *Lactobacillus* genera from centenarians, similar to their metagenomic data (Li et al. 2022). While Li et al. did not consider these isolates potential probiotics, their methods provide a valuable example of how pairing culturomics with metagenomic sequencing can result in the isolation of taxa of interest. Future studies could then further characterize those isolates and determine if they provide health-promoting benefits within the human gastrointestinal tract (Li et al. 2022).

To this point, Wang et al. performed culturomics on healthy human fecal samples in order to isolate potential probiotics, although they did not pair with culture-independent methods (Wang et al. 2021). They utilized six preincubations, media types, and dilutions. Using these conditions, obtained 1100 colonies, of which 31 were isolated, identified, and screened colonies. These isolates were screened for antimicrobial resistance and both bile salt and low pH resistance to determine if they could survive the gastrointestinal tract. The isolates' cell-free supernatant was screened for antimicrobial activity against *E. coli, S. aureus*, and *S. typhimurium*. The authors concluded that *Weisella confusa* isolates were potential probiotics (Wang et al. 2020).

The studies outlined above utilized high-throughput culturomics, and in doing so, they obtained a great number of isolates, many of which were novel microbes or had never been isolated from the niche of interest before (Tidjani Alou et al. 2017). These methods also allowed them to culture potential NGPs (Duquenoy et al. 2020; Li et al. 2022; Tidjani Alou et al. 2017; Wang et al. 2020) and potential members of the "rare biosphere" that were not detected by culture-independent analysis (Li et al. 2022). Moreover, Wang et al. (2020) and Duquenoy et al. (2020) performed further screening of isolates deemed potential probiotics. Screening isolates to confirm their health- or production-related benefits and determine their ability to colonize their administration site is crucial. In the following section, we discuss studies where the taxa of interest were not fastidious; therefore, high-throughput culture was unnecessary. These studies employed more high-throughput screening of the isolates than the ones described previously.

Studies utilizing intensive isolate screening

Wang et al. cultured lactic acid-producing bacteria from sow milk on three media types (MRS, trypticase phytone yeast extract, and glucose yeast extract peptone), resulting in 1240 isolates from 271 taxa (Wang et al. 2022). Additionally, 151 taxa belonged to previously identified microbes, while 120 were unidentified. Furthermore, 80 isolates belonged to the genera *Pediococcus*. *Pediococcus pentosaceus* isolates were then screened for antimicrobial activity against *Salmonella typhimurium*, Enterohemorrhagic *Escherichia coli*, Enterotoxigenic *Escherichia coli, Klebsiella pneumoniae, Aeromonas punctata* subsp. Caviae, *Staphylococcus aureus, Listeria monocytogenes*, and *Clostridum perfringens*. The top 10 strains based on the antimicrobial assays were then inoculated into *Drosophilia melanogaster*, which were then treated with paraquat. The flies that were colonized with *P. pentosaceus* SMM914 had an increased survival rate. Finally, *P. pentosaceus* SMM914 was fed to piglets before early weaning. The authors concluded that treatment with *P. pentosaceus* SMM914 alleviated oxidative stress and reduced liver injury (Wang et al. 2022).

Kang et al. paired microbiome sequencing with culturomics analysis to analyze the gut microbiome of dogs and its relationship with aging (Kang et al. 2022). They observed that *Lactobacillus* and *Enterococcus* greatly decreased as dogs aged. They then utilized three culture conditions and obtained 305 total isolates. As they were primarily interested in lactic acid-producing bacteria, they selected those colonies to screen for acid and bile tolerance, mucin adhesion, antimicrobial susceptibility, and antimicrobial activity against *Listeria monocytogenes, Staphylococcus aureus, Salmonella typhimurium*, and *Escherichia coli*. Then, they utilized a Fermentation of the Intestinal Microbiota Mode model. Using this model, they cultured young and aged canine feces with mGAM media and supplemented the aged feces with *Lactobacillus salivarius* and *Enterococcus hirae*. They observed that this supplementation affected the composition and increased the diversity of the microbiota, specifically increasing the Chao1 index of the aged group back to the level of the young group (Kang et al. 2022).

Chuang et al. utilized culture-independent and -dependent analyses to study the gut microbiota of calves with diarrhea (Chuang et al. 2022). Using LeFse analysis, they identified

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

health-associated microbial biomarkers, which they then utilized sample dilutions and one media composition (MRS + 0.05% L-cysteine hydrochloride monohydrate) to isolate these microbes. The isolates were identified using full-length 16S sequencing. The isolated health-associated microbes were then screened for antimicrobial activity against *Bacillus cereus, Escherichia coli, Salmonella enterica, Staphylococcus aureus*, and *Vibrio parahaemolyticus* and cytokine stimulation, specifically TNFα and IL-10) of the murine macrophage cell line RAW 264.7. Based on these results, they concluded that two strains of *Bifidobacterium longum* subsp. *longum* were NGPs for calf diarrhea (Chuang et al. 2022).

Amat et al. identified that lactic acid-producing bacteria were negatively correlated with *Pasteurellaceae* in the bovine nasopharyngeal tract (Amat et al. 2019b). To further evaluate the bovine nasopharyngeal microbiota, Amat et al. isolated 300 colonies on MRS and Rogosa agar (Amat et al. 2019a). These isolates were then identified using full-length 16S Sanger sequencing. The authors then screened 178 isolates for *Mannheimia haemolytica*, a BRD opportunistic pathogen, growth inhibition. The authors then screened 47 isolates for adherence to bovine turbinate cells *in vitro*. Based on adherence assay results, 15 isolates were selected for *M. haemolytica* competitive inhibition assay and antimicrobial susceptibility testing. From there, 10 isolates were screened for innate and adaptive immune stimulation *in vitro*. Based on these results, six isolates from four species of *Lactobacillus* were selected as the best candidates for a bacterial therapeutic cocktail (Amat et al. 2019a). This cocktail was then tested *in vivo* in two different studies (Amat et al. 2020, 2023).

