UNDERSTANDING ANTIMICROBIAL DISCOVERY AND RESISTANCE FROM A METAGENOMIC AND METATRANSCRIPTOMIC PERSPECTIVE: ADVANCES AND APPLICATIONS

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Running title: Microbiome: AMR & antibiotic discovery

Abstract

Our inability to cultivate most micro-organisms, specifically bacteria, in the laboratory has for many years restricted our view and understanding of the bacterial meta-resistome in all living and non-living environments. As a result, reservoirs, sources, and distribution of antibiotic resistance genes (ARGS) and antibiotic-producers, as well as the effects of human activity and antibiotics on the selection and dissemination of ARGs were not well comprehended. With the advances made in the fields of metagenomics and metatranscriptomics, many of the hitherto little-understood concepts are becoming clearer. Further, the discovery of antibiotics such as lugdinin and lactocillin from the human microbiota, buttressed the importance of these new fields. Metagenomics and metatranscriptomics are becoming important clinical diagnostic tools for screening and detecting pathogens and ARGs, assessing the effects of antibiotics, other xenobiotics, and human activity on the environment, characterizing the microbiome and the environmental resistome with lesser turnaround time and decreasing cost, as well as discovering antibiotic-producers. However, challenges with accurate binning, skewed ARGs databases, detection of less abundant and allelic variants of ARGs, and efficient mobilome characterization remain. Ongoing efforts in long-read, phased- and single-cell sequencing, strain-resolved binning, chromosomal-conformation capture, DNA-methylation binning, and deep-learning bioinformatic approaches offer promising prospects in reconstructing complete strain-level genomes and mobilomes from metagenomes.

Keywords: microbiome; single-cell sequencing; resistance genes; mobilome; meta-resistome; methylome; phased-sequencing.

Introduction

Antimicrobial resistance (AMR) continues to restrict treatment options for infectious diseases. Hence, the attributable mortality and morbidity rates of drug-resistant infections are estimated at hundreds to millions of people, with attendant healthcare-associated costs of millions of dollars annually around the world (Pehrsson et al., 2013; Sekyere and Asante, 2018). As a means to increase our antibiotic arsenals and enhance treatment options for drug-resistant infections, increasing calls are being made to accelerate the discovery of novel antibiotics (Sekyere, 2016; Somboro et al., 2018). Historically, the discovery and subsequent use of novel antibiotics have been followed by the emergence of antibiotic resistance genes (ARGs) and resistant bacteria (Davies and Davies, 2010; Osei Sekyere et al., 2015), begging the question of which came first: antibiotics or ARGs?

The concept of bacteria producing antibiotics to eliminate competitors was recently highlighted by Donia *et al*. (2014) with the production of lactocillin in the vaginal commensal *Lactobacillus gasseri* (Donia et al., 2014), and by Zipperer *et al*. (2016) with the production of lugdinin by *Staphylococcus lugdunensis* in the nasal cavities to inhibit methicillin-resistant *Staphylococcus aureus* (MRSA) (Zipperer et al., 2016). Except for these two antibiotics, most antibiotics were discovered from soil-inhabiting bacteria. Notably, several ARGs, such as clinically important metallo-β-lactamases, were initially found in soil bacteria, some of which were producers of β-lactam antibiotics, before their emergence in clinical pathogens. Numerous environmentally discovered ARGs are not clinically relevant even though they could confer resistance to antibiotics under certain conditions when challenged with antibiotics (Berglund et al., 2017).

Most bacteria are not cultivable under standard laboratory conditions, restricting our view and understanding of the bacterial meta-resistome, mobilome, species diversity, relative abundance and functional interactions within all living and non-living environments until recently. Traditionally, research in AMR and discovery focused on cultivable bacteria, which were usually isolated from clinical or soil specimen (Fig. 1), and the roles played by known ARGs in those species (Pehrsson et al., 2013; Aguiar-Pulido et al., 2016). Thus, culture- and PCR-based approaches have been used to study microbial communities expressing AMR characteristics. Both approaches have served microbiologists well and have led to important discoveries and strides in AMR research and antibiotics discovery (Pehrsson et al., 2013). However, culture-dependent techniques have intrinsic limitations because most bacteria do not grow axenically under standard laboratory conditions, making them undetectable by this approach, and resulting in under-sampling of ARGs from diverse microbial communities. In addition, PCR-based methods only detect known or previously described genes, which makes it biased and exclusive (Data S1) (Aguiar-Pulido et al., 2016)

Over the past decade, the emergence and subsequent advances in next-generation sequencing (NGS) applications in microbiome studies have revolutionized the search for ARGs and antibiotics producers as non-cultivable microbes can now be analysed *de novo* without prior knowledge of available genes for primer design (Penders et al., 2013)(Data S1). Whole-genome shotgun metagenomics can be undertaken via a sequence-based approach in which the genomes of all microbiota in a given environmental or clinical sample is directly sequenced after extracting the DNA (Lanza et al., 2018). It can also be undertaken by a functional metagenomics approach in which cultivable bacteria are used as producers or factories to translate DNA fragments directly extracted from environmental or clinical microbiota into proteins. Restriction endonucleases are used to fragment the foreign genomic DNA and host plasmid vectors into strands with complementary ends to enable integration and subsequent transformation into the cultivable host bacteria. This is followed by screening of the transformed bacteria for foreign proteins such as antibiotics (Fig. 2) (Martínez et al., 2017; Lanza et al., 2018)(See supplementary data S1 for more details on sequenced-based and functional metagenomics).

Due to the differential abundance of various species in a microbiota, less represented species and their ARGs are commonly undetected by sequencing platforms, particularly when the read coverage is low (Martínez et al., 2017). As well, allelic variants of ARGs are normally not detectable by short-read sequencers due to their shorter sequence reads, making it difficult to predict the phenotypic profile of the uncultivable host species as different alleles of an ARG can have different resistance profiles (Arango-Argoty et al., 2018; Beaulaurier et al., 2018). Furthermore, the inability to reconstruct draft or whole genome sequences of species and strains with their associated mobile genetic elements (MGEs) such as plasmids, bacteriophages, transposons, and integrons, the so-called mobilome, into genomic bins (i.e., categories of same or similar species/taxa) for further downstream bioinformatics analyses continue to plague microbiome research (Martínez et al., 2017; Beaulaurier et al., 2018; Lanza et al., 2018). Finally, prediction of ARGs in metagenomes leads to several false negatives as current ARG databases are composed of only known, specific and clinically important ARGs with high sequence homology cut-offs and best-hit search algorithms (Martínez et al., 2017; Arango-Argoty et al., 2018).

Metatranscriptomics

How does the microbiome react to stress, including antibiotic stress, from humans and surrounding antibiotic-producing competitors? How does exposure to these stresses lead to ARGs' emergence, prevalence and distribution? How does acquired ARGs affect fitness cost and host physiology? To answer these questions, metatranscriptomics comes in as a better tool than metagenomics. One of the ways by which the human gut microbiota affects host health is by metabolizing antibiotics. Even though some research has been conducted on the diversity of these microbial communities, it still remains unclear which microorganisms are transcriptionally active and what factors elicit their activity and gene expression (Aguiar-Pulido et al., 2016). It can be demonstrated by metatranscriptomic analysis, that exposing the active gut microbiome to antibiotics on a short-term basis can considerably affect gene expression, physiology and structure of the microbiome (Table 1; Data S1). Particularly, genes encoding drug metabolism, antibiotic resistance and stress response enzymes/pathways have been found across several bacterial phyla (Maurice et al., 2013). Furthermore, the effects of acquired ARGs on host physiology are best understood through metatranscriptomic analyses. For instance, changing gene expression in response to antibiotic challenge can affect bacterial physiology or morphology through efflux hyper expression and suppression of certain genes respectively (Martínez et al., 2017).

Brief description of microbiomic methods used in AMR & discovery research

All metagenomic studies essentially follow the same primary steps (Data S1): Metagenomic DNA extraction kits are first used to isolate purified DNA from environmental specimens. Metagenomic DNA extraction kits are specially designed to extract inhibitor-free DNA from non-cultivable or difficult-to-culture organisms in various environments such as soil and water, which cannot be equally achieved by other non-metagenomic DNA extraction kits; DNA contaminants such humic and fulvic acids are not eliminated by the latter. This is followed by either direct NGS or excision with endonucleases for subsequent cloning into plasmid vectors and transformation into bacterial hosts. These cloned transformants are multiplied to create libraries, which are then extracted and sequenced (Fig. 2). The function of genes within the clones can be assessed through their expression in the transformant(s) (Fig. 1-2; Supplementary data S1) (Thomas et al., 2012).

The metatranscriptomic process involves extracting and analyzing metagenomic messenger RNA (mRNA), thus providing information about genes that are actively being expressed under specific conditions (Quaresma et al., 2013). Processing of reads may involve (a) mapping unto a reference genome, in instances of specific species search, to deduce the relative expression of individual genes. Transcript expression can be normalized by using reads per kilobase of transcript per million mapped reads (Jiang et al., 2016). Alternatively, (b) reads may be assembled de novo into transcript contigs and supercontigs i.e., an ordered and oriented set of contigs still containing some gaps, which is particularly facilitated when PacBio's single molecule real time (SMRT) sequencing and Iso-Seq analysis is used (Nudelman et al., 2018). The first approach is limited by the reference genome database information whereas the second approach may be limited by the ability of bioinformatic software to correctly assemble short reads data into contigs and supercontigs (Aguiar-Pulido et al., 2016).

Bioinformatics tools used in microbiome studies

All NGS reads, including metagenomic sequence reads, are quality checked using tools such FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), trimmed, corresponding to an average Phred quality score of a minimum of 30 using applications such as Trim Galore! (www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and filtered to remove adaptor sequences using tools such as AdapterRemoval (v1.1) or Trimmomatic (Tables 1-3); host sequences are also removed (Lindgreen, 2012; Bengtsson-Palme et al., 2015; Caputo et al., 2015; Willmann et al., 2015; Hansen et al., 2016; Millan et al., 2016; Raymond et al., 2016).

The assembly of the metagenomic sequences per sample is performed with software tools such as Ray Meta version 2.3.1 (Boisvert et al., 2012) and Megahit (Li et al., 2015c), SOAPdenovo, MetaVelvet-SL, and metaSpades (Vollmers et al., 2017)(Tables 1-3). Open reading frames' prediction, which is integral in many annotation tools and precedes annotation, is done with softwares such as Prodigal (Hyatt et al., 2010; Caputo et al., 2015; Willmann et al., 2015; Raymond et al., 2016) Annotation of results may be done using KEGG, COG, MEGAN, GO, MG-RAST and Swiss-Prot (Aguiar-Pulido et al., 2016). Identification of ARGs is done using tools such as Resqu (http://www.1928diagnostics.com/resdb) and BLAST. Vmatch (http://www.vmatch.de/), is frequently used to scan metagenomic reads for ARGs, using for example the Resqu database as a reference.

