University of Veterinary Medicine Budapest

Doctoral School of Veterinary Science

Aujeszky Aladár Doctoral Programme of Theoretical Veterinary Sciences

A One Health approach study on sources in the process of animal-to-human antimicrobial resistance gene transfer

Doctoral dissertation

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List of abbreviations

ARG Antimicrobial Resistance Gene

AMR Antimicrobial Resistance

AMU Antimicrobial Use

ANI Average Nucleotide Identity

bp Base Pair

CARD The Comprehensive Antibiotic Resistance Database

CDC Centers for Disease Control and Prevention

CI Confidence Interval

CIA Critically Important Antimicrobial

CRT Cyclic Reversible Termination

CRE Carbapenem-Resistant Enterobacteriaceae

CTN Composite Transposon

DNA Deoxyribonucleic Acid

dsDNA Double-stranded Deoxyribonucleic Acid

EBI European Bioinformatics Institute

ECDC European Centre for Disease Prevention and Control

EMA European Medicines Agency

ENA European Nucleotide Archive

ESVAC European Surveillance of Veterinary Antimicrobial Consumption

FFP Nonfermented Food or Fermented Food or Probiotic

FPKM Fragments Per Kilobase per Million fragments

HGT Horizontal Gene Transfer

HIA Highly Important Antimicrobial

HPCIA Highest-Priority Critically Important Antibiotic

HTST High Temperature Short Time

ICE Integrative and Conjugative Element

ID Identification

iMGE Integrative Mobile Genetic Elements

IQR Interquartile Range

Mb Megabases

MGE Mobile Genetic Element

MIC Minimum Inhibitory Concentration

MRSA Methicillin-Resistant *Staphylococcus aureus*

NCBI National Center for Biotechnology Information

NGS Next Generation Sequencing

NMDS Non Metric Multidimensional Scaling

OIE World Organisation of Animal Health

ORF Open Reading Frame

OTU Operational Taxonomic Unit

PCR Polymerase Chain Detection

RGI Resistance Gene Identifier

RNA Ribonucleic Acid

rRNA Ribosomal Ribonucleic Acid

SARS Severe Acute Respiratory Disease

SAVSNET Small Animal Veterinary Surveillance Network

SBL Sequencing by Ligation

SBS Sequencing by Synthesis

ssDNA Single-stranded Deoxyribonucleic Acid

SD Standard Deviation

SMRT Single-Molecule Real-Time (sequencing)

SNA Single-Nucleotid Addition

SRA Sequence Read Archive

UHT Ultra-High Temperature

VCIA Veterinary Critically Important Antimicrobial Agent

VHIA Veterinary Highly Important Antimicrobial Agent

VIA Veterinary Important Antimicrobial Agent

WHO World Health Organization

WGS Whole Genome Sequencing

1. Summary

The spread of antimicrobial resistance (AMR) is one of the most significant medical challenges of the 21st century, ranked among the top 10 global health issues by many international organizations, including WHO (World Health Organization). Although, the constant use of antibiotics inevitably contributes to the growing detection rates of AMR, antimicrobial resistance genes (ARGs) that are primarily responsible for the appearance of AMR and of the negative healthcare effects are the natural elements of the bacterial genomes. These genes can be transferred with horizontal gene transfer. For the execution of horizontal gene transfer, some requirements must be fulfilled, out of which the presence of mobile genetic elements is one of the most crucial. Such mobile genetic elements facilitate the spread of various genes among bacteria. Therefore, in case of ARGs, being accompanied by such elements is a key factor of the spread of AMR. If ARGs, facilitated by mobile genetic elements, enter a pathogenic bacterium, a clinical consequence may be the decrease in the efficacy of antibiotics.

