Debunking the myth of unculturable bacteria: recent advances and considerations in the culture of the gut microbiota

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Abstract

The gut microbiota has a crucial function in human health. A disbalance in the microbiota composition can lead to a variety of diseases, including inflammatory bowel disease, neurodevelopmental disorders, and cancer. Therefore, it is important to study and identify the gut microbiota. In the past, the gut bacterial microbiota was studied using culture-based techniques. However, due to suboptimal methods and protocols, many bacteria were thought to be unculturable. Currently, new efforts to optimize culture-based techniques allowed the high-throughput cultivation of various previously uncultivated gut bacteria. This approach is called culturomics. The workflow of culturomics can roughly be divided into three steps: sample collection, culture, and bacteria identification. Here, we describe the recent advances and considerations in these culturomics processes, including sampling and preservation methods, the design of culture medium, and data collection and storage. Although there are many successful efforts in culturomics, there are still various aspects that need to be explored in the future. Further optimizing the culturomics protocol could potentially lead to the isolation and identification of novel, clinically relevant, bacterial species.

Lay summary

The human gut not only consists of human cells, but also of many different bacteria. These bacteria are very important for health, as an imbalance in gut bacteria can lead to various diseases, such as inflammatory bowel disease and cancer. Therefore, it is important to study these gut bacteria. In the past, gut bacteria were studied by isolating them from humans and growing them in the laboratory. This process is called ‘cultivation’, and it allows researchers to perform experiments on the gut bacteria. Unfortunately, a lot of bacteria could not be cultivated, because the available methods and techniques were not capable enough. However, recent research has successfully improved these methods, so that many more gut bacteria could be cultured. In this report, we describe the latest improvements and concerns in the different steps of gut bacterial cultivation. These steps include the collection of bacteria from a persons gut, the culture of these bacteria in the laboratory, and puzzling out which types of bacteria were actually cultured. With this information, we hope to further improve the process of bacterial culture, so that more bacteria can be grown in the laboratory. This will help in discovering which bacteria contribute to disease development, how they do so, and what we can do to prevent this.

Introduction

The human gastrointestinal tract is an important habitat for numerous microbes, including bacteria, fungi, archaea and viruses[1]. This collection of microbes is called the 'gut microbiota', and has a variety of functions, including the metabolism of nutrients and drugs, the modulation of the immune system, the maintenance of the gut integrity, and the protection against pathogen colonization[1]. A disbalance in the microbiota composition is strongly associated with several disorders, such as inflammatory bowel disease, diabetes, allergies and neurodevelopmental disorders[2]. The gut microbiota is thus crucial for human health, and the identification and study of the microbes residing in the gastrointestinal tract is therefore of great importance. Unfortunately, studying the functional and behavioral characteristics of the gut microbiota remains a major challenge.

The gut microbiota, and in particular the bacterial gut microbiota, was traditionally studied with culture-based techniques. However, only a small fraction of the gut bacteria could be cultured with the available techniques, leaving many gut bacteria labeled as 'unculturable'. There are two major reasons for this unculturability of gut bacteria. Firstly, many gut bacteria require an anaerobic atmosphere for proper growth and survival, and due to inconvenient methods, a large part of the bacteria did not survive the traditional process of cultivation. Secondly, the diversity of bacterial strains in the gut drives the need for a diversity in growth conditions, which complicated the culture of the *entire* gut bacterial microbiota. These convolutions in bacterial culture led to a paradigm shift from culture-based techniques towards the use of culture-independent techniques, among which 16s rRNA sequencing and high throughput metagenomic sequencing (*metagenomics*) are currently the most well-established techniques[3]. 16s rRNA sequencing and metagenomics allowed for a more rapid and high quality identification of gut bacteria, and further revealed the hidden and complex composition of the gut microbiota[4]. Yet, these sequence-based techniques have multiple downsides. Firstly, sequence-based methods have a relatively high detection threshold. Therefore, these techniques mainly capture the most abundant microbes within a sample, whilst minority populations, which may significantly affect human health, are missed[5,6]. This phenomenon is known as 'depth bias'[5,6]. Secondly, sequence-based techniques show discrepancies in sequencing data between different sequencing centra, which is probably due to differences and errors in library preparations, DNA extraction methods and computational methods[7]. Thirdly, sequence-based techniques can only identify bacteria on the level of genotyping, whilst culture-based techniques are required to characterize bacteria on the level of physiology and pathophysiology[8,9]. Therefore, the culture-independent paradigm is currently partially shifting back towards the culture-based paradigm, with 'culturomics' as a new approach[5,10].

Culturomics is the high-throughput isolation, culture and characterization of the bacterial microbiota, which allows the assessment of etiological and molecular microbial mechanisms[10]. This new approach required the optimization of traditional culture conditions, and underwent a major breakthrough after implementation of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), which is an accurate, rapid, and cost-effective method to identify bacteria[11]. Strikingly, several studies show that culturomics and metagenomics identify different bacterial species. For example, Lagier *et al*. showed that within stool samples, only 15% of the identified bacterial species overlap between the two techniques[5]. A few years later, Lagier *et al*. showed that culturomics identified potentially new bacterial species that were undetected by a metagenomics approach[12]. Thus, culturomics complements metagenomics[12,13]. Overall, culturomics is a promising technique that potentially renders the label of 'unculturable bacterium' to be inaccurate.

The process of culturomics can be roughly divided into three steps (Figure 1). Firstly, bacterial samples are collected from either healthy individuals or specific individuals of interest. To get a representation of the *gut* microbiota composition, samples are often obtained from the feces[14]. In order to properly culture the gut microbiota, the viability and diversity of the microbiota should be minimally affected by the sampling and pre-processing methods. Second is the actual cultivation step, in which samples are divided into various culture conditions. These culture conditions have different physio-chemical properties or have specific nutritional supplements, to allow the growth of particular species[5,15]. In the final step, bacterial species are identified using the MALDI-TOF MS or 16s rRNA

sequencing approach[15]. The identified bacteria are formally described and the data have to be properly stored.

During the whole workflow of this cultivation-based approach, systematic biases and inadequacies can occur, making culturomics less optimal. Moreover, there are still many bacteria that have not yet been cultured, despite the emergence of culturomics. In fact, it has recently been estimated that approximately 1900 bacterial species of the human gut microbiota are still uncultivated[16]. Finally, culturomics remains a time-consuming and labor intensive method. Many efforts have been made to overcome these difficulties and issues, in order to properly cultivate the 'unculturable' bacteria. In this review, we will discuss the latest improvements and optimization steps of bacterial culturomics. We chose to hereby focus on the entire workflow of culturomics, since the success of culturing uncultivated bacterial species is not only dependent on the actual cultivation step, but also on preceding and subsequent steps. Furthermore, we discuss some challenges, considerations, and research gaps in culturomics studies.