For metatranscriptomics analysis based on read mapping, BWA and Bowtie2 are used to map reads to specific references. Annotation of results is done using KEGG, COG, MEGAN, GO and Swiss-Prot. Tools such as SOAPdenovo, AbySS, MetaVelvet, Trans-Abyss and Trinity assemble reads *de novo* into contigs and supercontigs (Aguiar-Pulido et al., 2016). RNA-Seq reads are taken as input alongside a reference transcriptome by RSEM, which evaluates the normalized transcript abundance (Aguiar-Pulido et al., 2016).

Current ARG databases such as ResFinder, SEAR, CARD (Comprehensive Antibiotic Resistance Database, ARGs-OAP, ARG-ANNOT, ARDB (Antibiotic Resistance Database) are unable to distinguish between intrinsic and acquired resistance, and are mostly plasmid- (e.g. SEAR) and species-specific, using best-hit approaches with high sequence homology cut-offs to determine ARGs from query sequences (Martínez et al., 2017; Arango-Argoty et al., 2018; Lanza et al., 2018). This increases false negative outcomes. In addition, these databases only host clinically important ARGs, making it difficult to identify unknown ARGs (Tsukayama et al., 2018). Arango-Argoty et al. (Arango-Argoty et al., 2018) therefore developed a new ARG-prediction database and tool, called DeepArg, using deep machine learning instead of a best-hit approach, with flexible identity cut-offs to increase sensitivity.

Sequenced-based ARG prediction, particularly for unknown ones, is not necessarily confirmatory. This means their clinical importance or ability to cause resistance in their hosts should be established through functional studies (Tsukayama et al., 2018). Moreover, the inability of currently used short-read sequencers to determine the allelic variant of an ARG in metagenomic sequences makes it less informative as different allelic variants of the same ARG can have different resistance profiles (Tsukayama et al., 2018). Hence, functional metagenomics should be used to determine the susceptibility profiles of such predicted genes by cloning them into cultivable bacterial hosts. However, lack of resistance or low level resistance in the species used for functional screening does not necessarily imply that the gene is not an efficient resistance gene in its original setting (Tsukayama et al., 2018). Thus, though functional studies are important in determining gene function, caution should be exercised in their interpretation, as genes may behave differently in different bacterial hosts due to epigenetic factors and promoter mutations. The risk of such ARGs to human health may be confirmed by infecting animal models with strains transformed with such identified ARGs.

Binning & DNA methylation, long-reads, single-cell and phased-sequencing

A major challenge to metagenomics is the ability to reconstruct complete or draft genomes of individual species and strains from metagenomic data to obtain a complete picture of species or strains and their resistome (ARGs) and mobilome (MGEs) diversity and relative abundance (Martínez et al., 2017). These drawbacks are due to the low sensitivity and specificity of available NGS platforms for less abundant species and their genomes, making it difficult to comprehensively determine all ARGs and MGEs. This is due to the absence of NGS platforms that can concomitantly generate long reads with high coverage. Thus, bioinformatic sorting or extraction of species or strains' genomes from the metagenome sequences into categories (or bins), a process called binning, is affected. Without effective binning, downstream bioinformatic analyses to associate identified mobilome and resistome with species or strains is impossible. Short-read sequencers are unable to provide reads that can be easily binned into separate species and strains as their assembled reads cannot provide longer contigs that can be easily resolved into species- or strain-level bins. Long-read sequencers on the other hand, have more errors and shallower coverage, making it difficult to identify low-abundant species and ARGs (Beaulaurier et al., 2018).

Phenotypic differences in strains of the same species in terms of virulence, pathogenicity, resistance etc., makes strain-resolved binning i.e., categorizing metagenomic contigs to simultaneously determine both gene sets and exact sequence of strains, extremely important for clinical microbiome applications. Thus, DNA processing protocols, high coverage long-read sequencing and bioinformatic algorithms have been developed to improve upon strain-resolved binning (Alneberg, 2018). For instance, phased sequencing, a novel technique for resolving chromosomal alleles into their paternal and maternal progenitors in eukaryotes, can be adapted in prokaryotes to enable easy strain-resolved binning (Alneberg, 2018; Choi et al., 2018). A new droplet-based barcode sequencing technique for creating linked-read sequencing libraries by uniquely barcoding the information within single DNA molecules in emulsion droplets, without the aid of specialty reagents or microfluidic devices has been further introduced to enhance phased-sequencing at a cheaper cost (Redin et al., 2017). This barcoding technique allows easy binning of reads to identify allelic variants and can be used to resolve closely related strains of the same species.

Furthermore, Bishara et al. (2018) recently introduced a new technique using the 10X Genomics (barcoding and library construction) platform and Athena assembler to generate longer highly contiguous (>200kb N50, <10 contigs) draft genomes with at least 20X coverage. This synthetic long read approach proved better than Illumina's Truseq or PacBio's reads as it combined long reads with higher coverage to enhance effective binning (Bishara et al., 2018). The Illumina NovaSeq 6000 sequencing system which supports an enormous output, generating up to 6 Tb and 20 billion reads in less than 2 days, helps to improve upon read coverage albeit its shorter read length of 2 x 150 bp will impact efficient binning (Svensson et al., 2018).

Single-cell sequencing (SCS), in which DNA from single cells in the microbiota are isolated through special techniques for amplification and subsequent sequencing without a culturing step has been proposed to study under-represented or low abundance species to overcome metagenomic challenges (Hedlund and Deng, 2017). However, the amplification step can result in non-uniform amplicons and subsequent differences in read depth and abundance of genes. The amplification step, which is necessary obtain adequate material for downstream analyses can be challenging. These challenges may introduce biases and errors that can thwart data interpretation (Gawad et al., 2016). It is critical to obtain genetic information from single cells while circumventing challenges such as genome loss, amplification bias and mutations. PCR-based techniques of whole-genome amplification (WGA) such as degenerate oligonucleotide primed PCR (DOP-PCR) result in low coverage (Gawad et al., 2016). Isothermal methods are alternative WGA methods that generate a greater genome coverage with lower error rates than PCR-based methods, even though the former techniques also lack uniformity. Methods such as multiple annealing and looping based amplification cycles (MALBAC) and PicoPLEX, developed to surmount the limitations of PCR-and isothermal-based techniques merge the two methods, by using isothermal amplification followed by PCR amplification of the amplicons generated by the isothermal step (Gawad et al., 2016). SCS can be added to metagenomics to identify less abundant species in the microbiota by first using 16S rRNA sequencing to identify less abundant species, which can be selected for SCS; however, this elaborate technique is expensive (Alneberg, 2018). Notwithstanding, single-cell RNA-seq holds much promise to identify individual cell's reactions to antibiotics in the microenvironment.

Bioinformatic binning tools such as ABAWACA, COCACOLA, CONCOCT, GroupM, MaxBin, MetaBAT and Mycc use GC content, sequence composition, genome coverage, phylogenetic markers and genomic signatures alone or in combination to bin metagenomes into similar taxa and species (Alneberg, 2018). The efficiency of these tools is however limited by short and low read depths, which can negatively impact efficient species and strain binning. Short reads are difficult to be assembled into longer contigs, leading to shorter contigs that does not allow efficient differentiation of genomes to the species and strain level, which affects binning (Alneberg, 2018). Although co-assembly, i.e. the combination of all or several samples prior to sequencing, has been proposed as an effective means to increase the overall relative abundance of lower abundance species during binning, it was found to be practically inefficient than binning individual assemblies (Alneberg, 2018; Beaulaurier et al., 2018). To overcome the inherent challenges in individual binning tools, Sieber et al. (2018) recently developed a dereplication, aggregation and scoring strategy (DAS) algorithm that combines a flexible number of binning algorithms to overcome the inherent difficulties in each. The DAS tool was found to be better than individual ones (Sieber et al., 2018).

EPIC-PCR (Emulsion, Paired Isolation and Concatenation PCR), a technique that has been suggested to address the afore-mentioned drawbacks of NGS platforms by linking phylogenetic markers and functional genes in uncultivated single cells to provide a throughput of several thousands of cells, has been introduced (Spencer et al., 2016). The method uses emulsion-based techniques to segregate cells in emulsion droplets, thus allowing cell lysis before PCR, capturing them in a hydrogel matrix, which holds the genomes of the bacteria for subsequent amplification of targeted genes. The method therefore links the identity of microbial community members to their function. Spencer et al. (2016) used the technique to identify a new sulfate-reducing microbial population among the diverse microbial population of a freshwater lake (Spencer et al., 2016).

A breakthrough approach in metagenomic binning was recently reported by Beaulaurier et al. (2018) using DNA methylation signatures and PacBio’s SMRT platform to efficiently bin metagenomes into species, strains, and MGEs. DNA methyltransferases (MTases), which catalyze the addition of methyl groups to cell- or strain-specific sequence motifs in bacteria and archaea, are found on both MGEs and chromosomes. MTases methylate both plasmids and chromosomes to ensure that the same methylation signatures exist within the cell. The uniqueness in methylation signatures allows it to be used alongside current binning tools to efficiently bin strains and associated MGEs. Bacterial methylome diversity are driven by MGEs bearing MTases, which can lead to similar signatures among cells hosting same MTase-bearing MGEs. Thus, the resolution of this approach decreases with increasing microbiome complexity, although its combination with current tools will enhance strain-resolved mobilome and resistome binning (Beaulaurier et al., 2018).

Resistome and mobilome analysis

The mobilome plays an important role in shaping the resistome of the microbiome as it shuttles ARGs horizontally between cells, strains and species. A perfect characterization of the mobilome and resistome in the microbiome is challenging as current short read platforms are unable to generate longer assembled contigs that can enable efficient binning and associate the resistome and mobilome with their host genomes. Moreover, the relatively low abundance of the mobilome and resistome requires deeper sequencing coverage for better detection, a property lacking by current long-read sequencers such as PacBio (Beaulaurier et al., 2018; Bishara et al., 2018).