It is essential to know in which interfaces the requirements of horizontal gene transfer are fulfilled and which ARGs can enter the human body from the possible sources. According to recent publications, 70% of the global antibiotic-use can be related to the animal husbandry sector [1]. Such use of antibiotics puts constant selective pressure on bacteria in and around domesticated animals, increasing bacterial ARG assets. In the recent past, great scientific attention has been focused on the static examination of ARGs, namely if their presence or lack was detectable. Nevertheless, this aspect cannot really reflect on the actual public health significance that certain ARGs potentially have. More wide-spectrum studies, covering the dynamic aspects related to ARGs, can help deepening our understanding of ARG spreading potential. Such studies have gained a wider popularity in the past few years, owing to the development of various reliable, widely accessible high-throughput sequencing technologies.
Throughout our research, we aimed to identify sample sources of animal origin that may be involved in the animal-to-human ARG spread routes.

Our methods encompassed bioinformatic analyses based on shotgun next-generation sequencing. ARGs can be transferred from animals to humans in either direct or indirect ways, and we examined the broadest spectrum possible of ARG transfer interfaces. A priority was given to screening samples of animal origin with a great live bacterial content that may be contacted by a wide range of people on a daily basis. Several foods with animal origin are consumed with high viable bacterial counts (raw and probiotic products), while other animal-deriving products may contact the surface of food to be prepared and cooked, thus contributing to the indirect spread of the farm-borne bacterial gene content. Furthermore, close physical contact and common, regular veterinary interventions related to companion animals may also take place in the animal borne spread of AMR (e.g. saliva of dogs). Examinations of interfaces described above were either based on our own sequencing results or on sequencing datasets derived from publicly available data repositories. Analyses were performed with freely accessible softwares incorporated in bioinformatic pipelines.

By our analyses, the taxonomic composition and relative abundance of bacteria (bacteriome), the composition of the resistance gene content (resistome) and the set of mobile genetic el-

ements (mobilome), such as plasmids, phages and integrative mobile genetic elements (e.g. insertion sequences, transposons) were identified and studied.

Based on the results, different interfaces can be evaluated according to their role in AMR spread and to the public health significance that they may be associated with. Our findings may even serve as a baseline for infrastructural and industrial measures to be taken to control the advance of AMR.

1. Összefoglalás

Az antimikrobiális rezisztencia (AMR) korunk orvostudományának egyik legjelentősebb kihívása, melyet több nemzetközi gazdasági, politikai és egészségügyi szervezet, például a WHO (World Health Organization) is napjaink 10 legfenyegetőbb, világszintű közegészségügyi problémája közé sorol. Az AMR térnyeréséhez az antibiotikumok használata nagymértékben hozzájárul, a jelenség kialakításáért és a negatív hatások megnyilvánulásáért azonban az antimikrobiális rezisztenciagének (ARG-k) felelősek. Az ARG-k természetes módon, a mikroorganizmusok egymással folytatott versenye miatt is előfordulnak a mikrobiomban. Mivel ezek a gének baktériumok között horizontális géntranszfer útján átadhatók, ha kórokozó baktériumba jutnak, annak klinikai következménye az antibiotikumok hatékonyságának csökkenése. A horizontális géntranszfer bekövetkezéséhez számos feltételnek teljesülnie kell, melyek közül az egyik legfontosabb a mobilitást elősegítő genetikai elemek jelenléte. A mobilitást elősegítő genetikai elemek különböző mechanizmusok útján lehetővé teszik a gének baktériumok közötti elmozdulását. Ennek megfelelően, ARG-k esetében a mobilitást elősegítő genetikai elemek jelenléte az AMR terjedésének alapvető kellékei.

Annak az ismerete, hogy milyen közegekben teljesülnek a terjedés feltételei, és milyen ARG-k juthatnak az emberi szervezetbe a különböző lehetséges forrásokból, kulcsfontosságú lehet az AMR terjedési útvonalainak pontosabb feltérképezése és megértése, valamint a folyamat ütemének csökkentése érdekében. Egyes források szerint, a világ antbiotikum-felhasználásának 70%-a állattartással összefüggő tevékenységekhez köthető [1]. A jelentős antibiotikumfelhasználás folyamatos szelekciós nyomást gyakorol az állatokban, valamint az állatok közvetlen környezetében található baktériumokra, azok ARG-készletét gyarapítva. Erre a feldúsult ARG-készletre már a közelmúltban is nagy figyelem irányult, a rezisztenciagének statikus, csupán a jelenlétre vagy a hiányra alapozott vizsgálata azonban csak kevéssé alkalmas az egyes minták valós közegészségügyi kockázatának felmérésére. Az olyan nagyszabású, dinamikus tulajdonságokra is kiterjedő vizsgálatok, melyek segítségével pontosabb képet kaphatunk az egyes gének baktériumok közötti potenciális mobilitásáról, csupán az elmúlt pár évben terjedtek el. Ennek hátterében a nagy felbontású szekvenálási technológiák fejlődése és egyre megbízhatóbbá, elérhetőbbé válása állt.