The studies outlined above clearly illustrate that there is no "tried and true" pipeline for NGP development. Duquenoy et al. (2020), Chuang et al. (2022), Wang et al. (2022), and Amat et al. (2019a, 2019b, 2020, 2023) all illustrate the development of NGPs in veterinary medicine and animal agriculture. However, many of these studies did not focus on isolating fastidious bacteria and, as a result, did not utilize a wide array of culture conditions. Li et al. (2022) and Tidjani Alou et al. (2017) were true culturomics-based studies utilizing a wide array of culture conditions; however, they did not heavily screen their isolates-of-interest as was done by Wang et al. (2020, 2022), Chuang et al. (2022), Duquenoy et al. (2020) and Amat et al. (2019a, 2020, 2023). Such screening is necessary to confirm the NGPs' potential beneficial health effects observed from culture-independent analyses, their ability to successfully colonize the administration site and to determine their probable function within the microbial community. Therefore, combining the methods of the studies outlined above may provide a useful framework for increasing the culture conditions in NGP development and increasing mechanistic screening of isolates in culturomics studies. Additionally, utilizing a wider variety of culture conditions may be valuable even if the microbe of interest is not fastidious, as many studies (Angelopoulou et al. 2019; Li et al. 2022; Zehavi et al. 2018) have cultured taxa not detected by culture-independent analyses. As NGP development typically identifies taxa of interest from culture-independent studies, these microbes may have undiscovered probiotic potential. Utilizing only a few culture conditions focused only on the microbes of interest could result in missing these isolates.

Applications of culturomics in studying the roles of microbiota

The composition and functional role of the microbiota can vary based on ecological niche (Human Microbiome Project Consortium 2012). However, overall, the microbiota provides pathogen colonization resistance, converts indigestible food into metabolites that the host can utilize, removes toxic compounds, synthesizes vitamins, produces antimicrobial compounds, increases mucus production and barrier function, and modulates the immune system (Heintz-Buschart and Wilmes 2018; Plaza-Diaz et al. 2019). Most information regarding microbiota function is based on -omics data, including metagenomics, metatranscriptomics, metabolomics, and metaproteomics. However, many challenges exist, such as a lack of data regarding the function of many microbiome-associated genes. Depending on the data analysis tool, up to 40–70% of protein-coding genes' function cannot be predicted, and the function of many genes detected from metagenomic datasets is unknown. Moreover, many members of the microbiota still have yet to be cultured. As a result, their functional capacity has yet to be explored (Heintz-Buschart and Wilmes 2018). To prove that a microbe's genomic functional data corresponds to actual functionality, it is necessary for the microbe to be grown in pure culture. Further illustrating the importance of pure culture, previously unknown bacterial pathways have also been discovered from experiments performed using pure culture (Liu et al. 2022). Culturomics can help solve these challenges by bringing these fastidious microbes into cultivation, allowing them to be further characterized, providing data about their potential function. A few studies to this effect are described as follows.

Ghimire et al. combined metagenomic sequencing with culturomics to study the human fecal microbiota utilizing one base media (modified BHI) supplemented with different antimicrobials and pretreatment with chloroform or heat shock, resulting in 12 total culture conditions (Ghimire et al. 2020). Using these conditions, 1590 colonies were isolated. Combining culturomics and metagenomics identified many open reading frames missing from the human microbiome-integrated gene catalog, and whole genome sequencing of the cultured isolates identified additional genes missing from the metagenomic analysis. Ghimire et al. were then able to perform *Clostridioides difficile* growth inhibition assays and additional biochemical characterization of the cultured isolates, providing valuable information on the role of those microbes within the human gut as well as information on how the microbial community works together to increase host health (Ghimire et al. 2020).

As previously discussed, using media compositions with similar salt content to the niche of interest or including the sample type (rumen fluid, fecal slurry) has resulted in isolating novel, previously uncultured microbes (Kenters et al. 2011; Zehavi et al. 2018; Ziemer 2014). These microbes may require a growth factor or metabolite found in the environment. Strandwitz et al. (hypothesized that unknown or novel growth factors produced by other microbes in the community might be necessary to bring some fastidious microbes into cultivation and developed an assay to screen for this (Strandwitz et al 2019). Briefly, a human fecal sample was cultured on fastidious anaerobic agar + yeast, and colonies were recorded for a week. Slow growing colonies that grew next to an early growing colony were isolated. The authors identified one colony (KLE1738), which required *Bacteroides fragilis* KLE1758 to grow. Based on full-length 16S sequencing, KLE1738 is likely a member of a novel genus within the Ruminococcaceae family. *Bacteroides fragilis* KLE1758 supernatant was purified and underwent nuclear magnetic resonance analysis. KLE1738 was cultured individually with all supernatant components; however, only Gamma-aminobutyric acid (GABA) resulted in KLE1738 growth (Strandwitz et al. 2019). This study indicates a novel function of

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

GABA producers and GABA within the human gut microbiome and illustrates that GABA-producers could be keystone members of the gut microbiota as it is clear that *Bacteroides fragilis* KLE1758 was essential for KLE1738 growth. It is likely that utilizing Strandwitz et al. (2019)'s methods to study the microbiota of other niches could identify additional novel bacterial species and potential keystone members of the microbiota and determine the potential role of specific microbes within the microbial community.

Further screening of potential NGPs, as discussed in the previous section, illustrates how screening pure cultures can provide insight into the role of the microbiota in animal health. For example, Kang et al. further screened their potential probiotics isolated using culture-dependent/culturomics methods described in the previous section to determine their potential role in aging using a *Caenorhabditis elegans* model. These microbes were fed to *C. elegans*, worm lifespan, thrashing, and chemotaxis were measured. Feeding *C. elegans* the probiotics increased lifespan and decreased aging-related degeneration. Additionally, probiotic supplementation likely prevented degeneration due to an increased expression of *skn-1, ser-7,* and *odr-3, 7, 10* (Kang et al. 2022), indicating a potential role of the gut microbiota in aging and brain degeneration. Furthermore, Amat et al. (2019a), described in the section above (Applications of culturomics in probiotic development), screened the microbes they isolated from the bovine nasopharyngeal microbiota and found that many of the isolates inhibited *M. haemolytica* growth *in vitro*, a BRD opportunistic pathogen and member of the bovine respiratory tract microbiota, competitively inhibited *M. haemolytica,* and stimulated the host immune system *in vitro* (Amat et al. 2019a). This data indicates a potential protective role of nasopharyngeal microbiota.

