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Recent advances in culture-based gut microbiome research

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ABSTRACT

Gut microbes affect the physiology of their hosts. Studying their diversity and functions is thus of utmost importance as it will open new avenues towards the discovery of new biomolecules and the treatment of diseases. Gut microbiome research is currently boosted by the unification of metagenomics, which has dominated the field in the last two decades, and cultivation, which is experiencing a renaissance. Each of these approaches has advantages and drawbacks that can be overcome if used synergistically. In this brief article, we summarize recent literature and own studies on the cultivation of gut microbes, provide a succinct status quo of cultured fractions and collections of isolates, and give short opinions on challenges and next steps to take.

1. All in the same boat: molecular work and cultivation get along well

Ample evidence has shown that the gut microbiota, *i.e.*, the communities of bacteria, archaea, fungi, protozoa, and viruses colonizing the intestine of animals, plays an important role in regulating host health. New molecular approaches, especially sequencing technologies, have been instrumental in generating breakthroughs in microbiome research. One good example is the recent use of shotgun metagenomic data to explore the diversity of microbiomes at the resolution of species and strains at unprecedented scales and depths. Metagenome-assembled genomes (MAGs) can be obtained by reconstructing the genome of single microbes from sequencing reads covering the entire community using new bioinformatic workflows (Nielsen et al., 2014; Almeida et al., 2019; Pasolli et al., 2019; Lesker et al., 2020). These approaches are intrinsically bound to the limitations of sequencing methods (*e.g.*, dominant microbes are primarily captured; experimental protocols may be biased towards certain taxonomic groups, especially during early steps such as DNA extraction) and bioinformatic tools (*e.g.*, processing time; accuracy). Nonetheless, the increasing number of MAG-based studies can give, if unified, the opportunity to establish comprehensive atlases of the microbial diversity existing on earth and in the gut. The Genome Taxonomy Database (GTDB) for instance is a very useful resource (Parks et al., 2020).

Despite such breakthroughs, the limitations of molecular workflows have led the field to turn back towards cultivation during the last five years, as isolates that can be grown in laboratories open several avenues of research: (i) facilitating mechanistic experiments; strains can be used to dissect cellular mechanisms underlying potentially new physiological processes or to mechanistically study interactions with host species; (ii) detailed genome analyses can be performed at a precision that exceeds that of MAGs and new genes can be identified, cloned, and characterized; (iii) novel microbes can be described taxonomically, providing reference points for sequencing datasets. Nevertheless, until innovative cultivation approaches with enhanced throughout are developed and use broadly, cultivation remains laborious.

The latter point mentioned above (taxonomic description), which eventually leads to the proposition and validation of names for new taxa, is also a time-intensive process that follows strict rules governed by the bacterial code (ICSP, 2019). The field of taxonomy is under the fire of an ongoing debate as to whether genomes, and not only cultured strains, can be granted the status of type material, allowing the naming and validation of novel taxa based solely on genome-based analyses. The corresponding pros and cons go well beyond scope of the present paper; readers are re-directed to specific reviews on this topic

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(Overmann et al., 2019; Murray et al., 2020). No matter whether molecular or cultivation work is used as foundation to describe novel microbial diversity, material and data quality is of utmost importance. Contemporary studies based on MAGs or using large-scale cultivation approaches tend to favour individual races towards providing the largest possible resource, at the expense of coordinated efforts. Molecular studies would substantially benefit from harmonized guidelines for processing standards and quality thresholds that help preventing the spread of error-prone diversity (Shaiber and Eren, 2019; Chen et al., 2020; Evans and Denef, 2020). Regarding cultivation, too many isolates, including representatives of novel taxa, are still published without deposition in international collections, partly due to the lack of funding for a task that benefits the entire community but is not recognized as valuable enough in a performance-oriented scientific landscape.

To date, a large number of species still lack a cultured representative, although observed genomically many times. Our inability to isolate these as-yet-uncultured bacteria represents a major hurdle in cultivation-based analysis. The combination of cultivation and sequencing can help here: functional traits of unknown microbes can be inferred from MAGs and thereby help obtaining new taxa in culture (Pope et al., 2011). Targeting specific metabolic functions to allow the sorting and subsequent cultivation of microbes can also be very powerful, although bound to the expert use of specific equipment (Lee et al., 2019). Comparative multi-omics analyses of ever-growing repositories of isolates will soon help resolving the intra-species differentiation of gut microorganisms and thereby enhance our understanding of the link between genetic and functional strain-level diversity, in particular when including isolates of origins other than Westernized countries (Costea et al., 2017; Rabesandratana, 2018; Sorbara et al., 2020). These are nice examples of studies combining cultivation and omics from the last decade, and more will come in the very near future.

