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The pig gut microbiota analysis techniques, a comparison

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ABSTRACT

The gastrointestinal tract microbiota composition can be radically different among pigs with regards to their health, age, feed intake, breed, and local conditions making its investigation into an important and multifaceted tool for evaluating the effects of new breeding and feeding schemes and animal welfare. For a long time, scientists have relied upon classical microbiology techniques to identify the makeup of bacterial populations from the gastrointestinal tract. Since their conceptions, high throughput sequencing and other molecular techniques contributed immensely in better understanding the pig gut microbiota and its makeup. Since many techniques to identify and quantify the microbiota are based on bacterial gene targets, they can, be used to determine the relation between different bacterial taxons and developmental processes, how it influences the host metabolism or diseases impact. In our review, we offer an overview of the different methods employed in the assay of the gut microbiota of pigs along with differences and pitfalls.

Keywords: next-generation sequencing, culturomics, pig microbiome, molecular techniques, 16S sequencing, pig microbiota review

INTRODUCTION

The gastrointestinal tract of the swine has been evolving in time along with the symbiotic microorganisms that colonise it (Oh et al., 2010) "Although these communities are often postulated to have coevolved with their hosts, evidence is lacking, yet critical for our understanding of microbial symbiosis in vertebrates". These microorganisms, bacteria, viral particles, protozoans and fungi have adapted to prosper in the digestive tract conditions generating essential interactions among them and their host (Hillman et al., 2017). The community made up by these microorganisms is commonly known as microbiota, and the

relationship with the host can be commensal, mutualistic, or pathogenic of nature. Mutualistic microorganisms extend beneficial roles that are essential to the host wellbeing, such as helping in the fibre digestive process, preventing pathogen colonisation of the gastrointestinal tract and essential vitamin production (Nigam, 2015; Guevarra et al., 2019). Likewise, commensal bacteria modulate host immune processes that alter the composition of the gastrointestinal microbiota resulting in homeostasis of bacterial communities (Schokker et al., 2015). Thus, apart from the host inheritance, the complex relationships increase, with maternal microbiota, disease, age, feed and local environment being influencing factors towards the gastrointestinal microbiota.

The research methods for identifying and quantifying the microbiota colonising the gut of the swine evolved beginning with the classical microbiology techniques of bacterial cultivation and characterization and reaching the Microbiomics/Bioinformatics era (Allali et al., 2017; Gomez, 2019; Wang et al., 2020). Classical microbiology studies contributed greatly towards the progress done on the subject of the microbiota, however, the limitations inherent in the techniques employed offered an overall fragmentary image made up by frequent unknown taxonomic information and interspecies complex relations. Due to the large differences found between the species colonising the gastrointestinal tract and those that can be cultivated, classical methods were largely replaced by culture-independent techniques (Leser et al., 2002). Thus, microbiota fingerprinting and sequencing methods (like the 16S rRNA new generation sequencing technique) became essential tools for the identification of microorganism populations as well as for determining the relationships between communities present in the gastrointestinal tract (Crespo-Piazuelo et al., 2018; Gao et al., 2018; Zeineldin et al., 2018).

The current review intends to compare the different techniques of microbiota analysis that evolved in time, from the classical, microbiological phenotypical studies to present-day Microbiomics/Next Generation Sequencing/Bioinformatics techniques, with their appropriate advantages and disadvantages.

Overall, the analysis of the microbiota is essential in the improvement of animal nutrition, health-related issues regarding the modulation of microbiota associated with gut pathology (Ramayo-Caldas et al., 2016; Gresse et al., 2017).

Particulars of Sus scrofa domesticus

Due to the ever-increasing global demand for meat, species with high-efficiency feed conversion rates such as *Sus scrofa* became one of the most important species used as livestock, having a fundamental role in numerous economies (Secco et al., 2020). Pork represents one of the most consumed meats in Europe, with an annual consumption of nearly 40kg/capita, which is three times higher than the global average which accounts for about 12kg/capita thus

making the EU the second-largest producer and leading exporter globally (Bellini et al., 2016; Delsart et al., 2020).

Because of their similarities to humans in terms of physiology, anatomy and genetics pigs are frequently used as an animal model ranging from drug testing to physiological and other medical studies (Eeckhaut et al., 2013), of various microbial infectious diseases and even in the analysis of the human microbiota (Moon et al., 2016; Maradiaga et al., 2018). As such, experiments with pigs can reach a high level of predictive power compared to other animal models.