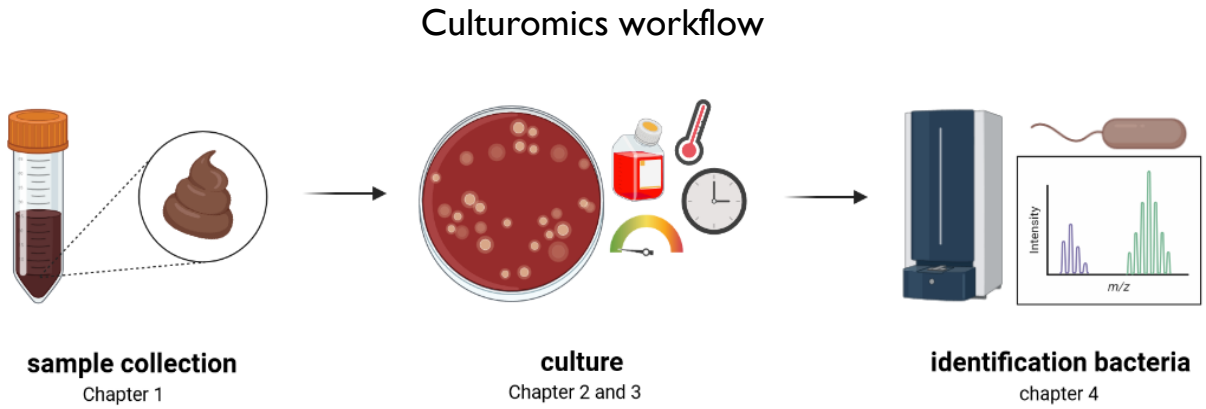

***Figure 1**: general overview of the culturomics workflow. First, samples are collected from individuals of interest. Then, the samples are cultured using a variety of culture conditions. Finally, bacterial colonies are identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry or 16s rRNA sequencing. In this report, advances and considerations in these individual steps are discussed. Figure is created in BioRender.*

1. Sample collection

1.1 sampling

The first important step in understanding the gut microbiota and its composition, is to consider a sampling method that obtains the most representative sample of the actual microbiota. There are various sampling methods used for the collection of the gut microbiota[17]. The most widely used method is fecal sampling, for fecal samples can be obtained in a non-invasive and convenient manner[17]. However, fecal samples are enriched for bacterial species that reside in the terminal colon, and hence the bacterial species of the upper gastrointestinal tract and small intestines are underrepresented or not even present in the feces. In addition, the microbes of the upper gastrointestinal tract that *are* found in the feces are mixed with bacteria originating from other parts of the gut. This makes it hard to trace back the exact gastrointestinal location of fecal derived bacteria[17,18]. Finally, the bacteria found in feces are predominantly representative for gut bacteria that reside in the gut lumen and only sparsely for mucosa-associated gut bacteria[17,19,20]. Thus, for the precise description of the gut microbiota, fecal sampling is suboptimal. Besides the use of stool samples, samples can be obtained by using several other methods, including endoscopic sampling of the terminal ileum, mucosal biopsies, luminal brushing, laser capture microdissection and catheter aspiration[17]. These sampling methods give a more accurate description of the gut microbiota

compared to fecal sampling, and have the advantage that the exact site of sampling is known[17]. Nevertheless, these methods are invasive, cause discomfort and have an inevitable chance of contamination[17]. From an ethical point of view, it is therefore debatable to use these methods in healthy individuals. Recently, an ingestible self-activating microdevice was developed to collect luminal and mucosa-associated bacteria from the upper gastrointestinal tract[21]. This technique is very promising for site-specific and non-invasive microsampling, but it is still far from optimal[21]. Future research is needed to address the safety of this sampling method in humans, and to demonstrate the viability and cultivability of the samples[21]. Altogether, there is a need for the further development of sampling methods that are both accurate and non-invasive, and until then, fecal sampling will remain the standard for gut microbiota research. Therefore, in the remainder of this chapter we will focus on fecal sampling. However, it is important to keep the shortcomings of fecal sampling in mind.

Besides the sampling method, it is also important to consider which study participants to include for sampling. Low abundance of bacterial species in participants and their feces is one of the reasons some bacteria are missed in culture[22]. There are a lot of different factors that affect the composition and the diversity of the human gut microbiota, including ethnicity, age, and diet[23]. Metagenomics data shows that uncultivated and novel bacterial species are most abundant in rural populations from non-Western countries[16,24]. Thus, using a wide range of individuals with different backgrounds and lifestyles, for example those from non-Western countries, might result in the cultivation of novel bacteria. In addition, computational methods might be a novel approach to link metagenomics data of uncultivated bacterial species to different outcomes, such as age and diet. This data can then be used to predict the specific study participants that are potentially needed to cultivate uncultured bacterial species[16].

Subsampling

There is a great variation in the distribution of gut bacteria within individual fecal samples. Local bacterial concentrations vary from 10^{11} bacteria/mL to concentrations that are undetectable[25,26]. Moreover, the amount of different bacterial taxa varies between the inside and the exterior of the feces. For example, the exterior surface has a lower concentration of *Firmucutes* and *Bifidobacteria spp.* compared to the inside region[27]. Thus, random subsampling from different spatial locations can hugely impact the composition and diversity captured by bacterial culture, and might therefore result in the misinterpretation of the microbiota profile[28,29]. Even though the impact of random subsampling on microbiota profiling is very high, many culturomics studies do not mention which fecal regions were used for culture (Supplementary Table 1). Therefore, a more detailed description of (sub)sampling regions by the authors will allow other researchers to better interpretate data and compare culturomics data between different experiments.

To overcome bacterial differences in spatial locations, homogenization of the feces prior to the culture might be beneficial. On the contrary, the bacterial differences in spatial locations might actually be useful to specifically culture bacterial taxa. For example, the surface of feces is likely to be in closer contact with the intestinal mucosa, and subsampling of this region might therefore yield more mucosa-related gut bacteria[29]. To implement this approach in practice, more research on possible correlations between bacterial compositions in fecal regions and original residence in the gastrointestinal tract would be important.

1.2 Sample handling

Sample preservation

The time between sample collection and culture should ideally be minimal. However, it is not always possible to start the bacterial culture of fresh stool samples directly after sampling, since sample collection often takes place outside the lab, for example in the participants' home[17]. In these cases, the fecal samples need to be stored, until further processing can take place. For the storage of feces, there are a several of options, including freezing in -20°C and -80°C or storage in preservations[30,31]. Freezing in -80°C is currently the most well-established method for the storage of feces[32]. Fouhy *et al*.

found no differences in the presence of culturable bacterial populations of fecal samples that were either fresh or stored in -80°C[33]. Moreover, rapid freezing on dry ice prior to -80°C storage did not significantly affect the culture bacterial population[33]. This finding is surprising, as one might expect that rapid freezing reduces ice crystal formation, which would be beneficial for the viability and cultivability of bacteria[33]. However, it is noteworthy that the study screened a predefined subset of bacteria, so there is a need to validate the findings in a wider range of bacteria[33]. Direct freezing of the feces is not always feasible. However, keeping fecal samples at room temperature or body temperature (37 °C), especially if the time between sampling and processing exceeds 24 hours, results in the extinction of anaerobes and the proliferation of aerobic and facultative anaerobic bacterial species, compared to immediate culturing[34]. Short-term storage at 4°C when processing is not earlier than 24 hours is therefore recommended[34,35].

Many studies on the storage of fecal samples use DNA analysis and bacterial abundance as outcome. However, for culture purposes, it is more important to include viability as outcome measure. Studies on fecal storage in the context of fecal microbiota transplantations, in which viability is important as well, may therefore provide helpful information[36]. In this clinical context, feces is often stored using cryopreservation or lyophilization. Since freezing might affect the viability of the gut bacteria, the use of cryoprotectants during the freezing of feces might be beneficial for fecal storage[34,36–39]. For example, maltodextrin, trehalose, and skimmed milk maintain bacterial viability (Table 1)[34,37–39]. Bircher *et al.* suggested that the effect of cryoprotectants is species-specific[39]. The currently performed *culturomics* studies notably used either fresh stool samples or cryopreserved feces samples (-80°C) without mentioning the use of cryoprotectants (Supplementary Table 1). Further research is needed to show the effect and importance of different cryoprotectants on cultivation. Besides cryoprotectants, anti-oxidants are also important for the preservation of feces, as will be discussed in the following section.