Several strategies have been developed to enhance plasmidome (mobilome) analysis in metagenomes. These include the use of detergents and exodeoxyribonucleases to respectively isolate plasmid DNA and degrade linear but supercoiled DNA, rolling-circle amplification using Φ29 polymerases to increase plasmid concentration, and genetic labelling that allows capturing of plasmid DNA in recipient cells, enable researchers to increase the quantity and quality of isolated plasmids for sequencing (Martínez et al., 2017; Krawczyk et al., 2018). Other methods include the introduction of competent cells into donor communities to receive plasmids and using transposon-aided capture (TRACA) to transform *Escherichia coli* cells with sample DNA that has undergone *in vitro* transposition reactions (Warburton et al., 2011; Zhang et al., 2011). While these methods allow for detailed characterization of isolated plasmids, not all plasmids can be studied as different plasmid sizes (larger ones) and plasmid types are not amenable to these techniques. Unlike plasmidome analysis, techniques for phageome, i.e. the complete repertoire of phages or phage DNA in an environment, isolation and analysis yield comprehensive results, albeit phage contamination with host chromosomes remains a challenge (Martínez et al., 2017).

Chromosomal conformation capture (3C), a method adapted from chromosomal DNA processing in which formaldehyde is used to crosslink chromosomal DNA to surrounding DNA (such as plasmids), is another major methodical advance in plasmidome/mobilome analysis. This technique allows the linking of plasmid to host chromosome into single DNA elements prior to sequencing, enabling efficient binning of the mobilome with host species (Martínez et al., 2017).

A novel targeted sequence capture technique to detect antibiotic, metal and biocide resistance genes towards overcoming the inherent deficiencies of metagenomics in terms of identifying less-abundant species and improve sensitivity and specificity was recently introduced. This technique, called ResCap (Resistome capture), further allows the simultaneous analysis of the presence and diversity of the mobilome, resistome and plasmid replicon genes using the NimbleGene technology in which constructed metagenomic libraries are hybridized and captured before sequencing on Illumina Nextseq (Lanza et al., 2018).

Bioinformatic tools that can efficiently assign the mobilome to their host genomes are limited, although promising results are obtainable with Recycler (Rozov et al., 2017), PlasFlow (Krawczyk et al., 2018), and the Plasmid Constellation Network (PLACNET) (Lanza et al., 2018). With the introduction of DNA-methylation-based binning, it is envisaged that better tools will be developed for mobilome analysis.

ARGs in the human microbiome

The role of the human gut microbiome in transporting ARGs between continents was studied by Bengtsson-Palme *et al*. (2015) using shotgun metagenomic sequencing on samples (fecal specimen) taken before and after exchange programs. It was observed that there was an increase in the relative abundance of ARGs; most notably sulphonamide ARGs (2.6-fold increase), trimethoprim (7.7-fold), and β-lactams (2.6-fold), even though no antibiotics were taken within the period (Table 1). Variations in resistance-encoding genes, particularly to widely used antibiotics such as tetracyclines, β-lactams and aminoglycosides were detected (Bengtsson-Palme et al., 2015). The study showed how travelling to different environments can affect the ARG profile of the microbiome and potentially result in colonization and possible infection with resistant microbes. However, low-abundant genes or taxa were undetected by this approach as ESBLs-encoding genes in *Enterobacteriaceae* were identified by culture (Bengtsson-Palme et al., 2015; Forbes et al., 2017). The effects of travel on the dissemination of ARGs has become an issue of interest in the light of this discovery, particularly as the world becomes increasingly globalized with sophisticated and fast modes of travel.

To investigate the effects of antibiotics on the microbiome, Willmann and colleagues (2015) observed the development of intestinal ARGs in two healthy individuals, without exposure to quinolone antibiotics in the previous year, over a 6-day course of treatment with ciprofloxacin (Willmann et al., 2015). Antibiotics affected ARG groups differently in the two subjects, particularly the class D β-lactamases. Increased intestinal ARGs also occurred in the subjects over the course of the antibiotic administration. The study found that the ARG composition in both subjects returned to their original composition four weeks after treatment, albeit to different degrees (Willmann et al., 2015)(Table 1). The use of the fixed- and random-effects models of calculating selection pressure, to calculate the amount of ARGs per daily dose of a particular antibiotic, when rightly adopted in clinical practice, can be used to determine the effects of therapeutic regimens on the intestinal microbiome. Clinical application of microbiomics to characterise the human microbiome for administering personalized therapeutic interventions with minimal dysbiosis was thus demonstrated. This is however only possible when the effect of antibiotics on the ARG pool is properly investigated. With a small sample size of 2 individuals, caution should be exercised in generalizing the findings as reproducibility is also not guaranteed. Again, it must be stressed that the study observed the shift in composition of the intestinal resistome due to the antibiotic administration. Thus, it has not been suggested that class D β-lactamases mediate ciprofloxacin resistance.

Raymond *et al*. (2016) further showed that the initial composition of the human gut microbiome influences the dysbiotic impact of antibiotics by administering cefprozil to healthy volunteers and analyzing stool samples before antibiotic exposure, at the end of treatment and three months after treatment (Raymond et al., 2016). *Lachnoclostridium bolteae* increased in most participants after antibiotic exposure, with a subgroup of the participants having an enrichment in *Enterobacter cloacae*. This effect was associated with lower initial microbiome diversity. Genes affected (increased) by antibiotic exposure included *arr2* (rifampicin), (bla_{CepA}) (beta-lactamase) and *mef*(G), even though the influence of antibiotic exposure on the microbiomes of the subjects remained largely individual-specific (Table 1) (Raymond et al., 2016).

While Willman et al. (2015) and Raymond et al. (2016) vouch for microbiomics in AMR screening, epidemiology and antibiotics prescription on a case-by-case basis, the cost, practicability and skills involved in such a concept should not be overlooked, particularly in resource-constrained settings. Nevertheless, the cheaper cost of the Oxford Nanopore (which is a fourth-generation biology-based NGS platform capable of sequencing DNA and RNA directly at the single-molecule level at a relatively cheaper cost) might make such propositions possible within a shorter time than imagined (Bertelli and Greub, 2013). On the other hand, the use of fecal microbiota transplantation (FMT), the process of transplanting fecal bacteria from healthy donors to recipients, to treat patients with drug-resistant *Clostridium difficile* infections, as shown recently by Juul et al. (2018) with the use of FMT and metronidazole, portends the potential benefits of using the microbiome in personalized medicine (Juul et al., 2018).

Millan et al. (2016) administered FMT from universal donors to 20 patients with recurrent RCDI through colonoscopy and observed them prospectively. Shotgun metagenomic sequencing and analysis showed that patients with RCDI had a larger number and diversity of ARGs, before FMT, than donors and healthy controls. β-lactamases, multidrug-resistant efflux pumps and fluoroquinolone ARGs were high in RCDI patients whereas donors mainly possessed tetracycline ARGs. Phylogenetic analysis revealed *Proteobacteria* as the dominant phylum in RCDI patients, with *Escherichia coli* and *Klebsiella spp.* being the commonest. It was observed that FMT decreased the number and diversity of ARGs, accompanied by decreased *Proteobacteria* but increased *Firmicutes* and *Bacteroidetes*. Furthermore, the resistome of the donor was similar to that of the recipient upon successful FMT, showing a change in microbiome consistent with healthy gut microbiome. (Table 1) (Millan et al., 2016).

Decreased ARGs correlated with resolution of RCDI symptoms, showing the importance of FMT and ARGS in RCDI. However, the observed decrease in *Proteobacteria* following FMT cannot be ruled out as contributing to the resolution of RCDI symptoms. FMT, whose effect on the microbiota is measured by metagenomics, presents a great treatment method for those in whom antibiotics have failed to work; a situation corroborated by Juul et al. (2018) (Juul et al., 2018). It may be more cost-effective than continued antibiotic use. It must be stated however, that FMT is not the result of metagenomics. However, the use of the method in sampling fecal samples before and after FMT, to investigate its effects on the composition and diversity of microbes and ARGs highlights its important place in FMT.

Identifying the sources, prevalence, diversity and hosts of ARGs is important in controlling and preventing AMR. Due to its ability to identify ARGs in non-cultivable bacteria, metagenomics is a useful tool for molecular epidemiologists fighting AMR. For instance, various other studies using metagenomics have identified various resistance determinants in the human microbiome, including β-lactamases, glycopeptide ARGs, *fosA*, *ant(6)-Ia*, *ermB, lnuB, tetL, tetU*, *CatB1* (Buelow et al., 2014; Caputo et al., 2015; Zaura et al., 2015; Jitwasinkul et al., 2016) and emerging ARGs, including a 16S rRNA methylase conferring aminoglycoside resistance and two tetracycline resistance proteins (Moore et al., 2013).

Pathogenomics & AMR

Pathogen diagnostics depends on the identification of already known aetiological agents (Miller et al., 2013; Osei Sekyere, 2018). Despite batteries of available tests such as culture-based investigations, microscopy, immunoassays, and molecular tests, aetiologies of many samples including nearly 40% of gastroenteritis and 60% of encephalitis cases sent to laboratories remain undiagnosed as the aetiologies may be novel or untargeted (Finkbeiner et al., 2008; Ambrose et al., 2011; Miller et al., 2013).

Metagenomics, being culture independent and pathogen-agnostic, presents a solution, in part, to the above-stated limitations, as the generated sequence data can be used to predict resistance determinants and virulence genes. Zhou *et al*. (2016) used metagenomics to detect pathogens without *a priori* knowledge (Zhou et al., 2016). In their study, they investigated diarrhea in stool samples, and identified β-lactamase and tetracycline ARGs as the most prevalent ARGs. Pathogens implicated in the infection included *Clostridium difficile*, *Clostridium perfringens*, norovirus, sapovirus, parechovirus, and anellovirus (Zhou et al., 2016). In a similar study comparing metataxonomic and metagenomic approaches to culture techniques in clinical pathology, Hilton and colleagues (2016) concluded that metagenomic analyses have the accuracy required as a clinical diagnostic tool in patients with ventilator-associated pneumonia (Table 1) (Hilton et al., 2016). However, metagenomics does not completely solve the puzzle of misdiagnosed or undiagnosed samples, warranting the development of improved pathogen diagnostics.

It has recently been shown that the introduction of diarrhea-causing enterotoxigenic *E. coli* (ETEC) into healthy persons resulted in a drastic change in the hosts' *E. coli* microbiome composition in that commensal *E. coli* were replaced with ETEC until the administration of antibiotics. The resistance of *E. coli* commensals to ciprofloxacin and β-lactams, to which the ETEC was susceptible, allowed the former to recolonize the gut 6-17 hours after antibiotics administration. Notably, no virulence or resistance gene exchanges were observed between the commenals and ETEC. The ability of ETEC to displace commensal *E. coli* and establish itself to cause diarrhoea confirms the role of pathogens in dysbiosis (Richter et al., 2018).