2. Cultivation makes important contributions at an accelerating pace

Since the renaissance of cultivation over the last five years, a substantial number of isolates have been collected worldwide. Alone at the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, the total number of bacterial isolates in the publicly available collection has increased at a rate of approx. 629 ± 372 every year between 2014 and 2019 and the total number currently (August 2020) amounts to 21,968 strains. In the same period of time, the number of publicly available bacterial strains from the human, mouse, and pig intestinal microbiome increased from 56 to 439 (Fig. 1).

Large-scale projects of the last years have investigated cultured microbes from the intestine of several host species (Browne et al., 2016; Lagier et al., 2018; Seshadri et al., 2018; Forster et al., 2019; Poyet et al., 2019; Zou et al., 2019). Considering the host-specificity of gut microbiomes (Frese et al., 2011; Seedorf et al., 2014; Gaulke et al., 2018), it is indeed essential to gather and further investigate microbes originating from different animals. Our own contribution to the field has been to study the diversity of bacteria in the intestine of mice (Lagkouvardos et al., 2016a) and, more recently, pigs (Wylensek et al., 2020), which are invaluable domestic and laboratory animals. In both projects, we strove to ensure public, long-term accessibility of all strains to facilitate the work of others and avoid losing valuable bacteria with local storage only. The mouse collection is currently being expanded and will soon deliver additional taxonomic and functional insights into this ecosystem. Liu et al. have followed this path and provided another valuable collection of isolates from ob/ob mice (Liu et al., 2020). Here too, harmonizing efforts are required to avoid creating multiple redundant resources over the years. Redundancy within collections has also been observed due to the use of a limited range of media leading to the repeated isolation of multiple strains of the same species. Whilst this facilitates the analysis of strain-level

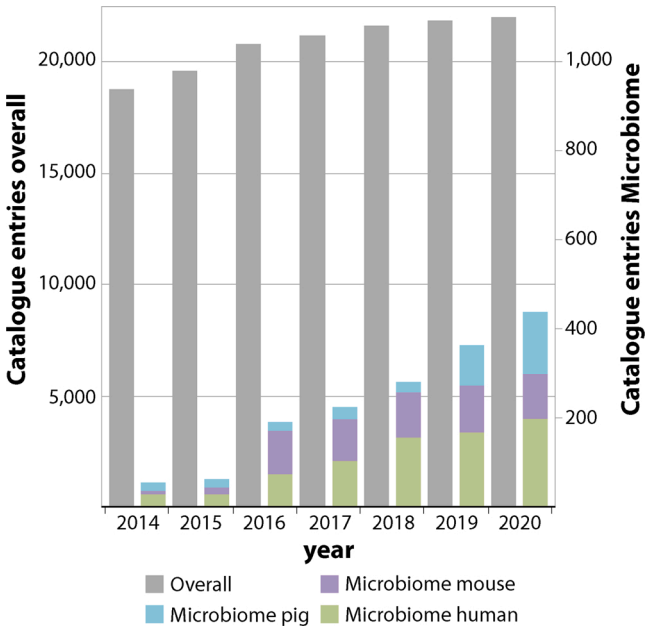


Fig. 1. Numbers of bacterial strains made publicly available by the Leibniz Institute DSMZ. Data are depicted for the time period between January 2014 and August 2020. Grey bars indicate total numbers of available bacterial strains, independent of their ecosystem of origin. Strains isolated from human (green), mouse (purple), and pig (blue) microbiomes are shown with stacked bar plots (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

diversity (Poyet et al., 2019; Sorbara et al., 2020), the use of a wide-range of media and various culture conditions (*e.g.*, atmosphere, co-culture design, *etc.*) helps capturing a greater phylogenetic diversity (Tramontano et al., 2018; Ito et al., 2019).