Table 1*: cryoprotectants and anti-oxidants used for fecal storage in clinical context*

Article	Freezing procedure	Cryoprotectants	Anti-oxidants	Outcome
A new protectant medium preserving bacterial viability after freeze drying (Bellali *et al.*, 2020)[37]	lyophilization	Mixture of sucrose, trehalose and skimmed milk	Mixture of ascorbic acid, uric acid and glutathione	The mixture of cryoprotectants and anti-oxidants is favorable for maintaining bacterial viability.
A Guide for Ex Vivo Handling and Storage of Stool Samples Intended for Fecal Microbiota Transplantation (Burz *et al.*, 2019)[34]	Cryopreservation and lyophilization	Mixture of maltodextrin and trehalose (in the ratios 3:1 and 1:3)	Mixture of ascorbic acid and cysteine	Maltodextrin and trehalose in a ratio of 1:3 is most favorable for maintaining the bacterial composition.
Successful Resolution of Recurrent Clostridium difficile Infection using Freeze-Dried, Encapsulated Fecal Microbiota; Pragmatic Cohort Study (Staley *et al.*, 2017)[38]	lyophilization	Sucrose, skim milk, trehalose, mannitol, or a mixture of these	None	Trehalose is most favorable for maintaining bacterial viability.

Oxygen atmosphere

Fecal exposure to oxygen has an enormous impact on the viability of the gut bacteria[40]. Only two hours of oxygen exposure to the fecal sample results in a 50% reduction of the number of cultured bacteria[40]. The oxygen sensitivity of many gut bacteria are related to the human physiology: the oxygen concentration in the gut varies from 100 mm Hg in the submucosa to a concentration of less than 0.01 mm Hg in the gut lumen[41]. In line with this, a relatively high concentration of aerobe and microaerophilic microbes is found in the mucosa, whilst a high concentration of anaerobes is found in the gut lumen[41]. In total, the number of anaerobic bacteria in the human gut substantially surpasses the number of aerobes[40]. However, a substantial part of the anaerobe bacteria residing in the *feces* are dead or injured, and consequently remain uncultivated[42]. Oxygen exposure is thought to be the cause for this high bacterial death[42]. The genomes of yet uncultivated bacteria have less genes

associated with redox and antioxidation processes in comparison to cultivated bacteria, indicating that the 'unculturability' of some bacterial species might indeed be affected by fecal exposure to oxygen[16,24]. Therefore, it is important to regulate the oxygen exposure during the workflow of culturomics.

Indeed, sample collection and pre-processing in an anaerobic atmosphere increases the culturability of gut bacteria[40]. However, due to practical reasons, it can be difficult to maintain a complete anaerobic environment throughout the first steps of culturomics. One solution for this, is to add antioxidants to the feces before storing the samples (Table 1). This increases the culturability of gut bacteria up to 67%[34,37,40]. Limiting the time between fecal sampling and bacterial culture is another approach to decrease oxygen exposure[32]. Still, there is a special need for oxygen-free storage and culture conditions. For this purpose, GutAlive was developed, which is a stool collection kit that minimizes the oxygen exposure to gut bacteria[32]. GutAlive can store stool samples at room temperature for more than 24 hours with limited reduction of the viability and diversity of anaerobic bacteria[32]. Other efforts for keeping an anaerobic atmosphere focus on culture medium, and will be discussed in chapter 2. A summary of considerations during the sample collection step of culturomics is shown in Figure 2.

sampling	considerations	future research
	• Which sampling method to use • Fecal sampling is simple, but not accurate • Diversity in study participants	• Need for accurate non-invase techniques for microbiota sampling
subsampling		
 vs	• Homogenization versus specific subsampling • Need for more detailed description of subsampling methods	• Examine possible correlations between bacterial composition in fecal regions and original residence in gastrointestinal tract
preservation		
	• Freeze samples as soon as possible • Short term storage at 4°C • Use of cryoprotectants, such as maltrodextin or trehalose • Regulation of oxygen exposure: limit time between sampling and culture, add antioxidants, use anaerobic devices (GutAlive)	• Determine best preservation method for culture purposes • Need for oxygen-free storage and culture conditions

Figure 2: *summary of considerations during the sample collection step of culturomics*

2. Culture: media design

The actual culture step in the culturomics workflow conventionally involves the extraction of microbial communities from feces and the subsequent cultivation on agar plates. However, fast-growing bacteria often occupy the majority of the culture plate, leaving less space for slow-growing species[43]. Indeed, many of the yet uncultivated bacterial species are relatively slow-growing microbes[44]. Moreover, some bacteria have complex growth requirements or nutritional preferences[43]. There are several approaches to overcome these problems, which will be discussed in this and the following chapter. In this chapter, we will discuss the different compounds that can inhibit or promote the growth of bacteria (Figure 3). In chapter 3 we will discuss other factors and techniques that affect the diversity and composition of bacterial culture.

2.1 Inhibition of fast-growing bacteria

Antibiotics

Various antibiotics have been used and tested to suppress the growth of highly abundant bacterial species[5,45,46]. For example, erythromycin, sulfamethoxazole, and ciprofloxacin provided the growth of rare and previously uncultured bacterial species by inhibiting the growth of other bacterial species[45]. Different antibiotics target either Gram-negative or Gram-positive bacteria. For example, the commonly used antibiotics colimycin and kanamycin inhibit the growth of Gram-negative bacteria, whilst vancomycin inhibits the growth of Gram-positive bacteria[5]. Combining multiple antibiotics with different target specificities may contribute to the improvement of selective culture media[47]. Antibiotics that are often used in high-throughput culturomics studies are listed in Supplementary Table 1. The use of antibiotics is notably hampered by the antibiotic resistance of certain gut bacteria. For example, *Bacteroidetes*, which are very abundant in the human gut, are relatively highly tolerant for antibiotics[45]. Thus, novel antibiotics or other medium supplements are needed to selectively inhibit the growth of these bacteria. In contrast, antibiotic resistance can also be utilized for the targeted culture of yet uncultured bacterial species. Using this approach, Versluis *et al.* cultured bacteria in media containing multiple antibiotics to isolate antibiotic resistance species[48]. As a result, the novel strain *Sellimonas intestinalis*, which was resistant to the antibiotics imipenem and metrodinazole, was isolated[48]. Several machine-learning software tools are developed to predict antibiotic resistance based on whole genome sequencing data[49–52]. This information can be used to specifically target antibiotic resistant bacterial species, or to supplement the culture medium with other bactericidal compounds, such as enzyme inhibitors or bacteriophages (described below).

Enzyme inhibitors

CHIR-090 is an inhibitor of the enzyme UDP-3-*O*-(*R*-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase (LpxC), which has a role in the biosynthesis of lipopolysaccharide[53]. Inhibition of LpxC suppresses the growth of numerous Gram-negative bacteria, amongst which the fast-growing *Escherichia coli*[54]. As a result, supplementation of culture medium with CHIR-090 allows the growth of slow growing species, and thus increases the bacterial diversity of microbiota isolates obtained from fecal samples[54]. Although CHIR-090 is the most potent LpxC inhibitor, there are more LpxC inhibitors available, that also have antibacterial properties (Table 2)[53]. The compounds listed in Table 2 are mainly tested on pathogens, so future research on the use of LpxC inhibitors on gut commensals would be of interest. Combining multiple LpxC inhibitors may help to inhibit a great amount of fast-growing Gram-negative bacteria. In contrast, choosing one inhibitor might inhibit a more narrow range of bacteria, so that potential bacteria of interest are not affected by the treatment.