Kutatásaink során olyan állati eredetű forrásokat vizsgáltunk shotgun újgenerációs szekvenálásra alapozott bioinformatikai módszerekkel, amelyekből ARG-k juthatnak az emberi szervezetbe. Az ARG-k közvetett és közvetlen módon is átadódhatnak az állatok és az emberek között, így a kutatások során az AMR terjedésében szerepet játszó közegek legszélesebb spektrumát tártuk fel. A célunk olyan baktériumokat nagy számban hordozó minták felmérése volt, amelyekkel számos hétköznapi szituáció során találkozhatnak az emberek.

Számos állati eredetű élelmiszer nagy mennyiségű élő baktériummal együtt kerül fogyasztásra (nyers és probiotikus termékek). A haszonállattartással összefüggő, feldúsult rezisztenciagénkészlettel rendelkező baktériumok más szállítóközegekben, így például a bélsárban is nagy mennyiségben lehetnek jelen, ez pedig a gazdák, az állatgondozók, valamint a telepek közelében élő lakosság egészségi állapotát is befolyásolhatja. A kedvtelésből tartott állatokkal sok gazda által fenntartott rendkívül szoros fizikai kontaktus, valamint az ezen társállatoknál egyre átlagosabbá, rendszeresebbé váló állatorvosi beavatkozások hozzájárulhatnak az ARG-k álla-

tokról emberre való átjutásához (pl. a kutyák nyála).

A fent leírt közegek vizsgálatát részben saját szekvenálási eredményeinkből, részben pedig nyilvános adatbázisokban tárolt szekvenálási adatokból (NCBI SRA, EBI ENA) kiindulva végeztük. Az elemzésekhez szabad felhasználású, ingyenes szoftvereket használtunk, folyamatosan frissített bioinformatikai pipeline-ok kidolgozása és alkalmazása mellett. Az elemzések során feltárható a vizsgált metagenomikai mintákban található baktériumok rendszertani hovatartozása, egymáshoz viszonyított gyakorisága (bakteriom), a rezisztenciagén-készlet összetétele (rezisztom), valamint az egyes rezisztenciagénekkel kapcsolatban álló mobilitást elősegítő genetikai elemek (mobilom), vagyis plazmidok, fágok és egyéb integratív genetikai elemek (inszerciós szekvenciák, transzpozonok stb.) jelenléte is. Az elemzések alapján következtethetünk a különböző minták szerepére az AMR közvetítésében, valamint a képviselt közegészségügyi jelentőség mértékére. Az eredmények akár különböző az AMR terjedését lassító ipari, vagy infrastrukturális lépések megtervezésében is szerepet játszhatnak.