Moreover, suppose a specific potential microbiota function is of interest. In that case, gene-guided enrichment can be paired with culturomics to isolate bacterial species harboring the gene of interest, as illustrated by Liu et al. (2023), who utilized gene-guided enrichment to isolate ureolytic microbes from the rumen. Once isolated, microbes containing the gene of interest can then undergo additional screening and characterization, confirming the function of the gene of interest and providing additional information regarding their function within the ecosystem. While culturomics is still in its infancy, it holds great promise for elucidating the role of the microbiota, especially when paired with metagenomic sequencing, as a pure culture of an isolate is required to confirm the role of a microbe and its genes within a community (Liu et al. 2022).

Considerations for designing culturomics studies

Isolation methods used in culturomics

There are several effective methods for microbial isolation through culturomics (Figure 5). Traditional isolation relied on randomly picking up the colonies in the bacterial culture plate. This method is low cost; however, it comes with inevitable drawbacks such as increased time and labor and uncertainty. In addition, fast-growing bacteria may inhibit slow-growing bacteria during culture processing. The 96-well plate-based isolation could solve these problems to some extent (Zhang et al. 2021). In this workflow, a two-sided barcode PCR system based on Illumina technology was used to target the bacterial 16S rRNA in each well of the plate. This pipeline relieves tedious effort in picking colonies one by one but cannot avoid repeated isolation of the same dominant bacteria. Recently, an automatic machine learning-based high-throughput isolation promotes the development of culturomics (Huang et al. 2023). Huang et al. developed the Culturomics by Automated Microbiome Imaging and Isolation (CAMII) platform. This machine's workflow can be divided into four functions: (1) an imaging and machine learning algorithm component that selects colonies based on colony morphology; (2) an automated robot that isolates the selected colonies; (3) a pipeline to sequence the isolates; and (4) a bank and database containing the physical isolate and colony morphology and genomic sequence. This tool can greatly reduce the labor and time required for a culturomics project as well as increase precision colony picking. Additionally, Huang et al. have developed a public biobank database based on their results (CAMII strain biobank [microbial-culturomics.com]) with culture, morphology, and genomic data that could help researchers when developing their own culture conditions (Huang et al. 2023). The approach is able to identify different colony morphology and achieve the prediction of phenotype–genotype integration in the isolation process. The authors obtained 26,997 isolates with 1,197 high-quality genomes from humans in this paper, pioneering a new and effective automated culturomics method.

Culturomics strategies and conditions

The National Institute of Health and the Human Microbiome Project have developed a Most Wanted List describing the taxa they deem the highest priority to bring into cultivation (https://www.hmpdacc.org/most_wanted/) (Fodor et al. 2012). In this section, we provide a framework for readers when designing culturomics studies and discuss some new methods that can be utilized to culture particularly fastidious microbes of interest.

To the authors' knowledge, the first culturomics study utilized 212 unique culture conditions, including various pretreatments, nutrient and antibiotic supplementation, oxygen conditions, and filtering steps, to culture microbes from the human gastrointestinal tract. The authors noted that all isolated bacteria could be cultured utilizing only 70 conditions (Lagier et al. 2012). Consequently, additional studies have focused on reducing these conditions to a more manageable number and expanding them to culture a wider variety of microbes. Utilizing the previous conditions and additional ones to cultivate a wider variety of microbes, Lagier et al. isolated 1057 unique microbial species. These included 197 novel species, 187 bacterial species, and 1 archaeal species that had never been isolated from humans, and 146 species that had never been isolated from the human gastrointestinal tract (Lagier et al. 2016). Furthermore, Diakite et al. reduced the culture conditions even further (from 58 to 25) and noted that 98% of cultured bacteria could be isolated using only 16 conditions (Diakite et al. 2020). These studies illustrate that pretreating samples with alcohol allow for isolating spore-forming bacteria (Afouda et al. 2020; Diakite et al. 2020) and that filtering and antibiotic supplementation allow for isolating low-abundant microbes (Lagier et al. 2012). Although these studies list the media compositions utilized, the specific culture conditions used should mimic the natural environment of the sample being cultured as closely as possible (Kaeberlein et al. 2002). This can include ensuring the salt composition of the media is similar to that of the sample (Kenters et al. 2011) or by providing undescribed growth factors from the environment, such as a sterilized sample (Zehavi et al. 2018; Ziemer 2014). It is possible that the microbe of interest may require an unknown growth factor produced by another microbe within the