Table 2: *LpxC inhibitors and their targets (based on Kalinin & Holl, 2016)*[53]

LpxC inhibitor	Compounds	Targets
Sulfonamides	BB-78485 BB-78484	Gram-negative bacteria, including *Enterobacteriaceae sp., Serratia marcescens, Morganella morganii, Haemophilus influenzae, Moraxella catarrhalis and Burkholderia cepacia*. Not active against *Pseudomonas aeruginosa*
Aryloxazolines	L-573,655 L-161,240 L-159,692	Gram-negative bacteria, including *E. coli, Enterobacter cloacae and Klebsiella pneumoniae.* Not active against *Pseudomonas aeruginosa and Serratia marcescen*
N-aroyl-L-threonine hydroxamic acids	CHIR-090 LPC-009 ACHN-975	Gram-negative bacteria, including *E. coli, P. aeruginosa, Aquifex aeolicus Neisseria meningitidis, Helicobacter pylori, and Yersinia enterocolitica*
Sulfone-containing compounds	LpxC-2 LpxC-3 LpxC-4	Gram-negative bacteria, including *Klebsiella pneumonia, Escherichia coli, Enterobacter aerogenes and Citrobacter freundii*

Bacteriophages

Bacteriophages are viruses that can infect and kill bacteria. In contrast to most antibiotics, bacteriophages are very specific. This specificity enables the bacteriophages to kill bacterial species with great precision, scarcely affecting species of interest[55]. Although most literature on bacteriophages focusses on therapeutic and clinical applications, bacteriophages can also be used in microbiota culture[5,55]. For example, Sillankorva *et al*. used phages to successfully kill *E. coli* strains obtained from the urothelium. In this study, the three lytic phages T1, T4 and phiX174 were tested, of which T1 was the most effective[56]. Lagier *et al*. used a mixture of T1 and T4 bacteriophages on microbiota samples derived from feces[5]. They successfully eliminated *E. coli* from the culture plate, resulting in the discovery of the novel species *Enterobacter massiliensis*[5]. Thus, bacteriophages are a promising method for the cultivation of yet uncultivated bacterial species. Future experiments are needed to test other phages, for example the ones that are currently described for therapeutical applications, in the context of culturomics. A putative phage would be VA-7, which targets *Bacteroides fragilis,* a major component of the gut microbiota[57,58].

2.2 Promotion of bacterial growth

General culture media

The high-throughput culturomics studies use numerous culture and media conditions for the growth of novel gut bacteria. For example, the group of Raoult (Aix-Marseille Université) tested over 200 culture conditions and successfully cultivated numerous previously uncultured gut bacteria. A major drawback for this approach is that many laboratories lack the capacity and means to generate this amount of culture conditions. Therefore, a list of 16 culture conditions was composed, that captured 98% of the total number of previously isolated gut bacteria[59]. These 16 culture conditions are based on six different media (Table 3), and additionally vary in blood culture preincubation, rumen fluid supplementation and active filtration, amongst other things (see sections below).

Table 3: overview of the growth media used in the top 16 most successful culture conditions (based on Diakite, 2020)[59].

Medium	**Main components**
R-medium	Casein hydrlysate, proteose peptone, ascorbic acid, glutathione, uric acid, haemin, ketoglutarate
CNA agar medium	Agar, colistin, nalidixic acid, sodium chloride, pancreatic digest of casein, peptic digest of animal tissue, yeast extract, beef extract, corn starch
Christensenella broth medium	Meat filtrate, casitone, yeast extract, sodium carbonate, D-glucose, maltose, cellobiose
Marine broth	Sodium chloride, magnesium chloride, calcium chloride, peptone, yeast extract
YCFA broth	Casitone, hemin, vitamin mix, resazurin, L-Cysteine, sodium bicarbonate, Yeast extract, volatile fatty acid solution
Sheep blood broth	Sheep blood

Antioxidants and vitamins

Antioxidants are used in culture medium to neutralize free radicals, so that anaerobic bacterial growth and viability are maintained. Indeed, the antioxidants glutathione, ascorbic acid (vitamin C), and uric acid allow the growth of strictly anaerobic bacteria in an aerobic environment[60,61]. Furthermore, medium supplementation with these antioxidants can be used to increase the viability of bacteria after freeze-drying (Table 1)[37]. Besides the beforementioned antioxidants, cysteine and riboflavin (vitamin B) promote the survival of the beneficial anaerobic bacteria *Faecalibacterium prausnitzii* under aerobic conditions[62]. Other antioxidants that might be beneficial for medium supplementation include folate, anthocyanin, carotenoids, glucosinolate, lipoic acid, ubiquinol and a-tocopherol (vitamin E).

Medium supplementation with vitamins affects the diversity of the bacterial composition[63]. For example, the vitamins B2, E, and B2 in combination with C, increases the number of A*ctinobacteria, firmicutes* and *Verrucomicrobia,* whilst these same vitamins decreases the number of *Bacteroidetes*[63]. When adding vitamin C (abcorbic acid) to the culture medium, it has to be considered that vitamin C

decreases the pH of the culture medium, thereby promoting the growth of acidophiles and inhibiting the growth of alkaliphiles[63]. Vitamin K (menaquinone), a component of the anaerobic respiratory chain, was identified as a universal growth factor for gut bacteria[64]. Therefore, adding menaquinone to culture media is beneficial to promote the growth of bacteria[64]. In various studies, menaquinone is indeed added to the culture medium to optimize culturomics and identify novel species[65,66]. Yet, the exact effect of menaquinone supplementation on gut bacteria cultures (e.g. diversity, composition) remains to be researched.

Specific nutrients

A very recent study of Gu *et al*. showed that different nutrients in different concentrations affect the diversity and composition of a bacterial community *in vitro*[67]. More specifically, beef powder positively affects the presence of *Alistipes*, *Proteus*, *Bacillus*, *Phyllobacterium*, *Bacteroides*, and *Enterococcus*[67]. Yeast extract powder and peptone positively promote the presence of *Clostridium* sensu stricto 7, *Brevundimonas*, *Sporosarcina*, *Parabacteroides* and *Muribaculum*[67].

Another nutrient that might be important for gut microbiota culture is mucin. Mucins are proteins that are produced by human epithelial cells and reside in the outer mucus layer of the large intestine. Mucins have O-glycans attached to their amino acid backbone, which serve as a nutrient source for intestine bacteria. Therefore, supplementation of the culture medium with mucins or O-glycans may enhance the viability and selection of certain bacterial species. Indeed, mucin stimulates the growth of *Akkermansia muciniphila*, a hard-to-culture but clinically relevant bacteria[68]. Furthermore, the growth of previously uncultivated members of the phylum *Synergistetes,* was stimulated by mucin in oral samples[22]. *Synergistetes* are also present in the gut, and some of its genera are on the 'most-wanted taxa' list of yet-to-be-cultured bacteria[69]. Therefore, mucin supplementation might positively affect the growth of uncultered intestinal *Synergistetes* bacteria[70].

An additional component that is able to increase the growth of bacteria is the neurotransmitter γ-aminobutyric acid (GABA)[71]. For example, the growth of the hard-to-culture KLE1738 bacterium is promoted when adding GABA to the culture medium[71]. GABA supplementation and the use of other neurotransmitters on other bacterial species remains to be studied, but might be useful to identify novel low-abundant bacterial species.