Environmental reservoirs of ARGs

AMR from soil microbiome

The evolution of antibiotic-producing microbes in the soil and other environments over the years has contributed to the menace of antibiotic resistance (Perry and Wright, 2013). Human activities such as the use of antibiotics in agriculture have led to an increase in selection pressure, which in turn can influence the environmental ecology, distribution and diversity of the meta-resistome (Fig. 1) (Perry and Wright, 2013). There are documented examples of environmental ARGs moving into human pathogens, which suggest that clinical resistance may have originated from the environment (Poirel et al., 2005; Pehrsson et al., 2013; Perry and Wright, 2013). Indeed, strong proof exists to imply that genes encoding resistance to β-lactams (*bla*$_{\text{CTX-M}}$), aminoglycosides, vancomycin and quinolones (*qnr*), have direct links with the environmental resistome (Pehrsson et al., 2013).

Xiao et al. (2016) detected and quantified a total of 16 ARGs types from paddy soils from South China, which were uniquely different in abundance and distribution from ARGs discovered in activated sludge and pristine deep ocean sediment, but similar to those of sediments from estuaries impacted by human activities. Multidrug-resistance genes (encoding multidrug efflux pumps) were found to be the most abundant (38–47.5% of detected ARG-like sequences) in this study (Table 2). Moreover, acriflavine, MLS (macrolide–lincosamide–streptogramin) and bacitracin ARGs were found. Three major resistance mechanisms, namely efflux, antibiotic deactivation and cellular protection were found (Xiao et al., 2016). Thus, uncultured soil bacteria represent a vast reservoir of ARGs that can potentially be transferred to pathogenic bacteria in humans and animals. It must be noted however, that apart from environmental factors, physicochemical properties of soil such as pH, soil organic carbon and moisture content can affect the composition of soil microbes and ARGs. For instance, soil pH affects nutrient availability or physiological activity, thereby applying selection pressure on soil microbes and affecting their abundance and diversity. Thus, the significant correlation between soil pH and microbiome distribution might have been influenced by differences in pH of the different soils (Xiao et al., 2016).

The prevalence and abundance of florfenicol and linezolid ARGs in soils adjacent to swine feedlots were investigated by Zhao *et al*. (2016). A high prevalence of florfenicol ARGs was found in soils close to farms where florfenicol was heavily used than other sites. Extensive florfenicol use in livestock and spread of swine waste-contaminated soils could potentially lead to dissemination of florfenicol ARGs (Table 2) (Zhao et al., 2016). The possible dissemination of

ARGs through HGT makes this observation worrying as ARGs can spread within the environment and to humans with attendant public health repercussions. Veterinary antibiotics use should be encouraged only where necessary to reduce AMR. In both oxic and anoxic paddy soil zones, ARGs and enzymes involved in production of secondary metabolite and organic matter degradation were highly expressed (Table 2)(Kim and Liesack, 2015).

The detection of ARGs in relatively pristine environments indicates that AMR is a widespread natural process that can occur without selection pressure from anthropogenic provocation, albeit their original function may not be to essentially mediate resistance. Thus, the original biological function of ARGs in bacteria is yet to be ascertained, although their use as protection against competition from antibiotic producers has been suggested. This is illustrated in a study in which Diaz and colleagues (2017) characterized various ARGs, mainly associated with efflux pumps, fluoroquinolone, vancomycin and sulphonamide resistance, in the pristine Artic Wetland (Diaz et al., 2017) (Table 2). Although there is little proof that AMR determinants are involved in natural processes besides conferring resistance to xenobiotics, they might be involved in vital cell processes including biosynthetic pathways' regulation, homeostasis, virulence, detoxification (Martinez et al., 2009; Allen et al., 2010) or growth and survival (Groh et al., 2007). Furthermore, not all naturally occurring ARGs threaten human health and the threat they pose might depend on whether they are carried by commensals or pathogens (Martinez et al., 2009).

A better understanding of the ecological role of AMR in the non-clinical setting can help forecast and reduce the occurrence and evolution of AMR (Martinez et al., 2009). Indeed, there remains a lot to learn about the effects of human-impacted changes of natural habitats/ecosystems on the evolution and spread of resistance in nature. Furthermore, with the rising detection of ARGs even in pristine environments, metatranscriptomic analysis has become imperative to determine whether or not these potential functional genes are partially or fully expressed and also to investigate their other functions in these environments apart from potentially conferring resistance (Aguiar-Pulido et al., 2016).

AMR from aquatic environments

ARGs have been discovered in aquatic environments such as oceans and rivers, which are a rich source of both cultivable and uncultivable microorganisms (Fig. 1) (Chen et al., 2013).

The prevalence, abundance and distribution of ARGs may differ from environment to environment depending on the level of impact of anthropogenic activities on the environment or the absence of it, as demonstrated by Chen *et al*. (Chen et al., 2013). In that work, comparative metagenomic profiling was carried out on samples taken from human-impacted environments, i.e. Pearl River Estuary in South China, and relatively pristine environments, i.e. deep ocean beds of the South China Sea. The most prevalent ARGs identified in the South China Sea were macrolide and polypeptide ARGs, with efflux pumps being the predominant mechanism. However, fluoroquinolone, sulphonamide and aminoglycoside ARGs were detected in the Pearl River estuary, which correlates with commonly used antibiotics in clinical medicine and animal farming (Table 2). Again, the pristine environment saw a lower diversity in both genotype and resistance mechanisms than that heavily impacted by human activities (Chen et al., 2013). The study presented a more inclusive description of the effects of urbanization on the microbial community, in this instance, freshwater ecosystems; this is in contrast with most studies, which previously focused on specific aspects of urbanization such as chemical pollution, microbial density and nutrient modification (YAMAGucHI et al., 1997; Paul and Meyer, 2001; Kroon et al., 2012). ARGs such as bla_{NDM}, bla_{VIM}, bla_{KPC}, $bla_{\mathrm{OXA\text{-}48}}$ and $bla_{\mathrm{IMP\text{-}type}}$ carbapenemases as well as *tet*(X) and *mcr*-1 genes that respectively confer resistance to carbapenems, tigecycline and colistin, which are last-resort antibiotics (Graham et al., 2014; Cerqueira et al., 2017), were found in a study investigating the effects of disposing untreated/partially treated sewage on the environmental resistome and bacterial communities of a river flowing through a city in India (Marathe et al., 2017). Developing countries have challenges in sewage management and a lack of adequate treatment and proper disposal can contribute to AMR by spreading antibiotic-resistant bacteria. Also, water-borne infections resulting from ineffective sewage disposal may lead to increased antibiotic use, further compounding the problem. Similarly, using functional metagenomics, Marathe et al (Marathe et al., 2018) found a novel mobile β-lactamase which hydrolyses carbapenems. The study found seven putatively novel ARGs, which include one amikacin resistance gene and six β-lactamases (Table 2).

Similar studies using metagenomics to investigate marine habitats led to the identification of sulphonamides, bacitracin, tetracycline, β-lactams, chloramphenicol, glycopeptides and macrolides ARGs (Table 2) (Port et al., 2012; Yang et al., 2013a; Guo et al., 2016).

AMR from wastewater treatment effluents

Wastewater treatment plants (WWTPs) are notable sources of diverse kinds of bacteria and ARGs, some of which are associated with human pathogens. WWTPs collect liquid and solid waste from communities, hospitals, industries etc. for treatment and subsequent disposal (Fig. 1), making them an important source of resistant pathogens (Li et al., 2015a). Activated sludge, digested sludge and influent can facilitate the spread of ARGs and metal ARGs through HGT due to the varying microbial populations present in these systems (Li et al., 2015a).

Li and colleagues (2015) reported on six plasmid DNA from two municipal WWTPs in which tetracycline and quinolone ARGs were the most abundant (Table 2). This culture-independent metagenomic approach provided more data in a shorter time at a reduced cost, circumventing the challenge of traditional plasmid analysis methods (Li et al., 2015a). Oxygen in WWTP environments may play a role in the occurrence and abundance of ARGs as observed by Wang *et al.* (2013). Tetracycline ARGs, most predominantly *tet33*, were highly abundant in the anaerobic sludge but was absent in the aerobic sludge, although the sulphonamide resistance gene, *sul1*, was found in both environments (Wang et al., 2013). Other studies have implicated WWTPs as important sources of environmental ARGs and putative novel plasmids including tetracycline resistance genes (Zhang et al., 2011; Yang et al., 2013b; Huang et al., 2014; Bäumlisberger et al., 2015; Rowe et al., 2016), sulphonamide resistance genes (Bengtsson-Palme et al., 2014; Tang et al., 2016; Tao et al., 2016) and β-lactam resistance genes (Staley et al., 2015)(Table 2).

The effects of antibiotics on WWTPs has been studied by challenging a WWTP with an antibiotic mix of norfloxacin, azithromycin, sulfamethoxazole and trimethoprim, and assessing their effects on the bacterial community and activity (Gonzalez-Martinez et al., 2018). *ermF*, *carA* and *msrA* (erythromycin), and *sul123* (sulphamethoxazole) ARGs were detected (Table 2). Resistance to norfloxacin was found to be mediated by mutations in *gyrA* and *grlB* (Gonzalez-Martinez et al., 2018).

Moreover, ARGs-containing effluents (from municipal hospital and dairy farm) affect the receiving environment i.e. a river catchment, which was shown by comparing gene abundance for both the source and receiving environment (Rowe et al., 2017). The correlation between the average ARG and their transcript abundances in both farm and hospital effluents, indicated that the identified genes were being expressed. Prolonged hospital antibiotic usage was associated with high abundance of β-lactam resistance gene transcripts. Effluents contributed to high ARG levels in the receiving aquatic environments. Significant ARGs' expression was associated with antibiotic use at the effluent source (Table 2) (Rowe et al., 2017), suggesting that antibiotics pollution directly increases ARGs expression and dissemination in the environment.

This study by Rowe and colleagues is particularly interesting because it combines metagenomics and metatranscriptomics and attempts to relate the expression of ARGs in the environment to antibiotic selection pressure, the first study to do so. Previous studies focused on anthropogenic effects on the resistome in receiving waters (Rowe et al., 2017). Although the study links the overexpression of ARGs to antibiotic use, it must be stated that other factors such as temperature of effluent and metabolic activity of samples may play a role. Hence, further studies may be required to buttress the association between antibiotic use at effluent source and ARG expression (Rowe et al., 2017).