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

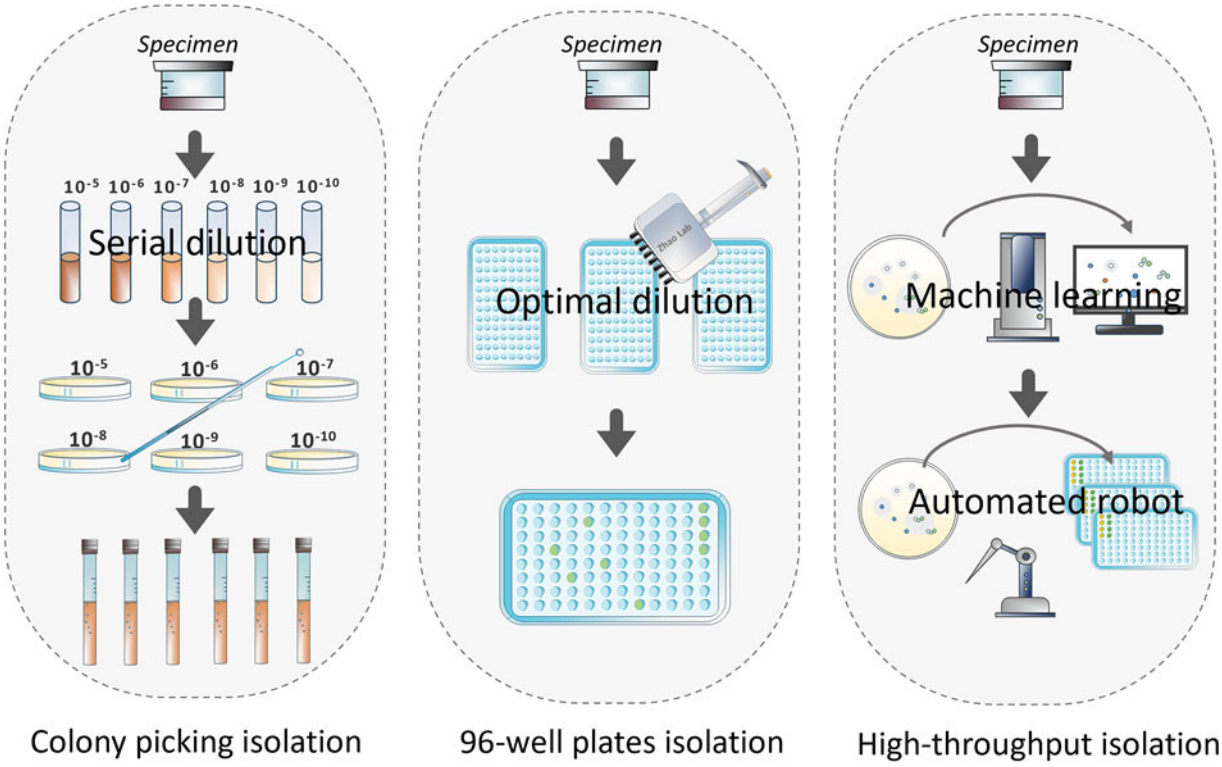

Figure 5. Different isolation methods for culturomics.

community. In this instance, coculture methods can be performed to isolate the microbe (Strandwitz et al. 2019).

Coculture can also allow for microbes that require intensive conditions for cultivation to be cultured more easily, as illustrated by Khelaifia et al., who cocultured *Bacteroides thetaiotaomicron* with *Methanobrevibacter smithii*. Using this method, *M. smithii* was able to be cultured aerobically (Khelaifia et al. 2016). Similarly, Wang et al. illustrated that many bacteria considered strict anaerobes, such as *Dorea, Clostridium, Megasphaera, Blautia, Mogibacterium, Prevotella*, and *Bacteroides* could be cultured under aerobic conditions on specific media compositions (Wang et al. 2021). Both the methods and conditions employed by Khelaifia et al. (2016) and Wang et al. (2021) can be used to investigate growth conditions that can be used to more easily culture other strict anaerobes aerobically. In addition, Khan et al. developed an oxygen adaptation protocol that increased *Faecalibacterium prausnitzii*'s oxygen tolerance (Khan et al. 2023). Similar methodologies may be utilized for other anaerobic species once cultured. Moreover, as metabolites and growth factors produced by host cells can affect the microbiota (Jensen et al. 2020), it is possible that a host cell from the niche of interest may be producing a metabolite required for growth. Jalili-Firoozinezhad et al. developed an "intestine-on-a-chip" model to culture and analyze community interactions between both anaerobic and aerobic microbes and the human intestinal epithelium. While they utilized this method to analyze the microbiota and its interactions (Jalili-Firoozinezhad et al. 2019), we speculate that, in the future, this model, or ones like it, may be tweaked to isolate fastidious microbes requiring unknown growth factors produced by host cells or multiple microbes.

As previously discussed, antibiotic supplementation and filtering are commonly used to isolate low abundant microbes (Lagier et al. 2012), dilutions (Zehavi et al. 2018), as well as single-cell isolation, can also be utilized to selectively culture microbes present in a sample at lower abundances that may be outcompeted by more abundant, less fastidious microbes. Bellais et al. (have illustrated that flow cytometry cell sorting can isolate fastidious microbes anaerobically. Briefly, authors generated antibodies for ATCC strains of *Faecalibacterium prausnitzii* and DSM strain of *Christensenella minuta*. These antibodies were then used with flow cytometry to sort *F. prausnitzii* and *C. minuta* from fecal samples. They observed that Live/Dead staining and antibody labeling did not affect the cultivability of the bacteria and that the antibody labeling and sorting enriched the microbe of interest (Bellais et al. 2022).

Culture-independent sequencing data can also be utilized to design culture conditions to isolate fastidious microbes. Metagenomic data can guide cultivation through gene-targeted isolation and stable-isotope probing guided Raman-activated microbial cell sorting (Liu et al. 2022). Liu et al. (2023) provide a detailed methodology for utilizing gene-guided isolation methods to isolate ureolytic bacteria from the rumen (Liu et al. 2023). Jing et al. illustrates the use and methodology of stable-isotope probing guided Raman-activated microbial cell sorting combined with culturing and sequencing (scRACS-Seq/Culture) to isolate phosphate solubilizing microbes from sewage (Jing et al. 2022). Additionally, the bioinformatics software Traitar can be used to analyze bacterial genomes and metagenomic assemblies, providing information on 67 traits related to oxygen requirements, carbon and energy sources, and antibiotic susceptibility, providing data valuable for formulating media compositions and developing culture conditions (Weimann et al. 2016). While this review has focused mostly on culturomics as it applies to bacteria, culturomics has also been utilized to study archaea and fungi, and the review by Tidjani

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

Alou et al. discusses considerations for utilizing culturomics for archaea and fungi, as well as bacteria (Tidjani Alou et al. 2020). To aid researchers, we have compiled a table of basal media used in the studies cited in this review (Supplementary Table S1). For brevity, we did not include the preincubations, nutritive additives, or antimicrobial additives used by these studies. If a specific culture condition is needed, researchers can see the corresponding citation. Additionally, the review by Tidjani Alou et al. also contains a table of media compositions utilized by their citations as well (Tidjani Alou et al. 2020).

Conclusions

In conclusion, this manuscript reveals the transformative impact of culturomics in microbiology. It has proven instrumental in expanding our understanding of the microbiota in humans and animals, offering novel insights into microbial diversity. This approach not only complements existing culture-independent methods but also paves the way for groundbreaking discoveries in microbial ecology and potential therapeutic applications. The integration of culturomics with other techniques underscores a holistic approach to studying microbial communities, crucial for advancing medical and environmental research. The future of microbiome studies, enriched by culturomics, holds immense promise for unveiling the intricate connections between microbes and their hosts, opening new horizons in health and disease management.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/anr.2024.6.