It is clear that different nutrients promote the cultivation of different bacteria. One consideration in the supplementation of specific nutrients to the culture medium is that there are some specific nutrients that not only promote the growth of bacterial species, but also inhibit the growth of (other) gut bacteria. For example, some bacterial species require short chain fatty acids, such as butyrate and propionate, and aromatic amino acids, whilst multiple other gut bacteria prefer the absence of short chain fatty acids[72]. The inhibitory effect of short chain fatty acids on some species is probably due to a decrease in pH or the accumulation of toxic intermediates during its metabolism[72,73]. Another example of nutrients with contrasting effects are polyphenols[74]. Many polyphenols promote the growth of *Lactobacillus* and *Bifidobacteria*, whilst inhibiting the growth of *Escherichia coli* and *Enterococcus caccae*, amongst others[74].

Other factors that affect bacterial growth

Blood is a commonly used substant of culture medium[47]. Blood contains many growth stimulating supplements, such as vitamins, lipids and minerals. In agreement with this, Gu *et al.* al showed that defibrinated sheep blood increases the diversity of a the cultured gut microbiota[67]. Pre-incubation of feces in blood culture bottles promotes the enrichment of the fecal samples[5]. In fact, from the previously mentioned list of 16 optimal culture media that was defined by Diakite *et al.*, 8 involve a pre-incubation step with blood[5]. Nevertheless, high concentrations of blood in culture medium might be harmful for bacterial growth. For example, excessive concentrations of heme, which is a component of blood, inhibits the growth of some bacterial species[75]. Besides blood, rumen fluid is also used as culture medium supplement, because it mimics the natural environment of gut bacteria. The addition of rumen fluid allowed the isolation of novel bacterial genera and species[5]. Consequently, blood and

rumen fluid are used in almost all currently performed high-throughput culturomics studies (Supplementary Table 1). Finally, there are some inhibitors of Gram positive and Gram negative bacteria, that indirectly promote the growth of Gram negative and Gram positive bacteria respectively (Table 4).

During culturing, the concentration of nutrients and other elements decreases because the nutrients are consumed by the bacteria. Therefore, regularly supplementing old medium with fresh media yields 22% more bacteria species, compared to cultures that are not supplemented with fresh medium[14]. Despite this, some bacterial species are only isolated from non-supplemented culture, indicating that culturing with and without fresh medium supplementation are complementary[14]. A summary of chapter 2 is depicted in Figure 3.

***Table 4**: compounds added to culture medium to inhibit or promote the growth of bacteria. Based on Chapin & Lauderdale, 2015[76] and Bonnet et al., 2020[47].*

Action	Compound
Inhibition Gram positive bacteria	Deoxycholic acids Bile salts Potassium tellurite Lauryl sulphates Tergitol 7 d-cycloserine Dyes: methylene blue, eosin, crystal violet, ethyl violet
Inhibition Gram negative bacteria	Ox gall Tergitol 7 d-cycloserine Lithium chloride
General growth factor	Purine and pyrimidine bases Amino acids Triglyceride, soy oil

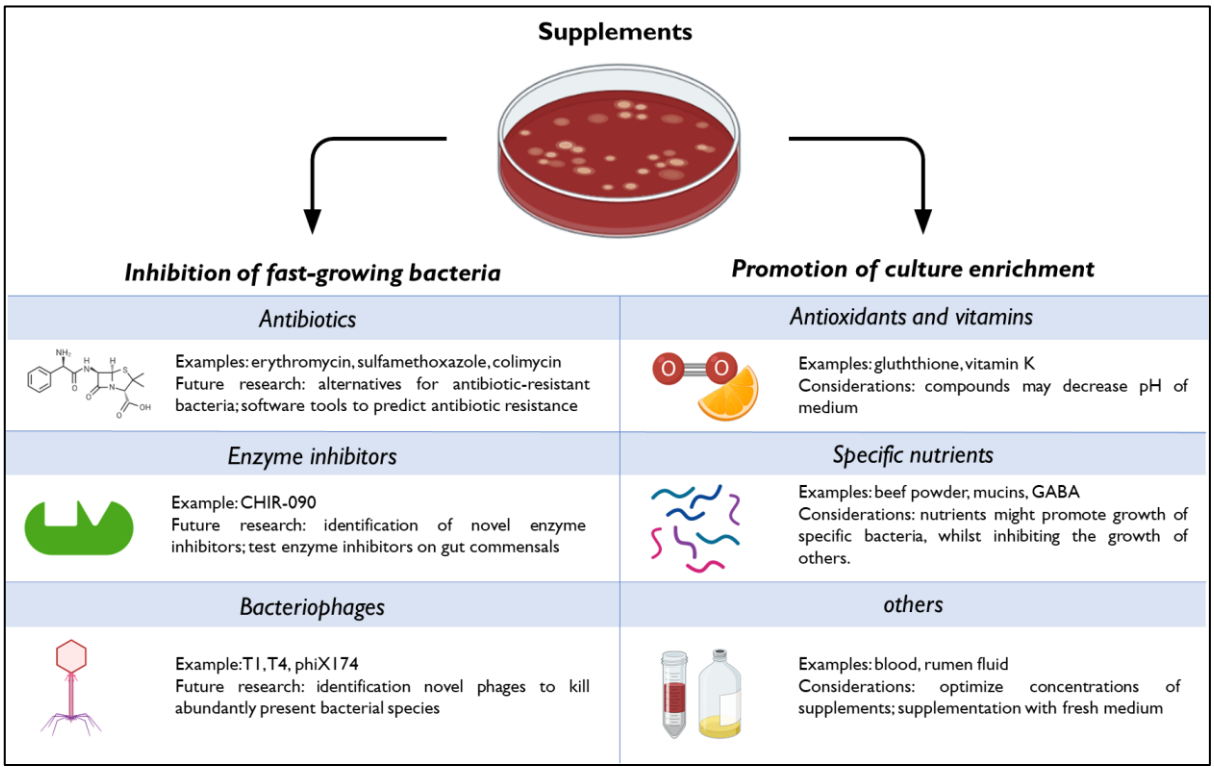

***Figure 3:** summary of medium supplements that can be used to inhibit fast-growing bacteria or to promote culture enrichment. Figure is created in BioRender.*

3. Culture: Tools & Techniques

3.1 Environmental factors

pH

The pH of the atmosphere affects the growth of bacteria, and the pH for optimal growth varies between bacterial species[14]. For example, Gram-positive bacteria often prefer an acidic atmosphere[77,78]. Therefore, the pH of culture medium partially determines which bacteria types are cultivated[14]. Many gut bacteria produce and secrete organic acids and metabolites that may lower the pH of the culture media[79]. On the other hand, bacteria can consume metabolites and organic acids from the culture medium, thereby increasing the pH[80]. Thus, by changing the pH of the culture medium, bacteria themselves can inhibit or promote the growth of other bacteria[79,80]. Therefore, it is important to monitor and/or control the medium pH. Remarkably, in the high-throughput culturomics studies, little attention is paid to the effect of pH on the culturability of gut bacteria (Supplementary Table 1)[5,12,81]. For example, in the study from Lagier *et al*., 2012, pH was kept between 5-7, but nothing is mentioned on which pH values were most optimal for high-throughput use[5]. A study from Diakite *et al*., 2020 did not mention the pH at all, and a study from Ghimeiere *et al*., 2020 only mentioned that the pH was above 5.5[13,82]. Only the study from Lagier *et al*., 2016 used media adjusted to a different pH, including a pH of 4, 4.5, 5, 5.5, 6 and 7.5[12]. The pH of the human gastrointestinal tract gradually increases from the upper to the lower parts of the gastrointestinal tracts, varying from a pH of 3 in the duodenum, to a pH of 6.2-7.5 in the small intestine and a pH of 4.5-7.5 in the colon[83]. Regarding this range in pH, the role of pH in bacterial culture receives insufficient attention in current culturomics studies, and it is therefore important that future studies retain awareness to medium pH, in order to optimize the culturomics workflow.