The gastrointestinal microbiota of a swine consists predominantly of bacteria with a large part of those being anaerobic *Gram-positive* species. The number of bacterial species that are estimated to be present ranges from 450 to 600, with population densities growing overall from the upper to lower gastrointestinal tract (Hui Yang et al., 2016). Colon contents and faeces can contain up to 10^{10}–10^{12} microorganism cells x g^{-1}(Moon et al., 2016). The microbiota is essential for its role in a series of nutritional, physiological, developmental, and immune processes of the swine influencing overall health and growth performance.

Studies concerning the comparison between conventionally reared versus bacteria-free pigs (Konstantinov et al., 2006; Fernando, 2012) shown that commensal bacteria play essential roles in organ, tissue, and immune system development, contributing digestive processes. Moreover, a healthy microbiome protects the pig from harmful bacteria colonisation, and overgrowth of non-pathogenic species (Konstantinov et al., 2006; Collado et al., 2007; Fouhse et al., 2016). The piglets' digestive tract is thought to be free of bacterial colonisation before birth, and constant exposure to various bacteria and other microorganisms after birth.

Constant contact and succession of microbial communities gave rise to evolutionary adaptations resulting into distinct physiological structures that cover special needs of different stages of development (Saraf et al., 2017; Maradiaga et al., 2018; Guevarra et al., 2019). The gut microbiota can be shaped by a multitude of internal and external factors (Korpela et al., 2018) such as dietary changes, the addition of antibiotics, probiotics or prebiotics (Kraler et al., 2016). These factors can lead to essential changes in the structure of microbial communities. Weaning can be another disruptive event toward the gut microbiota and also a great source of stress in the life of animals (Frese et al., 2015; Gresse et al., 2017).

The gastrointestinal tract microbiota composition is significantly different between pigs conditioned by the breed, health, age, feed, and local conditions (Xiao et al., 2016) making the investigation of the digestive tract microbiota a multifaceted technique for predicting the effects of breeding programs, feeding schemes with regards to animal welfare (Camarinha-Silva et al., 2017). Even so, several meta-analysis studies point out to the existence of a common core of

bacterial families and species shared among pigs of all breeds and locality (Slifierz et al., 2015; Hua Yang et al., 2018). This core of microorganisms is comprised by a majority of *Firmicutes* and *Proteobacteria* phyla, differing in comparison from those found at the caecum and mid-colon segments, where *Bacteroidetes* and *Spirochetes* have sizable communities (Niu et al., 2015). Other encountered phyla such as *Tenericutes, Fibrobacteres, Actinobacteria*, and *Synergistetes* have a lower presence making up less than 10% of the total microbiome (Niu et al., 2015). Bacterial orders such as *Lactobacillales, Spirochaetales, Clostridiales,* unclassified *Firmicutes, Bacteroidales*, and unclassified *Gammaproteobacteria* were found to be the most abundant (Crespo-Piazuelo et al., 2018).

Less than 1% of total 16S ribosomal RNA sequences were of *Archaea* origins and were dominated by the *Methanomicrobia* and *Thermococci* (Niu et al., 2015; Chen et al., 2017; Crespo-Piazuelo et al., 2018). At the genus level, this includes *Subdoligranulum, Prevotella, Clostridium, Blautia, Lactobacillus, Roseburia,* and *Ruminococcus* (Frese et al., 2015; Niu et al., 2015; Zhao et al., 2015; Chen et al., 2017).

Traditional Techniques

For a long time, scientists have relied upon classical microbiology techniques (selective media plating coupled with phenotypic-biochemical tests) to identify the makeup of bacterial populations from the gastrointestinal tract (Richards et al., 2005). Phenotypic-biochemical tests are usually classified into three main categories, universal, differential and specific (Ferrario et al., 2017; Fenske et al., 2019) . Universal tests usually guide the microbiologist to further sets of biochemical tests to ascertain a better identification. Examples range from hemolysis pattern to motility and enzymatic tests (Mi et al., 2019). Differential tests can further identify an isolate up to a species level with examples as triple sugar iron test (Casanova-Higes et al., 2019). Specific tests are performed to confirm an isolate at the subspecies level (Hiergeist et al., 2015). Tests included in this category are γ-Glutamyl aminopeptidase test and propyl aminopeptidase however neither of these tests alone are sensitive enough (Wang et al., 2020).