Acknowledgements. J.Z. contributed to conceptualization. S.H., Z.L., and B.Z. contributed to writing the manuscript. S.H., Z.L., B.Z., and J.Z. edited the manuscript. All authors read and approved the manuscript.

Funding support. This work was supported by Agriculture and Food Research Initiative Competitive Grant program (no. 20196701629869) from the USDA National Institute of Food and Agriculture, and in part by the funds administered by the Arkansas Biosciences Institute.

References

Afouda P, Hocquart M, Pham TP, et al. (2020) Alcohol pretreatment of stools effect on culturomics. *Scientific Reports* **10**(1), 5190.

Allison MJ, Robinson I, Bucklin J, et al. (1979) Comparison of bacterial populations of the pig cecum and colon based upon enumeration with specific energy sources. *Applied and Environmental Microbiology* **37**(6), 1142–1151.

Amat S, Alexander TW, Holman DB, et al. (2020) Intranasal bacterial therapeutics reduce colonization by the respiratory pathogen *Mannheimia haemolytica* in dairy calves. *mSystems* **5**(2), e00629–19.

Amat S, Holman DB, Timsit E, et al. (2019b) Evaluation of the nasopharyngeal microbiota in beef cattle transported to a feedlot, with a focus on lactic acid-producing bacteria. *Frontiers in Microbiology* **10**, 1988.

Amat S, Timsit E, Baines D, et al. (2019a) Development of bacterial therapeutics against the bovine respiratory pathogen *Mannheimia haemolytica*. *Applied Environmental Microbiology* **85**(21), e01359–19.

Amat S, Timsit E, Workentine M, et al. (2023) A single intranasal dose of bacterial therapeutics to calves confers longitudinal modulation of the nasopharyngeal microbiota: A pilot study. *mSystems* **8**(2), e0101622.

Angelopoulou A, Holohan R, Rea MC, et al. (2019) Bovine mastitis is a polymicrobial disease requiring a polydiagnostic approach. *International Dairy Journal* **99**, 104539.

Bara M, McGowan M, O'boyle D, et al. (1993) A study of the microbial flora of the anterior vagina of normal sows during different stages of the reproductive cycle. *Australian Veterinary Journal* **70**(7), 256–259.

Bellais S, Nehlich M, Ania M, et al. (2022) Species-targeted sorting and cultivation of commensal bacteria from the gut microbiome using flow cytometry under anaerobic conditions. *Microbiome* **10**(1), 24.

Bilen M (2020) Strategies and advancements in human microbiome description and the importance of culturomics. *Microbial Pathogenesis* **149**, 104460.

Bilen M, Dufour JC, Lagier JC, et al. (2018) The contribution of culturomics to the repertoire of isolated human bacterial and archaeal species. *Microbiome* **6**, 94.

Bloom SM, Mafunda NA, Woolston BM, et al. (2022) Cysteine dependence of *Lactobacillus iners* is a potential therapeutic target for vaginal microbiota modulation. *Nature Microbiology* **7**(3), 434–450.

Chai JM, Capik SF, Kegley B, et al. (2022) Bovine respiratory microbiota of feedlot cattle and its association with disease. *Veterinary Research* **53**(1), 4.

Chai J, Weiss CP, Beck PA, et al. (2024) Diet and monensin influence the temporal dynamics of the rumen microbiome in stocker and finishing cattle. *Journal of Animal Science and Biotechnology* **15**, 12.

Chang CJ, Lin TL, Tsai YL, et al. (2019) Next generation probiotics in disease amelioration. *Journal of Food and Drug Analysis* **27**(3), 615–622.

Chaudhari SN, McCurry MD and Devlin AS (2021) Chains of evidence from correlations to causal molecules in microbiome-linked diseases. *Nature Chemical Biology* **17**(10), 1046–1056.

Chen Y, Zhou J and Wang L (2021) Role and mechanism of gut microbiota in human disease. *Frontiers in Cellular and Infection Microbiology* **11**, 625913.

Chiers K, Haesebrouck F, Mateusen B, et al. (2001) Pathogenicity of *Actinobacillus minor, Actinobacillus indolicus* and *Actinobacillus porcinus* strains for gnotobiotic piglets. *Journal of Veterinary Medicine, Series B* **48**(2), 127–131.

Cho HW and Eom YB (2021) Forensic analysis of human microbiome in skin and body fluids based on geographic location. *Frontiers in Cellular and Infection Microbiology* **11**, 695191.

Chuang ST, Chen CT, Hsieh JC, et al. (2022) Development of next-generation probiotics by investigating the interrelationships between gastrointestinal microbiota and diarrhea in preruminant holstein calves. *Animals (Basel)* **12**(6), 695.

Çömlekcioğlu U, Jezierska S, Opsomer G, et al. (2024) Uterine microbial ecology and disease in cattle: A review. *Theriogenology* **213**, 66–78.

Creevey CJ, Kelly WJ, Henderson G, et al. (2014) Determining the culturability of the rumen bacterial microbiome. *Microbial Biotechnology* **7**(5), 467–479.

Deng F, Han Y, Huang Y, et al. (2024) A comprehensive analysis of antibiotic resistance genes in the giant panda gut. *iMeta* **3**(1), e171.

Deng F, Wang C, Li D, et al. (2023) The unique gut microbiome of giant pandas involved in protein metabolism contributes to the host's dietary adaption to bamboo. *Microbiome* **11**, 180.

Diakite A, Dubourg G, Dione N, et al. (2020) Optimization and standardization of the culturomics technique for human microbiome exploration. *Scientific Reports* **10**(1), 9674.

Diakite A, Dubourg G and Raoult D (2021) Updating the repertoire of cultured bacteria from the human being. *Microbial Pathogenesis* **150**, 104698.

Diop K, Dufour J-C, Levasseur A, et al. (2019) Exhaustive repertoire of human vaginal microbiota. *Human Microbiome Journal* **11**, 100051.

Dubourg G, Morand A, Mekhalif F, et al. (2020) Deciphering the urinary microbiota repertoire by culturomics reveals mostly anaerobic bacteria from the gut. *Frontiers in Microbiology* **11**, 513305.