One example of important bacteria from the mouse gut that we have recently described based on cultured isolates are members of the family *Muribaculaceae* within the phylum Bacteroidetes (Lagkouvardos et al., 2019). First reported in 2002 as MIB (mouse intestinal bacteria) (Salzman et al., 2002) and thereafter referred to as family S24–7 (Ormerod et al., 2016), this family is very prevalent and abundant in but not exclusive to the mouse intestine, where it likely plays an important role in the degradation of complex carbohydrates (Ormerod et al., 2016; Lagkouvardos et al., 2019) and interactions with the immune system (Graham et al., 2018; Kabbert et al., 2020). Out of an estimated number of more than 600 species within this family based on sequence analysis, we have been able to isolate and maintain in culture the three currently validly named species (from three separate genera; https://lpsn.dsmz.de/family/muribaculaceae), several additional species being currently under description. This unbalanced ratio towards yet uncultured *Muribaculaceae* clearly illustrates the need to continue cultivation work to be able to comprehensively archive the existing wealth of gut bacterial diversity. Nonetheless, the currently captured taxa can already help in performing functional studies, as demonstrated by the inclusion of *Muribaculum intestinale*, the first described species within family *Muribaculaceae*, in the synthetic community OligoMM (Brugiroux et al., 2016).

Prior to the release of the mouse intestinal bacterial collection (miBC) (www.dsmz.de/miBC) in 2016, we had estimated the cultured fraction and unknown diversity of the mouse gut microbiota (Clavel et al., 2016; Lagkouvardos et al., 2017). Although cultured fraction estimates vary a lot between ecosystems and depend on the approach used for calculation (Martiny, 2019; Steen et al., 2019), it is fair to say that more than a minority of mouse gut bacteria had already been cultured back then. Here, we aimed at providing an update of this analysis, thereby appreciating the progress made over the last four years.

Therefore, all 16S rRNA gene amplicon datasets from the mouse gut available in the IMNGS platform (n = 16,620) (Lagkouvardos et al., 2016b) were compared against reference sequences belonging to validly named species (from all kind of environments) as of September 2020, using SILVA (Quast et al., 2013) and LPSN (Parte et al., 2020). Note that our previous work (Lagkouvardos et al., 2017) considered sequences from any isolates and not only those with a valid name as done here, which is more restrictive. Those species isolated before 2016 were identified based on the date of their associated publication in the up-to-date list of prokaryotic nomenclature available at the DSMZ. The percentage of amplicon reads matching the corresponding reference sequences at a sequence identity >97 % was then considered as the cultured fraction within each sample. When calculated this way, the current median cultured fraction within the mouse gut is 31.0 % *vs.* 27.3 % before 2016 (Fig. 2). The distribution of cultured fractions around these median values shows a decreased prevalence of samples with very low coverage and an increased sample prevalence with high coverage: the proportion of samples with >50 % sequencing reads corresponding to cultured bacteria is 35.7 % in 2020 *vs.* 31.3 % before 2016.

We then complemented this gene-based sequence analysis by looking at the diversity of MAGs generated within the latest survey of the murine microbiome (Lesker et al., 2020). After filtering for high quality (>90 % completeness, <5 % contamination) and selection of a single representative per species (95 % ANI), the initial dataset of 20,937 MAGs was reduced to 830. Out of these 830, only 67 could be assigned to validly named and cultured species while the remaining 763 belonged to differing levels of unknown taxa. The taxonomic diversity of these, as yet, uncultured and named bacteria is shown in Fig. 3. Representatives of 9 phyla were identified to contain uncultured MAGs, stressing the need to continue efforts in cultivating bacteria. The majority (n = 309) belonged to the family *Lachnospiraceae* (phylum Firmicutes). Interestingly, the second highest number of unknown taxa were assigned to the family *Muribaculaceae* (n = 89), supporting the need for further cultivation of this family.

As already mentioned above, gut microbiomes are host-specific and the need to continue cultivation efforts is of course relevant also for host species other than mice. Multiple large collections of isolates from the human gut have been published recently (Forster et al., 2019; Poyet et al., 2019; Zou et al., 2019), each of which identified a wealth of previously uncultured taxa, including many previously identified as being of high interest to the community (Fodor et al., 2012). The use of ethanol treatment, as selection for spore-producing bacteria, has been applied multiple times, suggesting that ~50–60 % of bacteria within the human gut produce ethanol-resistant spores (Browne et al., 2016; Poyet