A large assortment of automated biochemical testing equipment are presently available which conveniently incorporate most of these tests providing necessary information regarding the composition of fastidious gut microorganisms to a large community of laboratories which don't have access to molecular methods.

Another technique used in the identification of bacterial species is the Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer. It has a widespread usage because of the rapid and precise identification on an extended range of gram-positive and gram-negative bacteria

(Nero et al., 2006) relying on recognizing a specific spectrum for the microorganism that is being worked on which is then compared with an extensive database within the instrument. The MALDI-TOF is not suitable for quantification work and can provide erroneous data when the species are closely related (Chaplin et al., 2015; Dobranić et al., 2016). A high volume of valuable data was generated using plating techniques (Guo et al., 2008). Several studies reported that the dominant microorganisms colonizing the pig gastrointestinal tract are made up by the following genera: *Bacteroides, Eubacterium, Lactobacillus, Fusobacterium, Streptococcus, Prevotella, Peptostreptococcus, Selenomonas, Clostridium, Ruminococcus, Bifidobacterium, Butyrivibrio*, and *Escherichia* (Leser et al., 2002; Guo et al., 2008; Fricker et al., 2019).

The selectivity of the media used also play an important factor as well as the gastrointestinal site targeted. A majority of the bacteria identified in colon belong to the Gram-negative group such as *Butyrivibrio, Selenomonas,* and most importantly *Bacteroides* (Isaacson & Kim, 2012). The remaining group identified were the Gram-positive bacteria with *Eubacterium, Peptostreptococcus*, and *Lactobacillus* being the most numerous (Lin et al., 2011; Isaacson & Kim, 2012).

Culturing techniques also evolved with the development of the culture-independent techniques with the employment of bacterial culture chips or microPetri dishes and gel microdroplets (Leicheng Zhang et al., 2019; Tovar et al., 2019) offering the culture of microorganisms that were previously uncultivable along with a high throughput performance.

Disadvantages

Despite plating and phenotypic-biochemical techniques being effective methods for the characterization of a microorganism phenotype, they are still being affected by drawbacks pertaining to the poor quantification of the microbiota populations (Zoetendal et al., 2004; Isaacson & Kim, 2012; Fricker et al., 2019). Several attempts were done in order to estimate the population proportions of various microorganism species colonizing the swine gastrointestinal tract. Such attempts are often deemed controversial, with population numbers heavily depending on the specific gastrointestinal sites from which the microbial probes are sampled, geographic location, the microbiological tests used, feed, and other factors (Zoetendal et al., 2004; Jian et al., 2020).

To assess the cultivability of a specific strain, microbiologists generally compare microscopic counts with total viable counts (Leser et al., 2002; Richards et al., 2005).

The number of viable colony-forming units (CFU) found in a gram of sample can be obtained through culturing on a nonselective agar (Namsolleck et al., 2004; Fricker et al., 2019). However the issue arising is that the microscopic counts are usually higher than the viable counts, because of the number of dead

microbial cells. It is suspected that a number as high as a third of the total bacterial counts are made up by dead cells in the gastrointestinal tract.

Recent molecular studies have shown that a high number of bacterial species counts that are specific to pig microbiome are different from the results obtained by plating (Namsolleck et al., 2004; Sieuwerts et al., 2008; Cassoli et al., 2016; Camarinha-Silva et al., 2017; Fricker et al., 2019).

Such distinctions between molecular and microscopic observations are on account of the impossibility of plating for a large part of the microbiota bacteria. This fact contributed to the discovery and evolution of culture-independent molecular methods in order to study the microbiome(Hiergeist et al., 2015). A growth selection is placed on a species when it is cultivated via plating techniques. More so, a large proportion of bacterial species (estimated between 40 and 75%) from the gastrointestinal tract raise problems due to unknown culture conditions, lack of media selectivity or anaerobic conditions (Zoetendal et al., 2004; Konstantinov et al., 2006). Of interest, microorganisms colonizing the intestinal tract often form symbiotic relationships between them as they rely on the metabolism of each other to flourish; this is also why pure-culture techniques can bring severe limitations to their approach (Cheng et al., 2019).

Due to the difficulties posed by achieving optimal growth conditions *in vitro* and because of the complexity and training required to reach a certain level of competence in the field of microbiology (McCaskey & LaRocco, 1995; Richards et al., 2005) the classical methods slowly gave way to molecular techniques in the microbial ecology field but even with these partial results, the impact of classical microbiology culturing techniques should not be understated.