AMR from drinking water

Shi *et al*. (2013) found that chlorination increases *ampC, aphA2,* $bla_{\text{TEM-1}}$, *tetA, tetG, ermA and ermB* ARGs, while considerably reducing *sulI* genes in drinking water. They confirmed that chlorination of drinking water could concentrate various ARGs, as well as MGEs (mobilome) (Shi et al., 2013). A greater percentage of the surviving bacteria after chlorination, most of which were *Proteobacteria*, was resistant to chloramphenicol, trimethoprim and cephalothin (Table 2). In addition, residual chlorine from chlorinated drinking water was found by another study to result in bacterial community shifts such that bacitracin resistance gene, *bacA*, and multiple ARGs were mainly carried by chlorine-resistant *Pseudomonas* and *Acidovorax* (Jia et al., 2015). Thus, while chlorination is widely used to sterilize water for drinking, the practice also selects for resistant bacteria and ARGs. Further research about chlorination is required before policy recommendations could be suggested.

AMR in veterinary and agricultural sources

Antibiotics are used extensively in animal husbandry for growth promotion, therapeutics, metaphylaxis and prophylaxis (Osei Sekyere, 2014). In China, it is estimated that 97,000 metric tons of the approximately 210,000 metric tons of antibiotics produced yearly are used in animal husbandry. Thus, a rise in the number of resistant bacteria in the animal gut has been observed (Zhao et al., 2016), compounded by the fact that more than half of administered antibiotics are not absorbed in the animal gut and are therefore shed in the faeces, exposing the environment to sub-therapeutic levels of antibiotics and contributing further to AMR (Osei Sekyere and Adu, 2015; Zhao et al., 2016).

Leclercq and colleagues (2016) (Leclercq et al., 2016) investigated the diversity of the tetracycline mobilome within a Chinese pig manure sample. Two new tetracycline ARGs (TRGs) namely, *tet*(59), encoding a tetracycline efflux pump, and *tet*(W/N/W), encoding mosaic ribosomal protection, were discovered together with 17 distinct TRGs (Table 3). The discovery of novel TRGs after decades of diligent studies shows our limited knowledge in AMR and the livestock meta-resistome.

The impact of antibiotic use in animal husbandry on human diseases was hotly contested until the recent emergence of the *mcr-1* gene, which showed transferability from veterinary to human medicine (Sekyere, 2016; Sekyere and Asante, 2018). To ascertain the effects of antibiotics on the swine intestinal microbiome, Looft and colleagues (2012) administered growth-enhancing antibiotics to one group of pigs but withheld antibiotics from another group, although both groups received the same diet. Increased abundance and diversity of ARGs and/or in *Proteobacteria* (mainly *Escherichia coli*) occurred in medicated pigs than in non-medicated ones (Looft et al., 2012). Various studies have also described the effects of antibiotics on animals, including mice, rats, and buffaloes (Chambers et al., 2015; Yin et al., 2015; Hansen et al., 2016), or have sought to characterize their microbiome (Table 3) (Durso et al., 2011; Bhatt et al., 2012; Singh et al., 2012; Guo et al., 2014; Reddy et al., 2014) and found fluoroquinolone resistance genes (Durso et al., 2011; Bhatt et al., 2012; Singh et al., 2012; Reddy et al., 2014) and tetracycline resistance genes (Guo et al., 2014).

The effects of composting (a biological treatment of animal manure) on the transcriptional response of ARGs and microbes found in manure, have been studied by relating changes in the resistome to the composting process (Wang et al., 2017), with the resistome found to contain various ARGs (Table 3). An observable reduction in the aggregated expression of these ARGs in the resistome was noticed by comparing metatranscriptomic and metagenomic data for the changing microbial community following composting (Wang et al., 2017). Specifically, composting reduced expression levels of TRGs, *tetM-tetW-tetO-tetS*, but had no effect on sulphonamide and fluoroquinolone resistance gene expression. Although the microbial population changed during the process, the core resistome endured. Again, the process reduced ARG-bearing pathogens of clinical relevance, RNA viruses and bacteriophages (Wang et al., 2017). Thus, composting reduced contaminants such tetracyclines and TRGs, consequently reducing the abundance of ARGs in manure and their spread thereof (Data S1).

Metagenomic analysis of multiple environments

Li *et al*. (2015) analyzed samples from various environments (including water, soil, sludge and fecal samples) and found an abundance of ARGs, corresponding to the level of anthropogenic activities in these environments, with the more impacted environments showing a higher abundance of ARGs than the less impacted environments (Table 3). ARGs for commonly used antibiotics in human and veterinary medicine were found: aminoglycosides, bacitracin, β-lactams, chloramphenicol, macrolide-lincosamide-streptogramin, quinolones, sulphonamides and tetracyclines (Li et al., 2015b). Resistance profiles and composition of bacterial communities from human, animal and environmental microbiomes have been profiled to provide extensive quantitative data on ARGs from multiple environments, (Pal et al., 2016). Resistance profiles and bacterial community compositions for the various types of environments were shown to be different, with microorganisms from human and animal communities showing limited taxonomic diversity: tetracycline, sulphonamide and metal ARGs were detected. The impact of human activities on the environment was further highlighted by the detection of high ARG abundances in environments polluted with antibiotics. The high abundance of MGEs found in environments polluted by pharmaceutical waste products should heighten concerns for transfer of resistance between bacteria (Pal et al., 2016).

A recent study of wild and captured baboons and human guts showed substantial differences between the microbiomes of wild and captured baboons as well as between baboons and humans (Tsukayama et al., 2018). This was suggested to be due to differences in habitat and lifestyle, which was influenced by contact with humans; suggesting the possible transfer of ARGs between humans and wild animals. Novel chloramphenicol resistance determinants were identified in wild baboons while human-exposed baboons harboured resistance to seven antibiotics including newer generation β-lactams and cephalosporins (Tsukayama et al., 2018).

Metagenomics applications in antibiotic discovery

It is estimated that about 90% of antibiotics currently in clinical use were obtained from cultivable microorganisms (Katz and Baltz, 2016). The discovery and introduction of novel antibiotics have stalled over the past 30 years, with only two novel classes of antibiotics being introduced onto the market in that period: daptomycin, the cyclic lipopeptide, and linezolid, the oxazolidinone (Fischbach and Walsh, 2009). Currently**,** traditional methods of antibiotic discovery involve the screening of natural sources, such as soil microorganisms for bioactive compounds of pharmacological interest. However, this approach of antibiotic discovery does not offer the promise of yesteryear, as demonstrated by the high rediscovery rates of known antibiotics, which has been shown to reach as high as 99.9% (Zaehner and Fiedler, 1995; Charusanti et al., 2012). However, metagenomics and genetic engineering can circumvent the limitations of cultivability to discover novel antibiotics in unknown microorganisms (Gomes et al., 2013).

The discovery of lactocillin, a thiopeptide antibiotic produced by a human vaginal commensal has kindled the hope of obtaining novel antibiotics from the human microbiota (Donia et al., 2014). It has long been known that bacteria produce natural antimicrobial chemicals to inhibit closely related competitors; however, species producing such inhibitory substances were mostly found in soil (Donia et al., 2014). By employing metagenomic and metatranscriptomic methods, biosynthetic gene clusters were identified in human-associated bacterial genomes, with the thiopeptides found to be extensively distributed in the metagenomes of human microbiota (Table 4). Lactocillin has been found to possess potent antibacterial activity against a number of Gram-positive vaginal pathogens. The production of such bioactive compounds by human commensals means humans may be constantly exposed to bioactive compounds, and it would be interesting to study how the microbiome responds to such exposure.

The characterization of the entire microbial diversity and genes of biotechnological interest, discovery of novel biosynthetic pathways and associated products, presents a potentially higher success rate in our search for natural antibiotics, particularly as an estimated 1% of microorganisms can be cultured axenically (Handelsman et al., 2007). The use of targeted metagenomics (The use of PCR, accompanied by Sanger's dideoxy chain termination sequencing in the analysis of the metagenome) in the discovery of new antibiotics was recently highlighted in a study (Hover et al., 2018). In that study, malacidins, a class of calcium-dependent antibiotics were discovered and found to be effective against multidrug-resistant Gram-positive pathogens (Table 4).

An example of the usefulness of NGS-based metagenomics in natural product discovery is the identification of '*Entotheonella*', a novel bacterial taxon, whose association with the Red sea marine sponge, *Theonella swinhoei*, produced more than 40 bioactive polyketides and modified peptides affiliated with seven different structural classes (Wilson et al., 2014). Polyketides are natural metabolites that make up the basic structure of many pharmaceuticals including anti-cancer agents, antibiotics and antifungal agents (Table 4).

Natural antimicrobial peptides (AMPs) have been found to be active against Gram-Positive and Gram-Negative bacteria, fungi, parasites and viruses (Huang et al., 2017). By inducing natural AMPs, green tea has been found in a study to possess antimicrobial activity against *E. coli* (Wan et al., 2016). Natural AMPs from bacteria obtained from oolong teas, a partially fermented tea widely used in Taiwan, with purported benefits including anti-allergic immune responses and anti-obesity among others, have been detected (Tables 2 & 4) (Huang et al., 2017) Metatranscriptomics, which can detect gene transcripts in such complex environments overcomes the limitation of functional gene microarrays, which only target specific species in complex environments. Again, metatranscriptomic sequencing resulted in more distinct and better defined output, facilitating the analysis of fine-scale variations in transcript sequences (Huang et al., 2017).

Limitations, future prospects and conclusions

Though considered a game-changer in the field of microbiology, NGS metagenomics is not without challenges. Virome assays for instance involve complicated sample and nucleic acid work-ups, although NGS of all DNA is possible in a given sample. A vast amount of taxonomically vague sequences is discarded. Taxa that are low in abundance may be tough to identify and strain-resolved binning can be challenging. Also, accessibility to thorough databases for all microbial groups and ARGs are limited. It is difficult to study the genetic environments of detected ARGs and the phylogeny of species that possess these functions (Martinez et al., 2009). Microbiomics can be expensive depending on the sample type, depth of sequencing and microbes of interest, coupled with the requirement of high technical expertise (Forbes et al., 2017). Again, obtaining high quality DNA is a challenge as they may be contaminated by environmental materials such as humic and fulvic acids, which are co-extracted with them. The use of high performance DNA extraction kits (e.g., kit Ultra Clean Mega Soil DNA from Mo Bio) however, can help partly evade this challenge, although their performance is influenced by the physicochemical nature of the environment (Gomes et al., 2013).

There are hindrances that restrict the large-scale application of metatrascriptomics despite the vast promise of this field (Aguiar-Pulido et al., 2016). Most of the collected RNA is from ribosomal RNA, the abundance of which can reduce the concentration of mRNA, which is the main target of metatranscriptomics (Aguiar-Pulido et al., 2016). Furthermore, distinguishing between host and microbial RNA can be a challenge, although commercial enrichment kits are available. Thirdly, mRNA is highly unstable, and this compromises the integrity of the sample prior to sequencing. Lastly, reference databases for transcriptomes are limited in terms of coverage (Aguiar-Pulido et al., 2016).