2. Introduction

The spread of antimicrobial resistance (AMR) is one of the most significant medical challenges of the 21st century, ranked as one of the top 10 global health issues by many international organizations, including WHO (World Health Organization) [2]. Interestingly, antimicrobial compounds and as a defense mechanism, AMR, have always been present as a means of natural rivalry among microorganisms [3]. However, the excess use of antibiotics inevitably contributes to the growing rates of AMR [4]. The primary elements, that are responsible for the appearance of AMR are antimicrobial resistance genes (ARGs). These genes can be transferred between bacteria, either pathogens or non-pathogens, with horizontal gene transfer (HGT). For the execution of HGT, some requirements must be fulfilled, out of which the presence of mobile genetic elements (MGEs) is one of the most crucial [5]. Such MGEs are the major facilitators of the spread of various genes among bacteria. Therefore, considering ARGs, the relatedness to such elements is a key factor for the efficient spread of AMR [6]. If ARGs, facilitated by MGEs, enter pathogenic bacteria, a clinical consequence may be that antibiotics that are commonly used in human and/or animal medicine decrease or lose their efficacy. On one hand, it is therefore essential to identify interfaces that fulfill the requirements for HGT. Furthermore, since the introduction and further elucidation of the term 'One Health' in 2003 [7] and 2022 [8], respectively, the question can be viewed from a broader perspective. Based on the One Health concept, the microbiota of humans, animals and the environment are interchangeable, transferable, and as such, closely related [9]. Thus, from a public health point of view, it is equally important to identify the possible source materials, that may act as interfaces for the flow of bacteria and bacterial genome fragments between various, originally unrelated bacterial populations. According to recent publications, 70% of the global antibiotic-use can be related to the animal husbandry sector [1]. However, the use of antibiotics puts a selective pressure on bacteria. Consequently, bacteria that colonize domesticated animals or their direct environment are more successful in their survival and propagation if their defensive mechanisms are more able to neutralize the effects of antibiotics. Within the pool of bacterial genomes (bacteriome), the set of ARGs is called the resistome [10]. Due to the increasing severity of AMR and the technological advances that facilitate the extensive study of the genomic material, scientific attention has been focused on the examination of the resistome. Studies that solely identify the ARGs that are present in a sample provide a static picture of the resistome without reflecting on the actual public health risk that certain ARGs potentially have. However, covering the ARG-associated elements that facilitate the HGT can deepen our picture. These dynamic aspects can help describing the ARG spreading potential [10]. High-throughput sequencing technologies facilitate the study of both the resistome, and the set of these MGEs, called the mobilome [11].

Throughout our research, we aimed to identify and describe sample sources of animal origin that may be involved in the animal-to-human ARG spread routes using shotgun next-generation sequencing. The sample types were chosen based on screening scientific literature for less studied interfaces. The regularity and the potential of human accessibility to the media were also considered. Thus animal derived or associated food ingredients, foods and other, non-food materials with high potential for regular human access were chosen. Several foods with

animal origin are consumed with high viable bacterial counts (raw and probiotic products). Other farm animal related transfer media, such as nasal and bronchial secretions or feces may also play a significant role in the animal-to-human spread of ARGs, and consequently the health-care expectancies of animal keepers, farmworkers and local residents living in the proximity of domesticated animal husbandry sites. Close physical contact and common, regular veterinary interventions related to companion animals may also take place in the animal borne spread of AMR (e.g. saliva of cats and dogs) [12].

Examinations of transfer media described above were aimed to be achieved by bioinformatic pipelines applied on either our own sequencing results or on sequencing datasets derived from publicly available data repositories such as the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) or the European Nucleotide Archive (ENA) from European Bioinformatics Institute (EBI).

By our analyses, we aimed to describe the taxonomic composition and relative abundance of bacteria (bacteriome), the composition of the resistance gene content (resistome) and the set of MGEs (mobilome), such as plasmids, phages and integrative mobile genetic elements (iMGEs) (e.g. insertion sequences, transposons). Moreover, another objective was the identification and genomic characterisation of pathogens of significant One Health concern.

The studied interfaces were evaluated according to their role in AMR spread and the public health significance that they may pose. Our findings could serve as a baseline for infrastructural and industrial measures to be taken to control the development of AMR.

The studies constituting the PhD work focus on interfaces with One Health significance, and are divided based on the nature of the sample types. Thus, two groups were formed. The first group includes studies of samples deriving from foods and dietary supplements, namely raw milk [13], kefir and yogurt [14], and two separate studies on probiotic dietary products [15, 16]. The second group consists of studies that describe various animal source media, such as canine saliva [17, 18] or pig feces [19].