Molecular Techniques

For more than 30 years, molecular techniques have evolved to bring a more exhaustive evaluation of the microbiota regarding the quantitative element of populations as well as qualitative one (De Gregoris et al., 2011). Isolation and analyzing the genetic material are the main aspects of these techniques with a few more modern ones also involving the 16S rRNA genes that offer several important advantages as well (Ott et al., 2004; Isaacson & Kim, 2012; Kraler et al., 2016) . The 16S rRNA genes are small ribosomal subunits and represent an ideal site for a molecular marker because they have kept their structure intact through the evolution of eubacteria species. It is no surprise that this method became to be regarded as the standard for taxonomic identification and classification (Hatt & Löffler, 2012).

Oligonucleotide probes serve as universal primers that recognize the highly conserved regions from any bacterial source, by amplifying and quantifying the 16S rRNA genes. In contrast, variable regions can serve as a base for oligonucleotide probes which can either be order, class, genus or even species-specific (Peng et al., 2003; Collado et al., 2007; Wang et al., 2012; Pedersen et

al., 2013). Identification at the level of species is sometimes problematic due to it relying on databases with sequences for strains and thus are limited to microorganisms that have already been classified by other microbiological methods (Hiergeist et al., 2015).

Fluorescent In-Situ Hybridization

Among the first molecular methods used for identification and quantification purposes was the Fluorescent In-Situ Hybridization (FISH). Fluorescent marked oligonucleotide probes such as 16S rRNA (or of other regions) were created to bind to the individual bacteria DNA of target species. In this manner, fluorescent bacteria can be visualized and microscopically counted or with the help of cytometry flow. A large assortment of probes are available for different species. The method can be automated and quantitative, and as an additional advantage, the target bacteria spatial distribution within the colonized medium studied can also be provided (Cui et al., 2016). As a disadvantage FISH represents a low-throughput method which delivers insufficient insight into the microbial makeup of a microbiota (Huber et al., 2018).

Terminal Restriction Fragment Length Polymorphism

Terminal Restriction Fragment Length Polymorphism (T-RFLP) relies on the electropherogram, the visualization of resulting bands from the amplicons fragmentation of the 16S rRNA gene with restriction endonucleases. T-RFLP is mainly used to compare between different microbial community components, or to assess microorganism variability in the gastrointestinal tract. As advantages this technique offer cheap, rapid and semi-quantitative results. It does not allow however for phylogenetic identification this being the main disadvantage of T-RFLP but it can be remediated by pairing 16S rRNA clone library analysis with T-RFLP (Samanta et al., 2019).

Quantitative Polymerase Chain Reaction

Another frequently employed method for microbiota evaluation is the quantitative PCR method (qPCR) (Pang et al., 2007; De Gregoris et al., 2011; Lin et al., 2011; Kraler et al., 2016). In this method, the molecular target (such as a gene encoding a toxin or 16S rRNA) is multiplied by copying and quantified with the use of fluorescence-labelled taxon-specific molecular probes or with a non-taxon-specific nucleic acid stain (SYBR® green) (Ott et al., 2004; Wang et al., 2012; Kraler et al., 2016). The hardware and software used in the qPCR systems function under the principle of measuring the fluorescence intensity from the samples. The chain reaction byproduct is monitored in real-time for each step of amplification process, as opposed to just at the endpoint, as occurs in traditional PCR. The capability of the PCR machine to measure the fluorescence and

at which cycle it increases over background allows the method to be quantitative (Hermann-Bank et al., 2013; Pedersen et al., 2013). This is due to the fact that fluorescent response is sizeable to the quantity of amplified target DNA, which is proportional to the bacteria target count.

In recent times qPCR has been successfully used alongside NGS (new generation sequencing) library preparation to provide an accurate quantification of absolute taxon abundances with the use of the 16S universal primers and species specific primers (Jimeno et al., 2018; Jian et al., 2020). Cycle threshold values were used to obtain standards of different 16S copy numbers (ranging from 10^4 to 10^8 number of copies) which were then used to identify the 16S copy numbers for a targeted species within a probe(Metzler-Zebeli et al., 2013; Jimeno et al., 2018; Jian et al., 2020). The results were similar to those obtained from NGS data that could also be used alongside high-throughput microbiome analysis and to verify each other. There were some limitations to this technique including the need for obtaining a high quality standard, the design and specificity of primers, and taking count of the fact that bacteria can have more than one 16S copy meaning that the gene copies is not equal to the cell count (Gratz et al., 2018). This can provide both a quantitative and qualitative info regarding the microbiome albeit an imperfect one, which can further be coupled with the NGS technology for further accuracy and clarity (Gratz et al., 2018; Tkacz et al., 2018; Wang et al., 2020).