Short-read and low-depth sequencing remains a major setback to effective binning of genomes, mobilomes and resistomes. Novel technologies and methodologies such as 3C, ResCap, DNA-methylation-based binning, phased-sequencing, SCS, and improved depths in long read or hybrid sequencing holds much promise in aiding the complete reconstruction of strain-specific genomes, mobilomes and resistomes from microbiomes.

Metagenomics will soon facilitate diagnosis of known and novel pathogens, cutting down cost and delay, enhance assessment of individual microbiomes for tailor-made therapeutic interventions (Miller et al., 2013), and spearhead the discovery of potent antibiotics (Hover et al., 2018). However, for clinical diagnostic purposes, improvement in metagenomics is needed to decrease turnaround time and costs. Microbiome research is a robust tool for effective surveillance of AMR in various environments, discovering novel ARGS and antibiotics (lactocillin, malacidins etc.) as well as ascertaining the dynamics of ARGs transfer between commensals and pathogens. Metagenomics and metatranscriptomics bridge the disconnect between bacterial identity and activity.

Funding: None

Acknowledgement: We are grateful to Miss Regina Esinam Abotsi of the Department/Division of Medical Microbiology, University of Cape Town, Cape Town, South Africa, for her role in data mining and collation in the preliminary periods of this manuscript.

Author contributions: Study design and supervision (JOS); Literature search (JA & JOS); Writing of manuscript (JOS & JA); Design of images (JOS).

Transparency declaration: The authors declare no competing interest in both the conception, writing and decision to publish this manuscript.

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Table 1. Antibiotic resistance genes and bacterial families identified through metagenomics/metatranscriptomics studies undertaken up to 2018 on humans, with next-generation sequencing

Year of study	Resistance gene found/expressed	Families, Class, Genus and Species found	Source(human)	Method/Platform used	Bioinformatics tool used	Reference
2012	Genes encoding drug metabolism, antibiotic resistance and stress response pathways	*Firmicutes*	Human gut	Illumina HiSeq	QIIME software (Quantitative Insights Into Microbial Ecology) (Caporaso et al., 2010)	(Maurice et al., 2013)
2013	Chloramphenicol, aminoglycoside and tetracycline resistance genes. Three novel resistance genes including a 16S rRNA methylase conferring aminoglycoside resistance, and two tetracycline resistance proteins nearly identical to a bifidobacterial MFS transporter	Bacteria	Human gut	Illumina HiSeq 2000	MUSCLE (http://www.drive5.com/muscle/, March 2012) , FastTree (http://www.microbesonline.org/fasttree/, March 2012), FigTree (http://tree.bio.ed.ac.uk/software/figtree/, March 2012). PSI-BLAST (Altschul et al., 1997).	(Moore et al., 2013)
2014	Aminoglycoside resistance genes (*aph(2")-Ib* and an *aadE*-like gene	*Bacteroidetes* and *Clostridium* clusters XIVa and IV	Human gut	Illumina HiSeq 2000	SOAPdenovo (http://soap.genomics.org.cn), BLAST, CD-HIT (Fu et al., 2012), soap.coverage (http://soap.genomics.org.cn)	(Buelow et al., 2014)
2015	CTX-M-15 gene, OXA-1 and TEM (beta-lactamases), *aph(3")-Ib* and *aph(6)-Id* genes,	*Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes*	Human gut	Illumina HiSeq 2000	Trim Galore! version 0.2.8 (www.bioinformatics.babraham.ac.uk/projects/trim_galore/), Resqu database version 1.1 (http://www.1928diagnostics.com/resdb), Vmatch (http://www.vmatch.de/)	(Bengtsson-Palme et al., 2015)

	and tetracycline resistance genes *tet*(Q) and *tet*(X)					
2015	Beta-lactamase classes A and D, multidrug resistance efflux pumps, ARGs mediating resistance to aminoglycosides, chloramphenicol, macrolides, glycopeptides, and tetracyclines.	Bacteria	Human gut	Illumina HiSeq 2000 platform	FastQC (available from http: //www.bioinformatics.babraham.ac.uk/projects/fastqc/), FASTX-Toolkit (available from http://hannonlab.cshl.edu/fastx_toolkit /index.html), SASS aligner (https://atom.io/packages/aligner-scss), Ray Meta version 2.3.1 (Boisvert et al., 2012), MetaGeneMark version 2.8, MALT (available from http://ab.inf.uni-tuebingen.de/software/malt/).	(Willmann et al., 2015)
2015	Beta-lactamases, glycopeptide, macrolide-lincosamide-streptogramin (MLS), sulphonamide and tetracycline resistance genes	*Akkermansia muciniphila*	Human gut	Roche/454 GS FLX Titanium platform	Deconseq (Schmieder and Edwards, 2011a), CLC workbench software (CLC bio, Aarhus, Denmark).	(Caputo et al., 2015)
2015	Erythromycin resistance genes, efflux pumps, chloramphenicol acetyltransferase (CatB1), beta-lactamases	Bacteria	Gut and oral microbiome	Illumina MiSeq	Trimmomatic (Bolger et al., 2014), Best Match Tagger v3.101 (K. Rotmistrovky and R. Agarwala, 2010), UBLAST from USEARCH v7.0.1090 (Edgar, 2010), HUMAnN (Abubucker et al., 2012).	(Zaura et al., 2015)
2016	Beta-lactam, multidrug efflux pumps, fluoroquinolone and tetracycline resistance genes	*Proteobacteria* with *Escherichia coli* and *Klebsiella* most prevalent	Human gut	Illumina MiSeq	FASTX-Toolkit (version 0.0.13; http://hannonlab.cshl. edu/fastx_toolkit/index.html), Bowtie2 (Langmead and Salzberg, 2012).	(Millan et al., 2016)

2016	*mecA*	Bacteria and fungi	Bronchial aspirates	IlluminaHiseq	PrinSeq- Lite v. 0.20.3 (Schmieder and Edwards, 2011b), Bowtie2.	(Hilton et al., 2016)
2016	*arr2* (rifampicin), beta-lactamases (*bla*$_{CepA}$) *mef*(G) (macrolide resistance gene)	*Lachnoclostridium Bolteae*, *Enterobacter cloacae,*	Human gut	Illumina Hiseq	Ray Meta 2.0 assembler, Prodigal 2.6, FASTA36, Integrative Genomic Viewer (Thorvaldsdóttir et al., 2013).	(Raymond et al., 2016)
2016	Beta-lactamase (Bl2e_cfxa), tetracycline resistance (*tetQ*) and macrolide resistance (*ermA*, *ermB*, *ermF*, and *ermG)* genes	*C. difficile,* norovirus, sapovirus, *Candida* spp., anellovirus and parechovirus	Stool sample	Illumina HiSeq	MBLASTX software (MulticoreWare) (Davis et al., 2015).	(Zhou et al., 2016)
2016	*bla*$_{TEM-124-like}$ (extended spectrum beta lactamase), *fosA* (fosfomycin), *ant(6)-Ia*, *ermB*, *lnuB*, *tetL* and *tetU* conferring resistance to aminoglycosides, macrolides, lincosamides, streptogramin B and tetracycline respectively	Bacteria	Human gut	454 pyrosequencing platform	Newbler software (Roche Diagnostics), ResFinder (Center for Genomic Epidemiology, Technical University of Denmark, Kgs. Lyngby, Denmark), BioEdit v.7.0.9.0 (http:// www.mbio.ncsu.edu/Bioedit/bioedit.html).	(Jitwasinkul et al., 2016)
2017	Genes involved in pH regulation and nickel transport	*Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria*	Stomach/gastric microbiota	Illumina HiScanSQ instrument	TrimGalore! version 0.3.5 (http:// www.bioinformatics.babraham.ac.uk/projects/trim_galore/) PrinSeq version 0.20.4 (Schmieder and Edwards, 2011b) DUST algorithm (Morgulis et al., 2006) Metaxa2 software version 2.1.1 (Bengtsson-Palme et al., 2015)	(Thorell et al., 2017)
2018	Aminoglycoside-, fluoroquinolone-, beta-lactam- and	*E. coli*	Human gut	Illumina HiSeq	MUMmer v.3.22, RAxML v.7.2.8 FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/	(Richter et al., 2018)

	macrolide-resistance genes	figtree/)	

Table 2. Antibiotic resistance genes and bacterial families identified through metagenomics/metatranscriptomics studies undertaken up to 2018 on the environment with next-generation sequencing

Year of study	Resistance gene found	Families, Class, Genus and Species found	Source(environment)	Method/Platform used	Bioinformatics tool used	Reference
2011	ARGs encoding tetracycline, macrolide and multidrug resistance genes	Bacteria (*Actinobacteria, Chloroflexi, Proteobacteria, Bacteroidetes*, and *Firmicutes)*	Activated sludge	Illumina Hiseq	SOAPdenovo (BGI, Shenzhen, China), BLAST, MetaGene, NCBI ORF Finder, Plasm software (ver 2.0.4.29) (http://biofreesoftware.com)	(Zhang et al., 2011)
2012	Tetracycline resistance genes	α-Proteobacteria (in particular Rhodobacterales sp.), Bacteroidetes	Puget Sound estuary (surface water)	Roche/454 GS FLX Titanium platform	Newbler v. 2.5.3 (Roche Diagnostics-454 Life Sciences), Meta Genome Rapid Annotation using Subsystems Technology (MG-RAST), BLASTN	(Port et al., 2012)
2013	Macrolide, polypeptide, sulphonamide, fluoroquinolone and aminoglycoside resistance genes	Bacteria	Deep ocean bed and river estuary	Illumina HiSeq 2000	BLASTX	(Chen et al., 2013)
2013	Aminoglycoside, tetracycline, sulphonamide, multidrug and chloramphenicol resistance genes	Bacteria	Activated sludge(WWTP)	Illumina Hiseq 2000	BLASTX (Altschul et al., 1997), BLAST	(Yang et al., 2013b)
2013	Aminoglycoside, tetracycline, beta-lactam, chloramphenicol, trimethoprim, glycopeptide, bacitracin, fluoroquinolone, macrolide, sulphonamide,	Bacteria (predominantly *Proteobacteria*)	Aquatic environment (marine sediment)	Solexa GAII sequencer (Illumina, San Diego, CA, USA)	Platform Galaxy (Blankenberg et al., 2010), Velvet (Zerbino and Birney, 2008), blastn, Blastx	(Yang et al., 2013a)