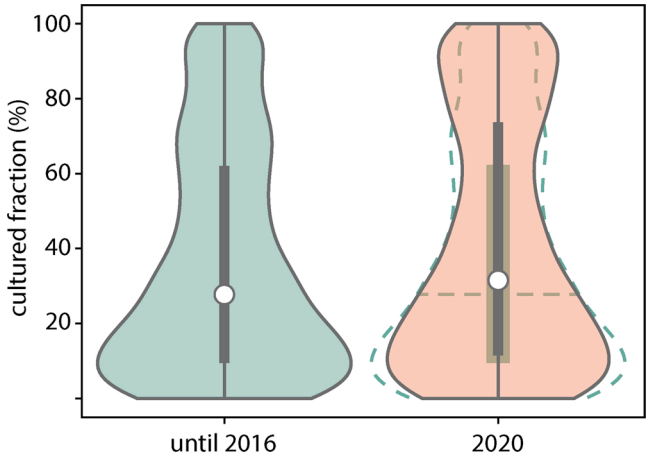
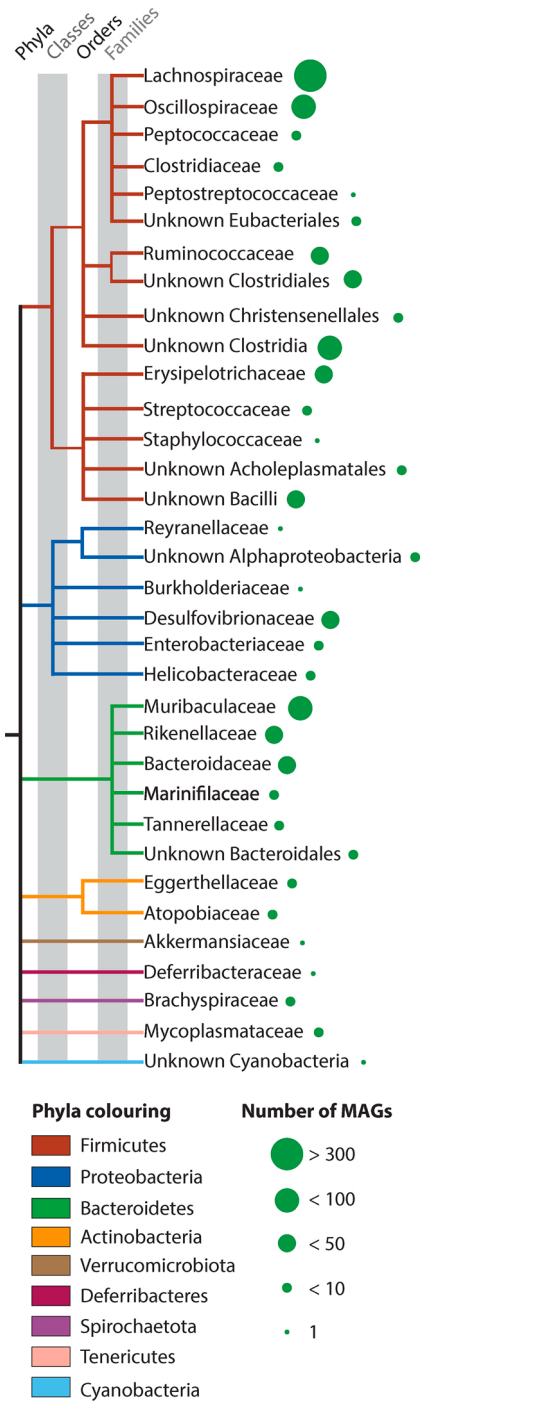


Fig. 2. Overtime improvement of prokaryotic cultured fractions within the mouse gut. Data were obtained as described in the text. For estimates in 2020, the violin plot for 2016 was shadowed below the actual data for rapid visual comparison.



`**Fig. 3.** Unknown, dominant bacterial taxa within the murine intestine. Dendrogram showing the taxonomic distribution (down to family level) of unclassified MAGs reconstructed from mouse gut metagenomes. Branches are coloured by phyla. Circles next to names are proportional to the number of MAGs assigned to that lineage.

et al., 2019). Larger and larger MAG collections have also been generated for the human gut (Pasolli et al., 2019; Almeida et al., 2021), each highlighting the lack of cultured representatives for the novel taxa they observe.

3. Challenges and outlook

Some ongoing global initiatives aim at preserving gut microbial diversity (Bello et al., 2018; Rabesandratana, 2018), highlighting the major interest in biodiversity protection by archiving microbes either in the form of complex communities, simplified consortia, or single strains. However, the dimension of such projects is colossal and stakeholder numerous, which translates into logistical, legal, and ethical obstacles (Chuong et al., 2017).