Advantages/Disadvantages

The main advantages of the qPCR techniques are that they are cheap, fast and reliable, can be automated and due to these aspects they became the standard to which other molecular techniques get compared (Peng et al., 2003; Pedersen et al., 2013). The main disadvantages are that it can analyze only a few batches of species since it is difficult for different primers to be used in the same time due to the apparition of hybrids or cross amplification, the DNA target sequence must also be known ahead of time and the quantities obtained can be small enough to provide ambiguous results (Cao et al., 2017). Although the qPCR technique has been a quantum leap forward in the molecular biology field, it also has limitations.

Each step from the analysis chain be it physical, chemical, or biological, from sample retrieval to the resulting 16S rRNA amplicons or chaperones (Chaban & Hill, 2012) represents a potential source of errors. For example, different nucleic acid isolation methods can indirectly influence the result of microbiota makeup; Gram-positive bacteria having thicker cell walls require more disruptive isolation conditions, conditions which may result in excessive fragmentation of Gram-negative DNA (Klaschik et al., 2002). Another major limitation is that qPCR alone is usually unreliable for quantification purposes since the number targeted genes are not always equivalent to the number of bacterial

species within a sample (Ott et al., 2004; Chaban & Hill, 2012). Nevertheless this technology can be a sensitive method for rapid quantification of bacteria from fecal samples, to validate different treatments or detect variations within a taxon population (Ramirez-Farias et al., 2008; Metzler-Zebeli et al., 2013).

Denaturing Gradient Gel Electrophoresis/ Temperature Gradient Gel Electrophoresis (fingerprinting)

Beside PCR amplification, DNA fingerprinting is another molecular technique that is used often.

The technique has been successfully used to characterize the gastrointestinal microbiota (Richards et al., 2005; Guo et al., 2008). With the fingerprinting technique, 16S rRNA genes molecular probes are used to amplify 16S bacteria sequences (Ott et al., 2004; Wang et al., 2012; Kraler et al., 2016). Using a polyacrylamide gel containing a DNA denaturing compounds (usually formamide or urea) gradient (DGGE) or a gradient of temperature (TGGE), realizes a separation between amplicons.

When migrating in the gradient gel, the amplicons with higher guanine and cytosine count are more resistant to denaturation and as such, a separation is done between species on the count of their guanine and cytosine ratio found in their genome rather than by the size of their products. Visualization is done by staining after electrophoresis, which provides a "fingerprint" for each microbial population contained in the sample target. The amplicons separated can also be recovered and then subjected to sequencing for species identification purposes (Niu et al., 2015; Hui Yang et al., 2016; Cao et al., 2017; Crespo-Piazuelo et al., 2018) .

DGGE/TGGE can also allow for the analysis of probes isolated using different techniques (Richards et al., 2005; Guo et al., 2008). The difference in DNA band intensity between different treatments can be used as a semi-quantitative discriminant to determine the treatment efficacy.

Advantages/disadvantages

The main advantage is represented by the semi-quantitative analysis of whole microbiota communities. The method is also highly sensible towards mutations and it can virtually detect most mutations from a sample rendering it ideal for fast genetic screening. Having an optimal fragment size of 500bp puts a limit on the genetic information found and on identifying PCR primers (Namkung et al., 2004). This also renders the method inadvisable in using it for quantitative comparisons. Other disadvantages include the lack of phylogenetic identification unless sequencing or probe hybridization is done a priori and also PCR associated bias (Richards et al., 2005; Petersson et al., 2009).

DNA microarrays

Also known as the DNA chip, phylogenetic microarray, and gene array, DNA microarray is a high throughput technology that can provide phylogenetic information about the gut microbiota. It is a highly sensible technique detecting very low microbial DNA concentrations from target sample (Gladney et al., 2004; Zhao et al., 2005). Microarray chips with the purpose of gut microbiota analysis have also been developed with some being commercially available. DNA microarrays are used primarily in comparative studies of microbiota between different populations (Salonen et al., 2010; Schokker et al., 2015).