	streptogramin, and multidrug efflux resistance genes					
2013	*sulI, tetA and tetG, ampC, aphA2*	Bacteria (*Proteobacteria*)	Drinking water	Illumina Hiseq 2000	SOAPdenovo (BGI, Shenzhen, China), MetaGeneMark (Noguchi et al., 2006), MEGAN 4 software (Huson et al., 2011).	(Shi et al., 2013)
2013	*sul1* (sulphonamide resistance gene), tet33,	*Proteobacteria, Firmicutes, Bacteroidetes* and *Actinobacteria*	Waste water	Illumina Hiseq2000	FASTX, MG-RAST QC pipeline, SEED established by Argonne National Lab (Argonne, USA), BLAST	(Wang et al., 2013)
2014	The *sul2* and *qnrD* genes	Bacteria	Polluted lake (Indian lake)	IlluminaHiSeq2000	FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc), Vmatch, Metaxa 2.0, Velvet, HMMER (http://hmmer.janelia.org)	(Bengtsson-Palme et al., 2014)
2014	Tetracycline resistance genes, sulphonamide resistance gene (*sul2)*	bacteria	Sewage treatment plant	Illumina, 454 pyrosequencing	Chimera Slayer (Haas et al., 2011), BLAST	(Huang et al., 2014)
2015	Ampicillin, cephalothin, and kanamycin resistance genes	Bacteria (predominantly *Proteobacteria*)	Aquatic environment (river)	Illumina MiSeq platform, HiSeq2000	Mothur ver. 1.29.2, SILVA reference database ver. 102 (Pruesse et al., 2007)	(Staley et al., 2015)
2015	Tetracycline, quinolone, beta-lactam, aminoglycoside and MLS resistance genes	Bacteria	Waste water treatment plant	IlluminaHiseq2000	MG-RAST, Statistical Analysis of Metagenomic Profiles (STAMP), BLASTX,	(Li et al., 2015a)
2015	Fluoroquinolone resistance genes including DNA gyrase subunit A (*gyrA*), B (*gyrB*), Topoisomerase IV subunit A (*parC*) and B (*parE*), Multidrug resistance efflux pumps, *rpoB,* tetracycline resistance genes	Bacteria, archaea and virus domains	Waste water	Illumina-HighSeq	MG-RAST (Meta Genome Rapid Annotation using Subsystem Technology, v3.2.2; website http://metagenomics.anl.gov; last access 16.06.2014)	(Bäumlisberger et al., 2015)
2015	Multi-drug resistance genes, *bac*A (bacitracin resistance), sulphonamide and aminoglycoside	*Proteobacteria*.	Drinking water	Illumina Hiseq	Galaxy (https://usegalaxy.org/), BLAST, Mothur (http://www.mothur.org)	(Jia et al., 2015)

	resistance genes					
2015	Aminoglycoside, bacitracin, beta-lactam, chloramphenicol, MLS, quinolone, sulphonamide and tetracycline resistance genes	Bacteria	Water, soil, sludge and fecal samples	Illumina Hiseq	BLASTX, MetaPhlAn (Segata et al., 2012)	(Li et al., 2015b)
2015	Antibiotic resistance, secondary metabolite production	*Cyanobacteria*, *Xanthomonadales*, *Myxococcales*, and *Methylococcales* (oxic layer); *Clostridia, Actinobacteria, Geobacter, Anaeromyxobacter*, *Anaerolineae*, and methanogenic archaea (anoxic zone)	Paddy soil	454 GS Junior system (454 Life Sciences) 454 GS FLX instrument (454 Life Sciences)	PRINSEQ (Schmieder and Edwards, 2011b), BLASTN, QIIME (Caporaso et al., 2010) INFERNAL (Nawrocki et al., 2009)	(Kim and Liesack, 2015)
2016	Multidrug resistance, acriflavine, MLS and bacitracin resistance genes	Bacteria	Soil	Illumina Hiseq 2000	MetaPhlAn (Version 2.0), BLASTX	(Xiao et al., 2016)
2016	Sulphonamide, bacitracin, multidrug, and MLS resistance genes	Bacteria and Archaea.	Marine coastal sediments	Illumina Hiseq2000 platform	BLASTn, MEGAN 4	(Guo et al., 2016)
2016	Multidrug transporters vancomycin, tetracycline, bacitracin, beta-lactam and MLS resistance genes	*Bacteroides*	Animal feces, manure, and soil samples collected from dairy farms	Ion Torrent	NextGENe V2.3.4.2, MGRAST	(Pitta et al., 2016)
2016	Aminoglycoside, sulphonamide, tetracycline, MLS, polypeptide and multidrug resistance genes	Bacteria	Pharmaceutical wastewater treatment plants (PWWTPs), sewage treatment plants (STPs)	Illumina Hiseq2500 platform	Galaxy (https://usegalaxy.org/), FASTQ Groomer, BLAST, MG-RAST) (http://metagenomics.anl.gov/),	(Tao et al., 2016)
2016	*tetC*, *tetW* and *sul2*	Bacteria	river	Illumina HiSeq2500	ARG-annot (Gupta et al., 2014), Search Engine for Antimicrobial Resistance (SEAR), Burrows-Wheeler Aligner (Li and Durbin, 2009)	(Rowe et al., 2016)

2016	Florfenicol resistance genes(*cfr, optrA*, and *fexA, floR*)	Bacteria	Soils Adjacent to Swine Feedlots	HiSeq 2500	BLAT (Kent, 2002)	(Zhao et al., 2016)
2016	Tetracycline, sulphonamide, beta-lactam resistance genes	Bacteria (predominantly *Proteobacteria*)	Waste water	IlluminaHiseq2500	Galaxy, MG-RAST	(Tang et al., 2016)
2017	ykfB, ylcT, leuL, rnhA	*Bacteroidaceae* (21.7%), *Veillonellaceae* (22%), and *Fusobacteriaceae* (12.3%), *Escherichia coli*, *Bacillus subtilis*, and *Chryseobacterium sp. StRB126*	Tea leaves (oolong teas)	Illumina Miseq	FASTX-Toolkit (a FASTQ/A shortreads pre-processing tools), Bowtie2, BLASTX, RSEM (RNA-Seq by Expectation-Maximization)	(Huang et al., 2017)
2017	*blaGES* and *blaOXA*	Bacteria	Effluents	Illumina HiSeq2500 (Exeter Sequencing Service, UK)	SEAR (Rowe et al., 2015) BWA-MEM (Li, 2013)	(Rowe et al., 2017)
2017	BepG, MdtC (efflux pump related genes, gyrA, VanA, DHPS	bacteria	Mire	Illumina HiSeq	Prokka (v.1.11), BLAST, HMMer (v3.0)	(Diaz et al., 2017)
2017	Carbapenemases (NDM, VIM, KPC, OXA-48, IMP, OXA-58 and GES types), *tet*(X), *mcr*-1,	*Acinetobacter*, *Proteobacteria*, *Bacteroidetes* and *Firmicutes*	Wastewater, river	Illumina HiSeq2500	Trim Galore, USEARCH (version 8.0.1445),	(Marathe et al., 2017)
2018	bla_{RSA1} and bla_{RSA2} (class A beta-lactamases), *tet*(A), *qnr* gene classes	Bacteria	River sediments	PacBio RS II system	BLASTx, Geneious,	(Marathe et al., 2018)
2018	*ermF, carA, msrA, sul123, gyrA, grlB*	*Alcaligenes*, *Paracoccus*, and *Acidovorax*	Waste water treatment systems	Illumina MiSeq	mothur v1.34.4 (Schloss et al., 2009) UCHIME v4.1 (Edgar et al., 2011)	(Gonzalez-Martinez et al., 2018)

Table 3. Antibiotic resistance genes and bacterial families identified through metagenomics/metatranscriptomics studies undertaken up to 2018 on animals with next-generation sequencing

Year of study	Resistance gene found	Families, Class, Genus and Species found	Source (animal)	Method/Platform used	Bioinformatics tools used	Reference
2011	Multidrug resistance efflux, fluoroquinolone and cobalt–zinc–cadmium resistance genes (14.09%).	Bacteria, archaea, eurkaryotes, viruses, and less than 1% unassigned plasmids.	Cattle faeces	Not specified	MG-RAST,	(Durso et al., 2011)
2012	*ermA, ermB, mefA, tet(32), and aadA*	Bacteria (*E. coli*)	swine intestinal microbiota	Roche/454 GS FLX Titanium platform	BLAST, PAST (Hammer et al.)	(Looft et al., 2012)
2012	multidrug resistance efflux pumps, fluoroquinolone and acriflavin resistance genes	Bacteria (*Firmicutes* predominant)	buffalo rumen	454 Life Sciences technology	MG-RAST	(Singh et al., 2012)
2012	Fluoroquinolone, copper and cobalt–zinc–cadmium, mercury, arsenic, erythromycin and fosfomycin resistance genes	*Escherichia coli, Pseudomonas aeruginosa, Pseudomonas mendocina, Shigella flexneri, Bacillus cereus, Staphylococcus aureus, Klebsiella pneumonia, Staphylococcus epidermidis*	Cattle milk	454 GS-FLX technology	GS Run Browser	(Bhatt et al., 2012)

2014	Tetracycline resistance genes (*tetQ*, *tetO* and *tetM*)	*Barnesiella, Lactobacillus, Bacteroides,* and *Clostridium* XIVa genera	Mouse gut	Illumina Hiseq 2000	MG-RAST, BLASTx	(Guo et al., 2014)
2014	Fluoroquinolone resistance genes, multidrug resistance efflux pumps, methicillin resistance (In *Staphylococci*)	Bacteria, viruses	buffalo rumen	Ion Torrent	MG-RAST, M5NR database (M5 non-redundant protein database, http://tools.metagenomics.anl.gov/m5nr/),	(Reddy et al., 2014)
2015	Beta-lactam resistance genes	Bacteria	Dairy cow feces	Illumina HiSeq	BLASTX, MG-RAST	(Chambers et al., 2015)
2015	Tetracycline, multidrug resistance genes	Bacteria (Bacteroidetes and Firmicutes) dominant	Mouse gut	Illumina Hiseq 2000 (Illumina, USA)	FASTX toolkit tools implemented in GALAXY, MG-RAST, BLAST	(Yin et al., 2015)
2016	*vanB* genes (vancomycin resistance gene)	*Enterococcus spp.*	*Rattus norvegicus* fecal samples	Illumina HiSeq 2000	AdapterRemoval (v1.1) (Lindgreen, 2012), Bowtie2, BLASTn	(Hansen et al., 2016)
2016	17 distinct tetracycline resistance genes. Two new *tet* genes: *tet*(59) (encoding a tetracycline efflux pump) and *tet*(W/N/W)	*Proteobacteria, Firmicutes*	Pig manure	Illumina HiSeq 125-bp pair-end sequencing and PacBio SMRT sequencing	PROKKA pipeline v1.10 (http://dx.doi.org/10.1093/bioinformatics/btu153), BLAST	(Leclercq et al., 2016)
2017	*vanR*, tetracycline, fluoroquinolonone resistance genes, APH(3''), *msbA, drrA, macB, macA*, MFS-1 and *emrB*	*Firmicutes, Actinobacteria, Bacteroidetes,* and *Proteobacteria*	Animal manure	Illumina MiSeq platform	bbduk tool in BBMap (V34: https://sourceforge.net/projects/bbmap/ CD-HIT (Li and Godzik, 2006), MEGAN (Huson et al., 2007), SOAPdenovo Assembler (Li et al., 2010)	(Wang et al., 2017)
2018	Beta-lactamases,	*Firmicutes,*	Baboon	Illumina MiSeq	PARFuMs (Forsberg et al., 2012), Resfams	(Tsukayama et