Temperature

The temperature of the gastrointestinal tract is approximately 36-39°C, and most clinically relevant bacteria grow at temperatures ranging from 25°C to 45°C[9]. Temperature can be a critical factor in the culture of bacteria[9]. Therefore, it has to be taken into consideration that low abundant and/or hard-to-culture bacteria might have an optimum temperature other than 37°C. Indeed, Lagier *et al.* used temperatures ranging from 4°C until 55°C[5,12]. In contrast, most other high throughput culturomics studies keep a temperature of 37°C (Supplementary Table 1).

3.2 Other techniques

Filtration

Filtration is a technique that is used to eliminate the predominant bacterial population by decreasing bacterial load, and to detect low-abundant bacteria[43]. Filtration can be performed using either an active or passive approach[43]. In the active approach, fecal samples are placed on filters with a successive pore size ranging from 5 to 0.2 µm, inoculating the filtrate of every filter step in agar and blood[5,43]. Active filtration is especially convenient for small sized bacteria, such as the hard-to-culture *Spirochaetes*[36,84]. Passive filtration is based on the motility of bacteria. Cell culture insert companion plates with microfilters are used, of which one side of the filter is inoculated with the fecal sample, and the other side with sterile broth[43]. Colonies detected by this method can then be subcultured and identified[43]. In general, filtration is not commonly used in high throughput culturomics studies (Supplementary Table 1). Moreover, studies that use filtration often do not address the impact of filtration on the amount of novel bacterial species that were cultured. Only Lagier *et al*. mentioned the latter: two novel bacterial species were isolated using filtration[5]. Therefore, future research should point out the (cost and labor) effectiveness of filtration.

Time control

Culturomics is a time consuming and labor intensive process. There are multiple ways to reduce the workload of culturomics. Firstly, the time-consuming task of colony picking can be improved. Chang *et al.* compared two techniques for colony picking: the 'experienced picking' and 'picking all' approach[14]. In the 'picking all' approach, all colonies on a plate are picked and subcultured, which is a comprehensive, but very time-consuming process. In the 'experienced picking' approach only a few colonies are picked, based on morphological similarity[14]. The 'experienced picking' method identifies 8.5% less bacterial species than the 'picking all' method, but reduces the work load for 85%[14]. In the future, automatic colony picking that is coupled directly to MALDI-TOF MS, might be even more accurate and low time-consuming[5]. Furthermore, the development of dyes that allow colony differentiation would make 'experienced picking' more accurate and less time consuming. Currently, there are very few dyes that facilitate colony staining[76]. Gram staining is the most well-known bacterial staining. However, these stainings discriminate only at a general level, and there is a need for more specific dyes that stain bacteria based on more distinctive bacterial properties.

Another way to deal with time-consuming tasks is the control of sampling time-points. As mentioned in chapter 2, prolonged pre-incubation is beneficial for yielding more bacterial species. However, picking samples every hour or day to obtain the highest number of bacteria species is extremely time-consuming. Therefore, Chang *et al*. calculated an optimal sampling rate during pre-incubation, and found that during a 30 day pre-incubation, sampling only at day 0, 3, 6, 9, 15, 27 and 30 was enough to isolate 90% of the bacterial species that were found when sampling at day 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30[14].

Colony density

The number of colony forming units (CFU) on one plate significantly affects the diversity and composition of the bacterial community[67]. The highest diversity is obtained at a moderate density. At a moderate density bacteria cooperate with one another, so that growth is promoted[67]. In contrast, the distance between colonies is too large for cooperation at low densities, while at high colony densities the cooperation between bacteria changes into competition[67]. A moderate bacterial density was defined by Gu *et al.* as 51-150 CFU per plate (remarkably, plate size was not mentioned)[67].

Gelling agents

For bacteria isolation, culture media is solidified through the addition of gelling agents[47]. The most conventional gelling agent that is used for this purpose is agar, which is also used in all the high-throughput culturomics studies performed so far (Supplementary Table 1). However, agar has some drawbacks. For example, a high concentration of agar in the medium can reduce the size of bacterial colonies and inhibit the growth of some bacterial species[47]. In addition, chemical components in the culture medium can interfere with the agar. For example, when autoclaving agar with phosphate, reactive oxygen species are formed, which inhibit the growth of bacteria[85]. Thus, autoclaving agar and phosphate separately, is beneficial for the number of colony forming units[85]. Finally, some bacteria secrete agarase, which breaks down the agar[86]. Therefore, there is a need for new gelling agents. Although alternatives for agar are not extensively studied, Bonnet *et al.*, listed examples of novel gelling agents, including carrageenan gums, sodium alginate, methoxyl pectins, and gellan gum[47,86]. The use of these gelling agents in culture medium might allow the growth novel bacterial species, that were previously uncultivated because of their inability to grow on agar[47].

Tools

Several tools are being developed to isolate gut bacteria. First, tools are developed for the high-throughput isolation of a diverse range of bacteria. To this extend, droplet based microfluidics devices were developed[87,88]. In this method, bacteria are encapsuled in droplets, which decreases competition between bacteria and consequently allows the growth of a wide range of (previously uncultivated)

bacterial species[87]. Secondly, there are tools focused on mimicking the *in vivo* environment of bacteria *in vitro*. An important example of such a tool is the isolation chip (Ichip), which was originally developed for the culture of bacteria derived from soil and seawater samples. The Ichip device contains numerous micro diffusion chambers in which single bacteria, that are obtained by diluting the soil or seawater sample, are loaded. Then, the device is placed into soil or seawater, where the diffusion chambers allow the bacteria to have continuously access to growth factors derived from their original environment or from other bacteria present on the chip[89]. Ichip successfully mediated the growth of many previously uncultivated bacterial species[89]. The use of diffusion chambers is now incorporated in the workflow of gut microbial cultivation. By separating the diffusion chambers, an aerobic or anaerobic environment can be maintained in each chamber allowing the growth of host cells and gut bacteria respectively[90]. More advanced tools to mimic the natural environment of gut bacteria include gut microbiota-on-a-chip devices[91–93]. These devices consist of multiple compartments, in which human intestinal epithelial cells and vascular endothelial cells, and gut bacteria are cultured[94]. Often, these compartments are separated by porous membranes that are coated with mucus and extra cellular matrix[94]. Progress is still made to further optimize the gut microbiota-on-a-chip culture systems, by for example establishing oxygen gradients across the chip[93]. The on-chip culture systems are extremely useful for studying the physiological characteristics of bacteria and the interaction between bacteria and host cells. Furthermore, these culture systems can be used for drug testing. A summary of chapter 3.1-3.2 is depicted in Table 5.