Advantages/disadvantages

DNA microarray technique is a fast, semi-quantitative technique, samples can be assayed for taxonomic identification en masse but the qualitative data is debatable (Schokker et al., 2015). Another limitation is given by the cross-hybridization probability (multiple sample hybridization) (Fricker et al., 2019). Low population species from the gastrointestinal tract can prove to be challenging to quantify although this can be remedied by using a reference sequence (Cheng et al., 2019).

Sequencing

The taxonomic golden standard for any kind of taxon, sequencing is dependent on the full-length 16S rRNA gene for information (>1,500 base pairs long), which is limited to clone library insert sequencing. Once obtained the sequence is compared to a database (GenBank https://www.ncbi.nlm.nih.gov/genbank/) or GreenGenes https://greengenes.secondgenome.com/), which contains nucleotide sequence lengths above the PCR requirements. Mounting demands for lower cost and higher-output sequencing made the Sanger sequencing technique to be replaced in the 1990s by the cheaper and higher volume 'next-generation' sequencing technologies (Xiao et al., 2016). An advantage over the next-generation sequencing techniques is that it can produce sequence reads of more than 500 nucleotides.

Disadvantages of the Sanger techniques are the laborious method involved, high costs related to output, and susceptibility to the same errors as the PCR technique (Xiao et al., 2016; Hua Yang et al., 2018).

16S rRNA Next-Generation Sequencing

In the present day, there is a preference from the scientific community toward the Next Generation Sequencing techniques of the 16S bacterial rRNA obtained from the amplification of the sample DNA (Hermann-Bank et al., 2013; Kim & Isaacson, 2015; Allali et al., 2017; Wang et al., 2019).

In contrast with other types of sequencing, next-generation sequencing provides independent sequence data from millions of individual DNA molecules allowing each fragment to be classified independently, with newer NGS technologies operating on a "sequencing by synthesis" chemistry rather than a "sequencing by ligation" process.

Further steps are needed in the process of analyzing the resulting raw sequencing data, such as quality filtering and denoising steps to eliminate background noise errors. Also included is the filtering of chimaera sequences to eliminate possible hybrid strands. After this step, the sequences are usually clustered into so-called operational taxonomic units (OTU), which make the taxonomic classification of each sequence easier (Pang et al., 2007; Pylro et al., 2014; Allali et al., 2017). Many bioinformatics tools can help interpret the data as well as process and analyze the 16S DNA information, for example, QIIME (http://qiime.org/), Mothur (https://mothur.org/), and MEGAN (https://bio.tools/megan) and the resulting data can either be interpreted in statistical packages like R-Studio (https://cran.r-project.org/), Primer-E (https://www.primer-e.com/) or QIIME (Haas et al., 2011; Mendes-Soares, et al., 2014; Hui Yang et al., 2016).

The pipeline software can give us the final picture of the microbiota community structure, which corresponds to the original raw data sampled. The final picture or the output data is exemplified into indices used in microbial ecologies such as alpha and beta diversity indices, similarity visualization methods such as the PCoA (principal coordinate analysis) method, and relative and absolute abundances indices (Lin et al., 2011; Wang et al., 2012; Eren et al., 2013; Korpela et al., 2018).

The most investigated part of the pig's gastrointestinal tract is the colon and the ileum, followed by the caecum (Leser et al., 2002; Konstantinov et al., 2006; Bokulich et al., 2016; Gibbons et al., 2016; Crespo-Piazuelo et al., 2018; Gao et al., 2018; Kraemer et al., 2018). The microbiota colonising each section was analyzed based on the extraction and amplification of the 16S rRNA genes of the V1-V4 highly conservative regions and sequencing the amplicons via next-generation sequencing technologies such as Illumina (https://www.illumina.com/), or Ion Torrent (https://www.thermofisher.com /ro/en/home/brands/ion-torrent.html) (Li et al., 2018; Kinstler et al., 2019; Wang et al., 2019;).

The ileum microbiota pointed to a lower diversity of abundances and richness indexes compared with the other gut segments with the dominance of only two phyla *Proteobacteria* and *Firmicutes*. In the colon and caecum, other phyla have been observed, mainly *Bacteroidetes, Proteobacteria*, and *Firmicutes* (Zhang et al., 2017; Tan et al., 2018).