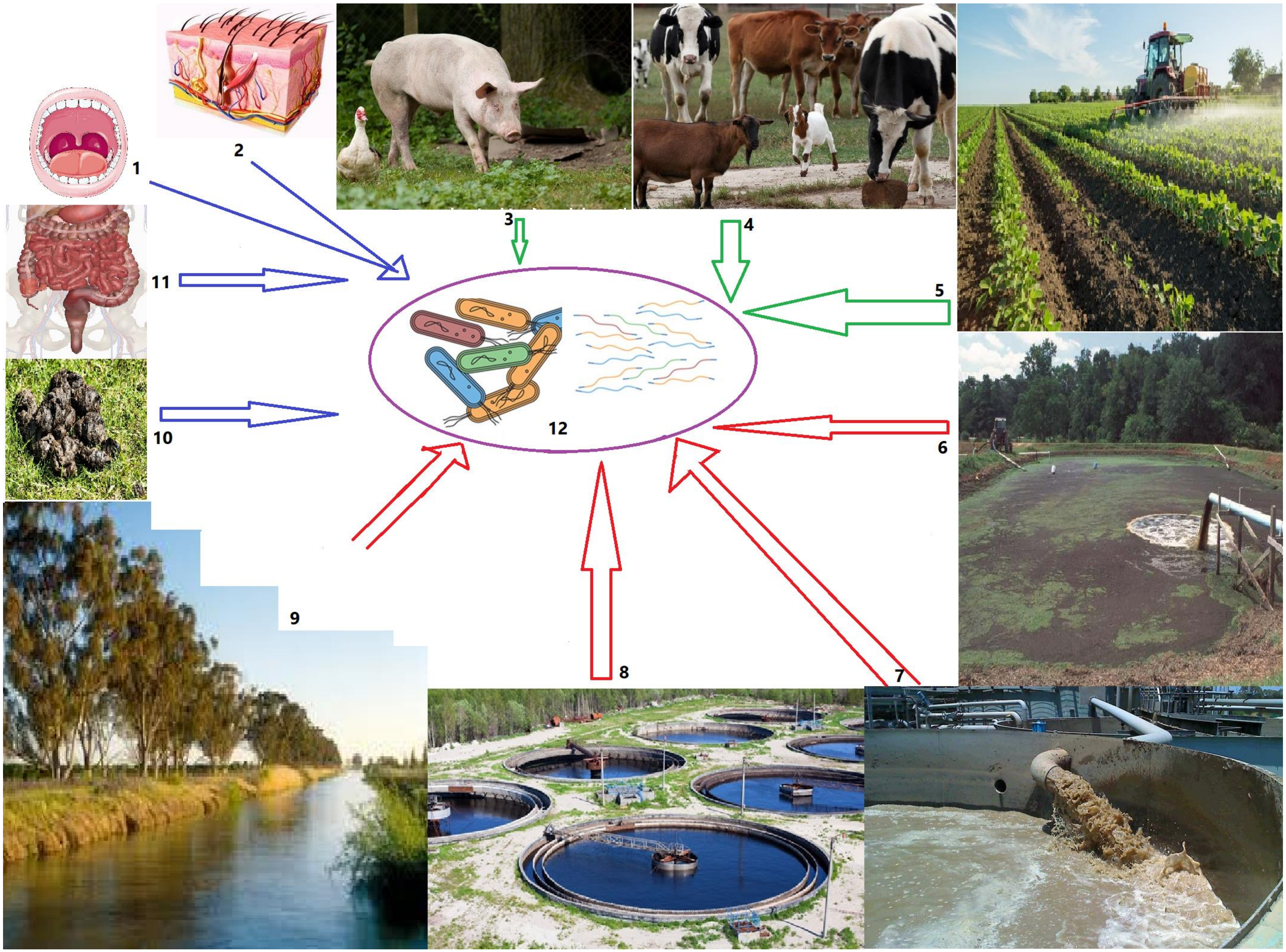
	chloramphenicol acetyltransferase, TetA efflux pump	*Lactobacillales, Actinobacteria*	gut		(Gibson et al., 2015), ShortBRED (Kaminski et al., 2015).	al., 2018)

Table 4 bioactive natural products identified through metagenomics/metatranscriptomics studies undertaken up to 2018

Year of study	Families, Class, Genus and Species found	Source	Natural product discovered	reference
2014	*Entotheonella* spp.	marine sponge *Theonella swinhoei*	Bioactive polyketides and peptides	(Wilson et al., 2014)
2014	*Firmicutes, Proteobacteria, Actinobacteria*	Human (vaginal microbiota)	lactocillin	(Donia et al., 2014)
2017	*Bacteroidaceae* (21.7%), *Veillonellaceae* (22%), and *Fusobacteriaceae* (12.3%), *Escherichia coli*, *Bacillus subtilis*, and *Chryseobacterium sp. StRB126*	Tea leaves (oolong teas)	Antimicrobial peptides	(Huang et al., 2017)
2018	Bacteria	Soil	malacidins	(Hover et al., 2018)

Fig. 1. Sources of metagenomes and bacterial resistomes. Microbiota from which metagenomes are obtained for microbiome studies include the oral cavity (1), skin (2), farm animals (3 and 4), farm crops and soils (5), farm waste and farm effluents (6), industrial effluents (7), sewage treatment plants (8), surface and underground water (9), faeces (10), and intestines (11). Genomic DNA from these sources, called metagenomes (12), are used for sequencing and microbiome analysis. The numbers 1-11 show the various sources for sampling metagenomes. The arrows show the sources of the samples: the green and blue arrows are for clinical (human and animal) sources, and the red-coloured arrow shows environmental sources.

Fig. 2. Sequence-based and functional metagenomics steps. Metagenomes are directly extracted from collected environmental and/or clinical microbiota samples (1) using metagenomic DNA extraction kits and taken through one of two steps: i. direct sequencing with a next-generation sequencer (2) followed by bioinformatic analysis (3 and 9); ii. Exonuclease-mediated excision (4) and cloning into plasmid vectors (5), followed by transformation into host bacteria for multiplication into metagenomic libraries (6). The multiplied host bacteria are grown on selective plates to identify the functions of the various cloned genes (7). DNA from selected colonies on the selective plates are extracted and sequenced (8), followed by bioinformatic analysis (9).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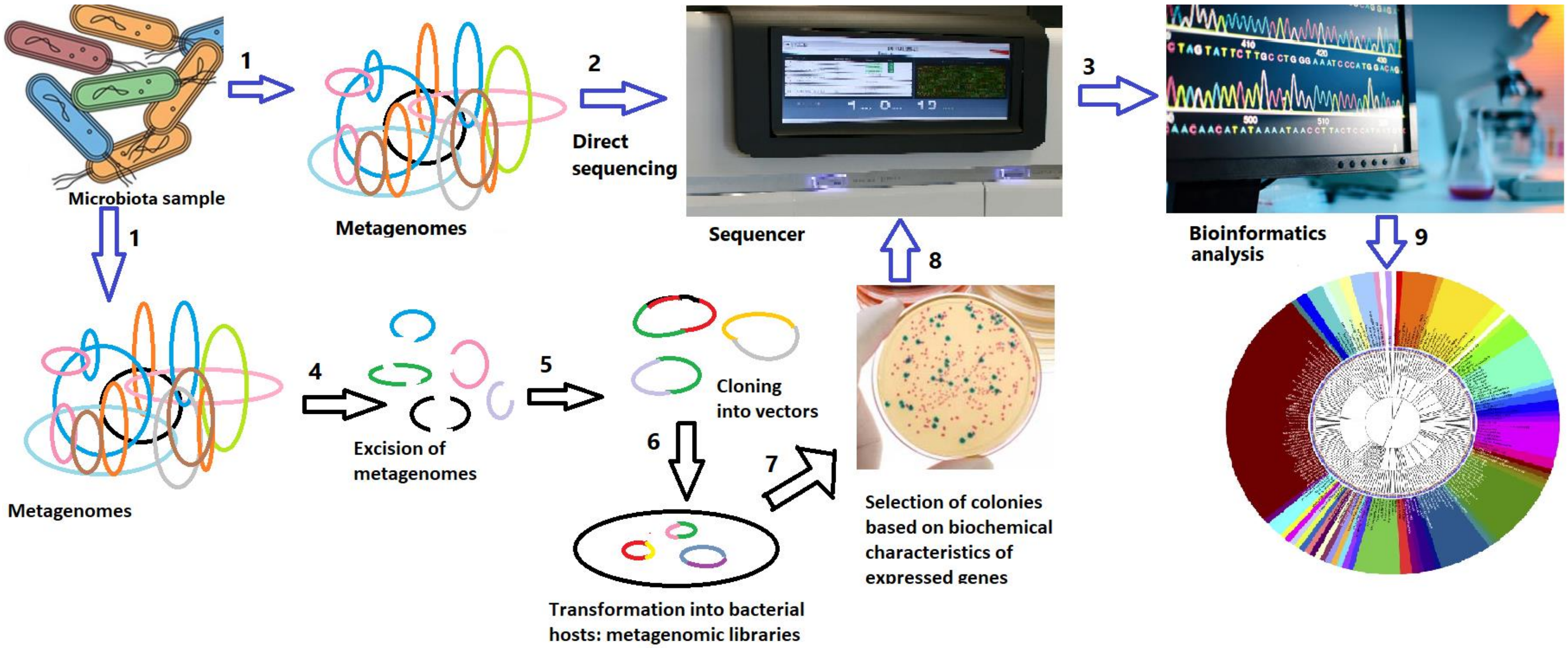
Microbiota sample
1
Metagenomes
2
Direct sequencing
Sequencer
3
Bioinformatics analysis
1
Metagenomes
4
Excision of metagenomes
5
Cloning into vectors
6
Transformation into bacterial hosts: metagenomic libraries
7
Selection of colonies based on biochemical characteristics of expressed genes
8
9