***Table 5**: summary of techniques and environmental factors that can be used to promote the diversity of bacterial samples, and suggestions for the optimization of these techniques.*

	Most practiced in high throughput culturomics studies	**Suggestions for optimization**
Environmental factors		
pH	Between 5-7 or not mentioned	• Use pH range from 3-7.5 (based on human physiology)
Temperature	37°	• Use temperature range from 4°C until 55°C
Techniques		
Filtration	Not commonly used	• Examine the effect of filtration on the amount of isolated previously uncultivated species • Use filtration in practice
Time-control	Picking method not mentioned; various incubation time points	• Use 'experienced' picking approach • Development of automatic colony picking • Limit the number of times of colony picking after pre-incubation • Development of differential dyes
Colony density	Not mentioned	• Keep colony forming units per plate at a moderate density (51-150 CFU/plate)
Gelling agents	Agar	• During agar preparation: autoclave agar and phosphate separately • Need for novel gelling agents
Tools	Droplet based microfluidics devices; on-chip culture systems	

3.3 Targeted cultivation

The techniques and media conditions that are described in this report are focused on obtaining a highly diverse sample, so that it can be used to identify and cultivate many (novel) bacterial species via untargeted high-throughput isolation. However, a diverse sample can also be used to specifically target a yet uncultivated bacterium, as will be shortly addressed in this section.

Sporulated bacteria

The majority of gut bacteria are able to form endospores, which are dormant and non-reproductive forms of bacteria that sporulate only under specific conditions[95]. These characteristics make endospores difficult to culture. Ethanol is a disinfectant which predominantly kills vegetative cells, but does not affect endospores[96]. Indeed, the pre-treatment of feces for 4h with ethanol yielded previously uncultivated spore-forming bacterial species[95–97]. Recently, it was discovered that ethanol not only selects for bacterial endospores, but also selects for some minority species that were not yet cultured by conventional culture methods, such as *Ruminococcaceae* and *Lachnospiraceae* species[97]. Another way to select for sporulated bacterial species is by performing a thermal shock. Lagier *et al.* heated stool samples to 65°C or 80°C for 20 or 30 minutes, respectively[5,15]. However, the effect of thermal shocks on the microbial diversity and cultivability was not mentioned. The germination and subsequent vegetative growth of endospores are induced by primary and secondary bile acids, such as taurocholate, glycocholate, deoxycholic acid, and cholate[95,98]. For several bacterial species, bile-acid induced germination is enhanced in by the co-supplementation of amino acids[99].

Media and nutrient prediction

Numerous different media are used to cultivate bacteria. However, the optimal culture conditions between bacteria are very divergent, making it difficult and time-consuming to specify convenient culture media[14]. Oberhardt *et al*. designed a database that links the growth of bacterial species with specific culture media[100]. This database, called KOMODO (Known Media Database), contains the GROWREC (growth medium recommendation) platform, which predicts the growth medium for specific bacteria, based on an 16s rRNA gene sequence input[100]. Using this approach, the growth of *Christensenella minuta,* a clinical relevant bacteria that resides in the gastrointestinal tract, was increased[101]. Although this database is very promising and extensive, it is not specific for the gut microbiota, and further efforts are needed to make this database more accurate. Furthermore, this prediction method has yet to be validated for the cultivation of uncultured species[100]. An attempt to culture uncultivated bacteria via prediction methods was performed by Lugli *et al.*, who used whole metagenome shotgun data of uncultured bacteria to asses their glycobiome and predict their nutritional requirements[102]. This method allowed the researchers to isolate two novel and hard-to-culture *bifidobacterial* strains from animal feces[102]. A similar approach was used by Nayfach *et al.*, who compared metagenome-assembled genomes and their functional annotation between cultivated and uncultivated bacteria[16]. The differences that were found were mainly in genes associated with the regulation of osmotic pressure and oxidative stress, which highlights the importance of oxygen regulation and electrolyte concentrations in the culture medium[16]. Yang *et al*. used genomic data to identify specific glycoside hydrolases in *Bifidobacteria*, and supplemented culture medium with the corresponding ligands of these enzymes, including raffinose and xylan, to successfully select for *Bifidobacteria* in fecal samples[103]. Finally, the software tool *gapseq* predicts metabolic pathways, based on genome and biochemistry data[104]. In addition, *Gapseq* can be used to predict metabolic interactions between bacterial species, such as cross-feeding, which is interesting when designing specific growth-media[104].

Besides the use of genomics data, culture experiments can predict growth factors or nutrient requirements. For example, Fenn *et al.* found that some bacteria, such as *E. coli* promote the growth of other bacterial species[64]. Using this information, a gene knockout library of *E. coli* was developed, and the genes and pathways that were important for the 'growth promoting' function of *E. coli* were determined[64]. Identification of these genes resulted in the discovery of the novel universal growth factor menaquinone. Future experiments with knockout libraries could help identify novel potential growth factors[64].

Cell-sorted targeted cultivation

Genomic data can be used to engineer antibodies, to isolate specific bacterial species for cultivation[105]. This technique was introduced by Cross *et al.* as 'reverse genomics'. During reverse genomics, bacteria

that are bound by the antibody are sorted out using flow cytometry, of which the workflow recently has been optimized for anaerobic cell sorting[106]. The antibody-sorted bacteria were viable enough to propagate in culture, and were used to isolate uncultured bacteria[105]. Reverse genomics would be especially useful for bacterial species that are present in low abundance[105]. However, a downside of reverse genomics is the need and requirement of, sometimes unavailable, genomic data[105].

Another approach for cell-sorted targeted cultivation is live-fluorescence *in situ* hybridization (FISH)[107]. During the standard FISH procedure, cells are fixed and permeabilized. Live-FISH is a variation of this standard protocol, in which centrifugation speeds are decreased and resuspension buffers are optimized amongst other things[107]. This allows the labeling of bacterial 16s rRNA with DNA probes in living cells. Hereafter, cells can be sorted using flow cytometry and cultured[107]. Unfortunately, the survival of cells when using live-FISH is still relatively low[107]. Therefore, further optimizing the live-FISH protocol might permit the culture of uncultivated low-abundant bacterial species[107].

4. Data analysis

Data collection

The identification of bacteria was conventionally performed using phenotypical and biochemical tests, which are time-consuming when processing large numbers of colonies. The emergence of MALDI-TOF mass spectrometry, which is an accurate, inexpensive, and rapid technique to identify bacterial colonies, was therefore a big advance for processing culturomics data[4311]. Moreover, MALDI-TOF MS allows the identification of morphologically indistinguishable bacteria, which are almost impossible to correctly identify with the traditional identification methods[5]. During the MALDI-TOF procedure, a bacterial colony is crystallized and ionized, after which the masses are measured in the mass spectrometer[43]. After obtaining the mass spectrum, the data is compared to a defined database consisting of numerous spectra, so that the bacteria can be identified on genus and species level[43]. The success of bacterial identification by MALDI-TOF is demonstrated by various research groups, as is reviewed in the article of Lagier *et al.*, 2015[43]. Currently, there are two major commercially available platforms for MALDI-TOF MS in which the user can add his own spectra: the Bruker BioTyper (Bruker Daltonics) and the VITEK-MS (bioMérieux). Both systems have a correct identification rate of above 85%, but BioTyper is slightly more accurate in the identification of species that are not yet in the database[108]. Therefore, BioTyper might be a more convenient platform to use when aiming to identify novel bacterial species[108].