Many studies performed on pig gut microbiota pointed to the high abundance of the members of *Bacteoridetes* phylum, representing almost half of the

colonic microbiota (Pedersen et al., 2013; Crofts et al., 2017; Argüello et al., 2018; Guevarra et al., 2018; Wang et al., 2019). These members are known to produce enzymes that help with the degradation of polysaccharide fibres and the production of short-chained fatty acids, both beneficial for the host. In contrast, the ileum was found to be abundant in *Firmicutes* members, many of them involved in the digestion of monosaccharide, amino-acids, and carbohydrates via ABC (ATP-binding cassette) transporting (Tian et al., 2017; Tröscher-Mußotter et al., 2019).

Regarding studies done on faecal sample microbiota, the same techniques were used, NGS sequencing, and analysis of the raw reads with the help of a pipeline software. The resulting microbiota communities picture was similar to that found in the colon, which association is making sense from a microbial point of view. *Firmicutes* and *Bacteroidetes* dominated at the phyla level, followed by smaller numbers of *Proteobacteria*. There was also a similarity between faecal samples and colon samples at the order level, with the abundance of *Clostridiales, Bacteroidales, Lactobacillales,* and *Spriochaetales* (Chen et al., 2018; De Rodas et al., 2018).

A shared genus core was also identified between many studies on swine faecal, comprised of some of the most frequently identified but not limited to *Clostridium, Ruminococcus, Blautia, Prevotella, Spirochaeta, Lactobacillus* and *Bifidobacterium* (Tsuchida et al., 2017; Arruda et al., 2019).

Next-Generation Sequencing has revolutionized the domain of microbial ecology. For the last decade, NGS has become a faster, more accurate, and cost-effective tool for the study of complex microbial communities. The advantages presented by the NGS method comprises of the magnitude of the sequences processed of each taxonomic level, identifying more than 95% of the bacteria present in the gastrointestinal tract, which was not possible to do before the appearance of this technique (Wang et al., 2019; White et al., 2019).

Because of the differing chemistry employed, sequencing platforms produce different phylogenetic distributions as a result.

Finding a more fundamental approach to sequencing complex microbial communities for the obtaining and analysis of data will remain a high priority for researchers focused on the field of microbial ecology. Biases appearing from the lack of using a standardized protocol for sample handling, preparation and data interpretation via pipeline software will be impossible to eliminate, unfortunately. Choosing the right sequencing platform and the appropriate pipeline software will contribute highly to the reduction of data acquisition and comparison biases between studies (Kuderer et al., 2017; Besser et al., 2018; Costello et al., 2018).

When it comes to the analysis methods of sequenced microbial communities, several revolutionary methods are worth mentioning, such as BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and UCLUST (http://www.drive5.

com/ usearch/) which attempt to group sequences with a 97% or above molecular similarity (Tsuchida et al., 2017; Han et al., 2018; Tan et al., 2018). They differ from other conventional methods by suppressing sequencing errors and calculating their probability distribution.

These issues are especially important when it comes to differentiating between single-nucleotide variations within the sequencing data which need appropriate analysis methods and protocols involved in the study of microbial ecology.

Among the other advantages mentioned are the microbiota ecological indices that can be derived only from this data, from both relative and absolute abundances, calculated with high precision (97%) for every taxonomic rank up to the species level (Wang et al., 2019).

One of the severe disadvantages that the method presents is the high price involved per probe analysis. As it usually tends to happen with the advancement of technology, the price per sample is bound to get lowered (Frese et al., 2015; Zhao et al., 2015; Yasukawa et al., 2017; Wang et al., 2020).

Discussions

The usefulness of microbiome studies

The largest surface in the swine body is the gastrointestinal tract, which harbours an incredible variety of microorganisms. This dynamic is fragile to external and internal imbalances, as it represents complex niches and, thereby, contributes towards the swine wellbeing. Until recently, microbial ecology comparative studies sampling was done between a few points of time; sometimes, pooled samples being used often. Considerable differences were seen between results, in part due to the swine subjects breed, gender and age and experimental testing, as well as biomolecular methods used for DNA extraction and sequencing, usually performed at the 16S rDNA gene level (Mendes-Soares et al., 2014; Patel et al., 2017; Pylro et al., 2014; Allali et al., 2017; Rintala et al., 2017) making the task of finding correlations from study comparisons to be a tedious task.