Duquenoy A, Ania M, Boucher N, et al. (2020) Caecal microbiota compositions from 7-day-old chicks reared in high-performance and low-performance industrial farms and systematic culturomics to select strains with anti-Campylobacter activity. *PLoS One* **15**(8), e0237541.

El Hage R, Hernandez-Sanabria E and Van de Wiele T (2017) Emerging trends in "Smart Probiotics": Functional consideration for the development of novel health and industrial applications. *Frontiers in Microbiology* **8**, 1889.

Fan Y and Pedersen O (2021) Gut microbiota in human metabolic health and disease. *Nature Reviews Microbiology* **19**(1), 55–71.

Fei N, Bruneau A, Zhang X, et al. (2020) Endotoxin producers overgrowing in human gut microbiota as the causative agents for nonalcoholic fatty liver disease. *Mbio* **11**(1), e03263–19.

Fenske GJ, Ghimire S, Antony L, et al. (2020) Integration of culture-dependent and independent methods provides a more coherent picture of the pig gut microbiome. *FEMS Microbiology Ecology* **96**(3), fiaa022.

Fewins BG, Newland L and Briggs C (1957) The normal intestinal flora of the pig. III. Qualitative studies of lactobacilli and streptococci. *Journal of Applied Microbiology* **20**(2), 234–242.

Fodor AA, DeSantis TZ, Wylie KM, et al. (2012) The "most wanted" taxa from the human microbiome for whole genome sequencing. *PLoS One* **7**(7), e41294.

Fonkou MD, Dufour J-C, Dubourg G, et al. (2018) Repertoire of bacterial species cultured from the human oral cavity and respiratory tract. *Future Microbiology* **13**, 1611–1624.

Fournier PE, Raoult D and Drancourt M (2017) New Species Announcement: A new format to prompt the description of new human microbial species. *New Microbes and New Infections* **15**, 136–137.

Ghimire S, Roy C, Wongkuna S, et al. (2020) Identification of *Clostridioides difficile*-inhibiting gut commensals using culturomics, phenotyping, and combinatorial community assembly. *mSystems* **5**(1), e00620–19.

Goodwin S, McPherson JD and McCombie WR (2016) Coming of age: Ten years of next-generation sequencing technologies. *Nature Reviews Genetics* **17**, 333–351.

Gresse R, Chaucheyras-Durand F, Fleury MA, et al. (2017) Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. *Trends in Microbiology* **25**(10), 851–873.

Hajishengallis G, Liang S, Payne MA, et al. (2011) Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host and Microbe* **10**(5), 497–506.

Heintz-Buschart A and Wilmes P (2018) Human gut microbiome: Function matters. *Trends in Microbiology* **26**(7), 563–574.

Henderson G, Cox F, Ganesh S, et al. (2015) Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Scientific Reports* **5**, 14567.

Hilt EE, McKinley K, Pearce MM, et al. (2014) Urine is not sterile: Use of enhanced urine culture techniques to detect resident bacterial flora in the adult female bladder. *Journal of Clinical Microbiology* **52**(3), 871–876.

Hol WH, Garbeva P, Hordijk C, et al. (2015) Non-random species loss in bacterial communities reduces antifungal volatile production. *Ecology* **96**(8), 2042–2048.

Holman DB, Timsit E and Alexander TW (2015) The nasopharyngeal microbiota of feedlot cattle. *Scientific Reports* **5**, 15557.

Howe S, Kegley B, Powell J, et al. (2023) Effect of bovine respiratory disease on the respiratory microbiome: A meta-analysis. *Frontiers in Cellular and Infection Microbiology* **13**, 1223090.

Huang Y, Sheth RU, Zhao S, et al. (2023) High-throughput microbial culturomics using automation and machine learning. *Nature Biotechnology* **41**(10), 1424–1433.

Hugon P, Dufour JC, Colson P, et al. (2015) A comprehensive repertoire of prokaryotic species identified in human beings. *The Lancet Infectious Diseases* **15**(10), 1211–1219.

Human Microbiome Project Consortium (2012) Structure, function and diversity of the healthy human microbiome. *Nature* **486**(7402), 207–214.

Hungate R and Macy J (1973) The roll-tube method for cultivation of strict anaerobes. In *Bulletins from the Ecological Research Committee* Stockholm: Oikos Editorial Office. 123–126.

Jalili-Firoozinezhad S, Gazzaniga FS, Calamari EL, et al. (2019) A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip. *Nature Biomedical Engineering* **3**(7), 520–531.

Janda JM and Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *Journal of Clinical Microbiology* **45**(9), 2761–2764.

Jensen EA, Young JA, Mathes SC, et al. (2020) Crosstalk between the growth hormone/insulin-like growth factor-1 axis and the gut microbiome: A new frontier for microbial endocrinology. *Growth Hormone and IGF Research* **53–54**, 101333.

Jiao JY, Liu L, Hua ZS, et al. (2021) Microbial dark matter coming to light: Challenges and opportunities. *National Science Review* **8**(3), nwaa280.

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), 106.

Jousset A, Bienhold C, Chatzinotas A, et al. (2017) Where less may be more: How the rare biosphere pulls ecosystems strings. *ISME Journal* **11**(4), 853–862.

Kaeberlein T, Lewis K and Epstein SS (2002) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science* **296**(5570), 1127–1129.

Kang AN, Mun D, Ryu S, et al. (2022) Culturomic-, metagenomic-, and transcriptomic-based characterization of commensal lactic acid bacteria isolated from domestic dogs using *Caenorhabditis elegans* as a model for aging. *Journal of Animal Science* **100**(12), skac323.

Kenters N, Henderson G, Jeyanathan J, et al. (2011) Isolation of previously uncultured rumen bacteria by dilution to extinction using a new liquid culture medium. *Journal of Microbiological Methods* **84**(1), 52–60.

Kenworthy R and Crabb W (1963) The intestinal flora of young pigs, with reference to early weaning, *Escherichia coli* and scours. *Journal of Comparative Pathology* **73**, 215–228.

Kernaghan S, Bujold AR and MacInnes JI (2012) The microbiome of the soft palate of swine. *Animal Health Research Reviews* **13**(1), 110–120.

Khan MT, Dwibedi C, Sundh D, et al. (2023) Synergy and oxygen adaptation for development of next-generation probiotics. *Nature* **620**(7973), 381–385.