For obtaining, describing, and archiving single strains, it is obvious that novel cultivation workflows with increased throughput and accuracy are needed. Robotized anaerobic pipelines integrating the purification, identification, and storage of cultures would help reaching an experimental speed appropriate to capture microbial diversity at the scale of additional tens of thousands. Some of the still undescribed taxa in the gut represent major phylogenetic holes in the current cultured landscape, as exemplified *via* the above MAG-based analysis.

Although culture-based analysis of gut microbiomes at the species level requires already considerable efforts, strain-level diversity has been shown to play an important role within gut microbial ecosystems. This includes the ability to interact with the immune system (Yang et al., 2020) and to modify the activity of pharmacological agents, as some strains of *Eggerthella lenta* contain the cardiac glycoside reductase (*cgr*) operon which facilitates the inactivation of the drug digoxin (Haiser et al., 2014). Thus, cultivation also needs to explore strain diversity, especially to help estimate the extent to which diversity, as detected by sequencing, translates into experimental functional differences. For example, genomic analysis of the prevalent human gut bacterium *Prevotella copri* identified that this species consists of four distinct clades (Tett et al., 2019), with strains differentially occurring based on their hosts eating habits (omnivore, vegetarian, vegan) (De Filippis et al., 2019). While specific polysaccharide utilisation genes were predicted to be key in the functional variation of these clades, it was only with the isolation of strains that *in vitro* testing confirmed the differential ability of members from each clade to grow on various glycans (Fehlner-Peach et al., 2019). The combination of both cultivation and metagenomic analysis was key in studying this species ecological niche and the drivers forcing its evolution. As well as bacteria, other microbes, especially fungi, and viruses, including bacteriophages, will need to be included in future collections (Richard and Sokol, 2019; Lourenco et al., 2020; van Tilburg Bernardes et al., 2020).

Studying mechanisms underlying the host-specificity of microbiomes is also fascinating. The establishment of host-specific collections and corresponding genome databases serves this purpose well. Gene catalogues that include the genetic information from uncultured taxa as obtained by shotgun sequencing are valuable resources (Qin et al., 2010; Xiao et al., 2016; Lesker et al., 2020). Strikingly though, our current ability to provide meaningful annotations to (meta)genomes is still low (Fig. 4) (Thomas and Segata, 2019). In other words, a lot of money is still spent on generating tremendous amounts of sequencing data that cannot be interpreted. This is partly due to the fact that identifying and describing novel genes is a tedious task. Hence, the field direly needs further mining of genomes for yet undescribed functionality and the development of new tools and approaches that allow genetic engineering of commensals (Neuhaus et al., 2016; Inda et al., 2019; Hitch et al., 2021).

Until further substantial progress has been made in describing new species and genes, and we are thereby able to setup a comprehensive map of structural and functional diversity within the gut microbiota, projects that develop simplified models mimicking complex ecosystems, without the drawback of containing unknown members, are very valuable. With the exception of strict symbiosis, microbes do not come alone in their native environment and understanding trophic chains and other types of interaction among them is very important. The design and development of minimal microbial consortia (also referred to as synthetic communities) is thus very important but a moving target: based on some of the models already available (Schaedler et al., 1965; Becker et al., 2011; Brugiroux et al., 2016), systems of increased complexity that better resemble their ecosystems of origin will soon be developed.

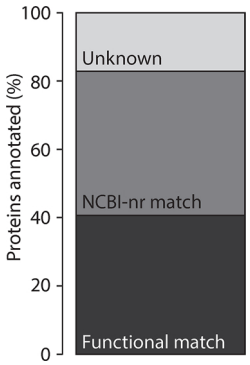


Fig. 4. Functional unknowns. Annotated fraction of proteins within the integrated mouse gene catalogue (Lesker et al., 2020). Those annotated and assigned to either a KEGG orthologue or CAZyme family are termed 'Functional match'. Proteins matching those in the NCBI-nr database, referred to as 'NCBI-nr match', have varying levels of functional annotation, as these include proteins seen in sequencing datasets but not studied and thus without a confirmed annotation. The remaining proteins (unknown) lack sequence similarity with any previously studied proteins.

In conclusion, after 15 years of intensive metagenomic work, microbiome research is further boosted by the renewed interest in obtaining and studying isolates.

Authors contributions

TCAH and AK performed bioinformatic analyses. TCAH, TR, JO, and TC analysed data. AA performed experimental work. TR and IL curated data. DH, IL, and JO provided access to essential materials. DH, JO, and TC secured funding. TC supervised the work. TCAH and TC wrote the manuscript. All authors reviewed the manuscript and agreed with its final content.

Declaration of Competing Interest

None.

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