Presently the Genomics methods have the distinct advantage of offering reliable measurements in contrast to the high volume of samples taken into account, at a competitive cost per sample when compared to other microbial ecology methods like classical or qPCR, exceeding the depths at which the microbiota can be described, with raw reads reaching the number of millions. Due to the broad comparison of samples being obtained, PCoA analysis along with alpha and beta diversity (indices reflecting different aspects of community heterogeneity) are employed to describe the microbial communities fingerprint, accounting for the external and internal effects on pig microbiota (Tian et al.,

2017; Besser et al., 2018; De Rodas et al., 2018; Lu et al., 2018; Muurinen et al., 2021).

The complex requirements to describe the phylogenetic diversity lead to the optimization of DNA extraction methods and amplification strategies such as within the 16S rRNA region. The Metagenomic analysis of the active bacterial population genetic sequences from within the microbiota of the swine gastrointestinal tract, especially of those prokaryotes which were proven to be fastidious or uncultured, is still at an incipient stage (Schell et al., 2002; Haas et al., 2011; Saraf et al., 2017; Tsuchida et al., 2017). Progress is expected to be made within the next years with the rise of prominence of the Metatranscriptomic and Metaproteomic studies, which in turn will grant deeper awareness on the relationship between the microbial communities and the host. With the support of collected data through labelling, accurate taxonomic, and microbiota role descriptions can be made to single members and of whole communities (Chen et al., 2018; Guevarra et al., 2018; Kumar et al., 2018). This issue can also contribute to modelling the microbiota response mechanisms towards change in the environment or feeding regimen and with relation to pathogens and antibiotics.

The studies on the swine gastrointestinal tract have risen noticeably in the past years in comparison to those done on human microbiota, contributing to the notion that the swine is a valid experimental model for the study of specific human afflictions (Lin et al., 2011; Zhao et al., 2015; Chen et al., 2018; Kumar et al., 2018; Maccari et al., 2018).

Towards a possible future of microbiota molecular studies

Due to the limited sensitivity of culture-dependent techniques employed in the study of microbial ecology, Next Generation Sequencing tactics are increasingly employed for microbial communities taxonomy and characterization either from the gastrointestinal tract or from other complex environments (Kim & Isaacson, 2015; Yasukawa et al., 2017; Fenske et al., 2019; Kraemer et al., 2018).

Concerning swine microbiota analysis, there are several directions which were taken among which 16S rDNA sequencing and other Omics like Metagenomics and Metatranscriptomics being the most often applied (Hui Yang et al., 2016; Saraf et al., 2017; Tröscher-Mußotter et al., 2019; Wang et al., 2020). These approaches accurately describe the relations between compositional and functional characteristics of the swine gastrointestinal microbiota. Along with discussing multiple microbiota analysis techniques available presently, this review has also presented a general outlook of the current state of the art pertaining swine microbiome studies, focused either on classical methods or molecular methods in general, with a focus on the next-generation approaches. Even if the NGS platforms are the most often metagenomics strategies used to analyze

DNA, mixed genomic techniques are also routinely used for microbiota assays. Lately, the next-generation sequencing approach was employed to sequence full-length 16S rDNA to verify the data obtained from classical methods for microbial species with varying relative abundances.

The NGS based profiling 16S rDNA correctly associated whole amplicons to both species as well as their abundance profiles which could then be correlated with abundances obtained through classical plating techniques (Recharla et al., 2019; White et al., 2019). Some improvement in terms of the depth of data were obtained with the latter techniques when compared to the current 16S rDNA strategies through species-level description of the bacteria. Generally, the potential benefits of multi-omic approaches in describing and possibly improving the microbiota composition in order to provide or maintain a healthy microbial balance, while also improving the pig's growth parameters were emphasized (Sieuwerts et al., 2008; Frese et al., 2015; Fouhse et al., 2016; Bottacini et al., 2017; Chen et al., 2017; Maradiaga et al., 2018; Tröscher-Mußotter et al., 2019).

In a short while it can be expected that Next Generation Sequencing molecular techniques to gain broader applications inside the microbial ecology field. In time, the obtained results can be carefully analyzed to design integrated (classical and molecular) and multidisciplinary techniques (especially from Omics) that can positively contribute towards the breeding, raising and wellbeing of pigs and food safety of pork products (Kim & Isaacson, 2015; Ramayo-Caldas et al., 2016; Gresse et al., 2017; Lu et al., 2018; Tan et al., 2018; Hua Yang et al., 2018; Arruda et al., 2019).

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