Khelaifia S, Lagier JC, Nkamga VD, et al. (2016) Aerobic culture of methanogenic archaea without an external source of hydrogen. *European Journal of Clinical Microbiology and Infectious Diseases* **356**(6), 985–991.

Knight R (2022) Cattle and Beef: Sector at a Glance. 2023. https://www.ers.usda.gov/topics/animal-products/cattle-beef/sector-at-a-glance/ (accessed 26 December 2023).

Kronfeld H, Kemper N and Hölzel CS (2022) Vaginal and uterine microbiomes during puerperium in dairy cows. *Agriculture* **12**(3), 405.

Lagier JC, 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.

Lagier JC, Dubourg G, Million M, et al. (2018) Culturing the human microbiota and culturomics. *Nature Reviews Microbiology* **16**, 540–550.

Lagier JC, Hugon P, Khelaifia S, et al. (2015) The rebirth of culture in microbiology through the example of culturomics to study human gut microbiota. *Clinical Microbiology Reviews* **28**(1), 237–264.

Lagier JC, Khelaifia S, Alou MT, et al. (2016) Culture of previously uncultured members of the human gut microbiota by culturomics. *Nature Microbiology* **1**, 16203.

Larsen B (1993) Vaginal flora in health and disease. *Clinical Obstetrics and Gynecology* **36**(1), 107–121.

Lee WJ, Ryu S, Kang AN, et al. (2022) Molecular characterization of gut microbiome in weaning pigs supplemented with multi-strain probiotics using metagenomic, culturomic, and metabolomic approaches. *Animal Microbiome* **4**(1), 60.

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**, 28.

Liu S, Moon CD, Zheng N, et al. (2022) Opportunities and challenges of using metagenomic data to bring uncultured microbes into cultivation. *Microbiome* **10**, 76.

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), 76.

Lomonaco S, Decastelli L, Bianchi D, et al. (2009) Detection of *Salmonella* in finishing pigs on farm and at slaughter in Piedmont, Italy. *Zoonoses and Public Health* **56**(3), 137–144.

Lynch MD and Neufeld JD (2015) Ecology and exploration of the rare biosphere. *Nature Reviews Microbiology* **13**(4), 217–229.

Malla MA, Dubey A, Yadav S, et al. (2018) Understanding and designing the strategies for the microbe-mediated remediation of environmental contaminants using omics approaches. *Frontiers in Microbiology* **9**, 1132.

Man WH, de Steenhuijsen Piters WA and Bogaert D (2017) The microbiota of the respiratory tract: Gatekeeper to respiratory health. *Nature Reviews Microbiology* **15**(5), 259–270.