One drawback of MALDI-TOF is that a mass spectrum of a particular bacterial strain needs to be present in the MALDI-TOF database, in order to increase correct identification of the micro-organism. If identification using MALDI-TOF MS is unsuccessful, bacteria need to be identified using 16s rRNA sequencing, which is more expensive and time-consuming than MALDI-TOF MS[109]. In practice, rare bacterial species decreases the validity of MALDI-TOF MS identification[43]. Furthermore, there are still some challenges to differentiate certain bacterial genera or species[43]. Expansion of the MALDI-TOF database and the development of algorithms that detect species-specific ion peaks have successfully resulted in the correct distinction between previously indistinguishable *Streptococcus* and *Bacillus* species[110,111]. Therefore, enlargement of the MALDI-TOF MS database is required to optimize the successful identification rate for culturomics. Moreover, it would be of value to create a freely accessible MALDI-TOF MS database in which researchers can add and/or use spectral data to help in the identification of bacterial isolates[112,113].

Data storage

The formal description of novel identified bacterial species is also part of the culturomics workflow. Currently, taxonomy is based on phenotypic and genotypic data, but a clear universal method for classification is still lacking[114]. The most recommended methods are based on 16s rRNA sequencing and DNA-DNA hybridization[114,115]. However, these assays are labor-intensive and require specific tools, that are only available in a few laboratories [114,115]. With the rise of large scale of culturomics and

genome sequencing, and the decline of phenotypic data based on biochemical assays, there is a need for simpler, faster and more comprehensive bacterial classification methods[114,115]. Hence, Fournier *et al*. introduced a polyphasic method for the description of new bacterial taxa, named taxono-genomics[115,116]. Taxono-genomics combines the data from genome sequencing, MALDI-TOF MS, and some universally available phenotypic assays, all cost-effective techniques, to describe novel species[115]. Still, with the faster classification of bacteria using taxono-genomics, it takes two till five years to publish the whole description of a novel strain[117]. Therefore, the platform 'New Microbes and New infections', was launched in 2016. On this platform, raw information, including 16s rRNA sequencing and basic phenotypic data, is made available, so that the time between a scientific discovery and the official description of the new bacteria is shortened[117].

Currently, in the majority of publications about the description of novel bacterial species, much of the information is repeated in the abstract, result, and description sections[118]. Furthermore, many authors only state the information that meet the minimal requirements for bacterial description, but do not elaborate on the biological significance of the novel identified bacteria[118]. Therefore, there are initiatives taken to link phenotypic and molecular data into a more redundant and tabular format, so that the information can directly be shared in publicly available databases[118]. A summary of chapter 4 is depicted in Table 6.

Table 6: *summary of advances and optimization steps in the data analysis step of culturomics.*

Data collection	
Advances	**Suggestions for optimization**
• Emergence of MALDI-TOF MS	• Expansion MALDI-TOF MS databases • Freely accessible MALDI-TOF MS databases • Algorithm development to detect species specific ion peaks
Data storage	
Advances	**Suggestions for optimization**
• Polyphasic description of polyphasic taxa • Shorten time between species discovery and official description (New Microbes and New Infections platform).	• Publicly available database for description bacterial taxa • Redundant and tabular format of data description

Discussion & concluding remarks

The culture of gut bacteria appeared to be exceedingly difficult in the past, leaving many bacteria labeled as 'unculturable'. Due to major advances in cultivation protocols during the past decade, many gut bacteria that were once thought to be unculturable have successfully been isolated and cultured, as we described in this report. Testing a wide range of culture conditions significantly contributed to this success, and by combining different conditions, a list of the 16 most optimal culture conditions was recently established[59]. The use of these culture conditions greatly reduces the cost and workload of culturomics experiments[59].

Despite the great success of culturomics, it was recently estimated that the majority of the bacterial species is still not yet cultivated[16]. Therefore, it is important to test even more culture conditions and complement the list of most optimal culture conditions. Studying the *in vivo* environment of gut bacteria in the host might provide suggestions to optimize the culture conditions. Questions as: 'what are the pH values in different sides of the gastrointestinal tract? What is the effect of immune cell activity on the growth of bacteria? What part plays the presence or absence of mucosal binding sites in a culture situation? Which metabolites and secondary metabolites are present in the different parts of the gut?' will give information on the growth preferences of bacteria, and might therefore be helpful for culture medium design. As an example, the pH range in the gastrointestinal tract is not properly represented in any of the culturomics studies performed so far (Supplementary Table 1). Thus, using culture conditions that vary in pH might yield many not-yet-cultivated bacterial species. In addition, machine learning is a new and promising approach to predict the optimal growth conditions for bacteria. Nevertheless, machine learning techniques remains a challenge, since accurate

predictions for cultivation are often based on whole genome sequencing, whilst whole genome sequencing is, for its part, often dependent on cultivation.

The far majority of culturomics optimization studies that are performed so far, predominantly focus on the design of culture medium, whilst the pre-processing steps receive very little attention. However, optimizing the sampling methods and pre-processing steps are at least as important as expanding the number of culture conditions, since these pre-culture steps hugely affect the diversity and viability of isolated gut bacteria. Thus, more research on (optimizing) pre-processing steps is indispensable for the cultivation of novel bacterial species. Some optimizations will be difficult and time consuming, such as the development of non-invasive and accurate sampling methods. Although these optimizations are very important, there are other optimizations that are relatively easier to study and that can be implemented at a shorter notice. An example of such, is the addition of cryopreservants and anti-oxidants during sample freezing.

When designing new culturomics experiments that aim to culture many yet-uncultured gut bacterial species, it is important to realize that culturomics can be related to other research areas. Research on optimizing the culturomics pre-processing steps share some common goals with the research on fecal microbiota transplantations, in which maintaining the viability and diversity of the microbial community is also crucial. In addition, much can be learned from domains outside clinical microbiology when regarding the cultivation step of culturomics. For example, many machine learning methods that are used in nutrient predictions were originally developed for environmental samples. Now, the field of culturomics can adopt these methods, make them more accurate for gut bacteria, and use them to design novel culture media. Thus, it is important to monitor the optimization steps in these research areas to potentially implement them into the culturomics workflow. Similarly, novel optimizations in the culturomics workflow might be of importance to these other research areas.

It is remarkable that most of the research on culturomics is performed by the group of Raoult (Aix-Marseille Université), as shown in supplementary Table 1. As a consequence, many culturomics experiments are based on the same protocol. On the one hand, this is beneficial to compare the outcomes of different experiments. On the other hand, this makes it harder to draw reliable and correct conclusions, since there is little validation by other laboratories. Thus, it is important to gain culturomics data from other laboratories.

The culture of gut bacteria is extremely important to properly characterize bacterial strains, and decipher physiological features and virulence properties. Furthermore, bacterial cultures are needed to screen antibiotics or other antimicrobial agents and determine antibiotic resistance[45]. Finally, bacterial cultures can be used to study host-bacterial interactions[119,120]. In conclusion, culturomics is an effective technique to capture the great diversity of the human gut microbiota. Despite the success of culturomics, and the recent optimization steps in the culturomics workflow, culturomics still misses many yet uncultured gut bacteria. By further studying some aspects regarding sample collection and culture (Figures 2 and 3 & Table 4 and 5), culturomics has the potential to identify even more novel bacterial species, and study clinical relevant bacteria residing in the gut.

Supplementary information

Supplementary Table 1: *Overview of the methods and techniques that are used in the high-throughput culturomics studies performed in the past decade. This table is based on literature search in Pubmed and Google Scholar. Search terms included cultivation, culturomics, high-throughput, gut, intestine, bacteria, microbiota. Inclusion criteria: publication date maximally ten years ago; article is about gut bacteria. Exclusion criteria: non high-throughput cultivation studies.*

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