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

Marois C, Le Carrou J, Kobisch M, et al. (2007) Isolation of *Mycoplasma hyopneumoniae* from different sampling sites in experimentally infected and contact SPF piglets. *Veterinary Microbiology* **120**, 96–104.
Maruvada P, Leone V, Kaplan LM, et al. (2017) The human microbiome and obesity: Moving beyond associations. *Cell Host & Microbe* **22**(5), 589–599.
Ma L, Tao S, Song T, et al. (2024) *Clostridium butyricum* and carbohydrate active enzymes contribute to the reduced fat deposition in pigs. *IMETA* **3**, e160.
Mendes R, Garbeva P and Raaijmakers JM (2013) The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. *FEMS Microbiology Reviews* **37**(5), 634–663.
Morand A, Cornu F, Dufour J-C, et al. (2019) Human bacterial repertoire of the urinary tract: A potential paradigm shift. *Journal of Clinical Microbiology* **57**(3), e00675–e00618.
Nearing JT, Douglas GM, Hayes MG, et al. (2022) Microbiome differential abundance methods produce different results across 38 datasets. *Nature Communications* **13**(1), 342.
Newbold CJ and Ramos-Morales E (2020) Review: Ruminal microbiome and microbial metabolome: Effects of diet and ruminant host. *Animal* **14**(s1), s78–s86.
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.
O'Hara E, Neves ALA, Song Y, et al. (2020) The role of the gut microbiome in cattle production and health: Driver or passenger? *Annual Review of Animal Biosciences* **8**, 199–220.
O'Toole PW, Marchesi JR and Hill C (2017) Next-generation probiotics: The spectrum from probiotics to live biotherapeutics. *Nature Microbiology* **2**, 17057.
Paiano RB, Moreno LZ, Gomes VTM, et al. (2022) Assessment of the main pathogens associated with clinical and subclinical endometritis in cows by culture and MALDI-TOF mass spectrometry identification. *Journal of Dairy Science* **105**(4), 3367–3376.
Parte AC, Sarda Carbasse J, Meier-Kolthoff JP, et al. (2020) List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. *International Journal of Systematic and Evolutionary Microbiology* **70**(11), 5607–5612.
Plaza-Diaz J, Ruiz-Ojeda FJ, Gil-Campos M, et al. (2019) Mechanisms of Action of Probiotics. *Advances in Nutrition* **10**, S49–S66.
Pleguezuelos-Manzano C, Puschhof J, Rosendahl Huber A, et al. (2020) Mutational signature in colorectal cancer caused by genotoxic pks(+). *E. Coli. Nature* **580**(7802), 269–273.
Poor AP, Moreno LZ, Monteiro MS, et al. (2022) Vaginal microbiota signatures in healthy and purulent vulvar discharge sows. *Scientific Reports* **12**(1), 9106.
Pust MM, Wiehlmann L, Davenport C, et al. (2020) The human respiratory tract microbial community structures in healthy and cystic fibrosis infants. *NPJ Biofilms and Microbiomes* **6**(1), 61.
Rinke C, Schwientek P, Sczyrba A, et al. (2013) Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**(7459), 431–437.
Robinson IM, Allison MJ and Bucklin JA (1981) Characterization of the cecal bacteria of normal pigs. *Applied and Environmental Microbiology* **41**(4), 950–955.
Robinson IM, Whipp SC, Bucklin JA, et al. (1984) Characterization of predominant bacteria from the colons of normal and dysenteric pigs. *Applied and Environmental Microbiology* **48**(5), 964–969.
Russell EG (1979) Types and distribution of anaerobic bacteria in the large intestine of pigs. *Applied and Environmental Microbiology* **37**(2), 187–193.
Salanitro J, Blake I and Muirhead P (1977) Isolation and identification of fecal bacteria from adult swine. *Applied and Environmental Microbiology* **33**(1), 79–84.
Satam H, Joshi K, Mangrolia U, et al. (2023) Next-generation sequencing technology: Current trends and advancements. *Biology (Basel)* **12**(7), 997.
Seng P, Drancourt M, Gouriet F, et al. (2009) Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases* **49**(4), 543–551.
Service UNAS (2022a) Cattle, Cows - Inventory.
Service UNAS (2022b) Cattle, Calves - Inventory.
Seshadri R, Leahy SC, Attwood GT, et al. (2018) Cultivation and sequencing of rumen microbiome members from the Hungate1000 Collection. *Nature Biotechnology* **36**(4), 359–367.
Shah T, Shah Z, Baloch Z, et al. (2021) The role of microbiota in respiratory health and diseases, particularly in tuberculosis. *Biomedicine and Pharmacotherapy* **143**, 112108.
Singh BR and Ebibeni N (2016) Aerobic microbiome of vagina of apparently healthy pregnant large black sows in Nagaland and antimicrobial resistance in isolates. *Global Journal of Pathology and Microbiology* **4**, 8–17.
Sorbara MT and Pamer EG (2022) Microbiome-based therapeutics. *Nature Reviews Microbiology* **20**, 365–380.
Strandwitz P, Kim KH, Terekhova D, et al. (2019) GABA-modulating bacteria of the human gut microbiota. *Nature Microbiology* **4**(3), 396–403.
Suez J, Zmora N and Elinav E (2020) Probiotics in the next-generation sequencing era. *Gut Microbes* **11**(1), 77–93.
Tidjani Alou M, Million M, Traore SI, et al. (2017) Gut bacteria missing in severe acute malnutrition, can we identify potential probiotics by culturomics? *Frontiers in Microbiology* **8**, 899.
Tidjani Alou M, Naud S, Khelaifia S, et al. (2020) State of the art in the culture of the human microbiota: New interests and strategies. *Clinical Microbiology Reviews* **1**, e00129–19.
Tonpitak W, Rohde J and Gerlach G-F (2007) Prevalence of "*Actinobacillus porcitonsillarum*" in porcine tonsils and development of a diagnosis duplex PCR differentiating between "*Actinobacillus porcitonsillarum*" and *Actinobacillus pleuropneumoniae*. *Veterinary Microbiology* **122**, 157–165.
van der Gast CJ, Walker AW, Stressmann FA, et al. (2011) Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. *ISME Journal* **5**(5), 780–791.
Van Houte JGR (1966) Studies of the cultivable flora of normal human feces. *Antonie, Van Leeuwenhoek* **32**(2), 212–222.
Vartoukian SR, Palmer RM and Wade WG (2010) Strategies for culture of 'unculturable' bacteria. *FEMS Microbiology Letters* **309**(1), 1–7.
Wagener K, Prunner I, Pothmann H, et al. (2015) Diversity and health status specific fluctuations of intrauterine microbial communities in postpartum dairy cows. *Veterinary Microbiology* **175**, 286–293.
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), e0047721.
Wang L, Liu Q, Chen Y, et al. (2022) Antioxidant potential of *Pediococcus pentosaceus* strains from the sow milk bacterial collection in weaned piglets. *Microbiome* **10**(1), 83.
Wang W, Liu W and Chu W (2020) Isolation and preliminary screening of potentially probiotic *Weissella confusa* strains from healthy human feces by culturomics. *Microbial Pathogenesis* **147**, 104356.
Wang X, Tsai T, Deng F, et al. (2019) Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome* **7**, 109.
Webb EM, Holman DB, Schmidt KN, et al. (2023) Sequencing and culture-based characterization of the vaginal and uterine microbiota in beef cattle that became pregnant or remained open following artificial insemination. *Microbiology Spectrum* **11**(6), e0273223.
Weimann A, Mooren K, Frank J, et al. (2016) From genomes to phenotypes: Traitar, the microbial trait analyzer. *mSystems* **1**(6), e00101–e00116.
Welch CB, Ryman VE, Pringle TD, et al. (2022) Utilizing the gastrointestinal microbiota to modulate cattle health through the microbiome-gut-organ axes. *Microorganisms* **10**(7), 1391.
Wolfe AJ and Brubaker L (2019) Urobiome updates: Advances in urinary microbiome research. *Nature Reviews Urology* **16**(2), 73–74.
Wolfe AJ, Toh E, Shibata N, et al. (2012) Evidence of uncultivated bacteria in the adult female bladder. *Journal of Clinical Microbiology* **50**(4), 1376–1383.
Wylensek D, Hitch TC, Riedel T, et al. (2020) A collection of bacterial isolates from the pig intestine reveals functional and taxonomic diversity. *Nature Communications* **11**(1), 6389.
Zagato E, Pozzi C, Bertocchi A, et al. (2020) Endogenous murine microbiota member *Faecalibaculum rodentium* and its human homologue protect from intestinal tumour growth. *Nature Microbiology* **5**(3), 511–524.

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press

Zehavi T, Probst M and Mizrahi I (2018) Insights into culturomics of the rumen microbiome. *Frontiers in Microbiology* **9**, 1999.

Zhang J, Liu YX, Guo X, et al. (2021) High-throughput cultivation and identification of bacteria from the plant root microbiota. *Nature Protocols* **16**(2), 988–1012.

Zhao L and Zhao N (2021) Demonstration of causality: Back to cultures. *Nature Reviews Gastroenterology and Hepatology* **18**(2), 97–98.

Zhu B, Tao Z, Edupuganti L, et al. (2022) Roles of the microbiota of the female reproductive tract in gynecological and reproductive health. *Microbiology and Molecular Biology Reviews* **86**(4), e00181–00121.

Ziemer CJ (2014) Newly cultured bacteria with broad diversity isolated from eight-week continuous culture enrichments of cow feces on complex polysaccharides. *Applied Environmental Microbiology* **80**(2), 574–585.

https://doi.org/10.1017/anr.2024.6 Published online by Cambridge University Press