3. Literature review

3.1 Antimicrobial resistance: background and significance

Antimicrobials – encompassing antibiotics, antivirals, antifungals and antiparasitics – are pharmaceuticals employed for the prevention and treatment of infections in humans, animals and plants. Antimicrobials are classified into various categories based on their target of action, with different antimicrobials affecting different types of microorganisms. For instance, antibacterial agents (antibiotics) are used against bacteria. Antivirals are used against viruses, and antifungals are used against fungi. Lastly, antiparasitics are used against parasites. AMR is defined as the ability of bacteria, viruses, fungi and parasites to change over time and no longer respond to medicines in the previously expected way [20]. The alteration in response may be manifested as either undiminished microbial reproduction rates or the absence of a decline in the quantity of viable microorganisms, that is to say, the absence of elimination of the targeted viable microorganisms [21]. Both of these phenomena can lead to infections that are more difficult to treat and can increase the risk of disease spread, severe illness and death. The development of drug resistance can render antibiotics and other antimicrobial medicines ineffective, thereby complicating the treatment of infections [20]. In light of the aforementioned health concerns, the World Health Organization (WHO) has identified AMR as one of the top 10 global public health challenges confronting humanity. It is particularly alarming that there is a rapid global spread of multi- and pan-resistant bacteria that are resistant against multiple drug classes [20]. In 2019, an estimate of 1.27 million deaths (95% CI = 0.911 - 1.71) were attributable to antibiotic-resistant bacteria, with further 5 million associated deaths (95% CI = 3.62 - 6.57) [22]. In the same year, the WHO identified 32 antibiotics in clinical development that address any of the 15 families of the WHO list of priority pathogens that are the most concerning for public health [23]. However, only six of these were classified as innovative [20]. Thus, it is of utmost importance to comprehend the mechanisms of emergence and spread of AMR to formulate effective control strategies. A pivotal aspect of this endeavor involves the analysis of the genetic determinants of AMR.

ARGs are the genetic elements in the background of AMR [24]. AMR can occur naturally, as a consequence of intrinsic or induced resistance or due to acquired ARGs [25]. Intrinsic AMR is always expressed, independent of previous antibiotic exposure and is based on a natural trait of the species that inhibits the action mechanism of a drug class [26]. An example for intrinsic resistance is reduced permeability of the outer membrane, specifically the lipopolysaccharides, in Gram-negative bacteria [25]. By induced resistance, such as in the case of several multidrug efflux pumps, the genes are naturally occurring in the bacterial genomes, but are only expressed after exposure to an antibiotic [27]. The temporary or permanent acquisition of genetic material that confers AMR is possible through HGT and mutations. HGT is the process by which a DNA strand is passed from one bacterium to another. The transferred gene sequence is nevertheless incorporated into the genome of the recipient bacterium, so that it can later be transferred from bacterial generation to generation [28]. The means of HGT are transformation, transposition, and conjugation. Apart from transformation, by which any gene can be taken up by the bacterium from its environment, the routes of HGT require special active delivery pro-

cesses. These include MGEs. MGEs are sequential structures that are capable of relocation within the bacterial genome or between bacterial cells [29]. By conjugation, which is the most common route for the acquisition of genetic material, cell-to-cell contact provides the opportunity for a copy of a plasmid or an Integrative and Conjugative Element (ICE) to translocate to a recipient bacterium [30–32]. In contrast, transduction negates the necessity for cell-to-cell contact, as in this case bacteriophages act as a conduit for shuttling genes among bacteria [33]. Furthermore, certain ICEs, such as transposons and insertion sequences are capable of the intracellular relocation of genetic elements [32]. Mobility is also promoted by the presence of genes encoding enzymes that are responsible for the reactions required for recombination (e.g. phage integrases, resolvases) [34]. If many ARGs are located in close proximity to some MGEs, it is likely to indicate a higher chance of HGT [35].

Furthermore, as a means of acquisition, bacteria may develop mutations in their own chromosomal DNA with an average mutation rate of 1 for every 10^6 to 10^9 cell divisions. Even though, most of these mutations will be deleterious to the cell [36, 37], a few occasions, such as the ones in the case of mutations by ARGs for drug targets, ARGs for drug transporters, ARGs for drug transporter regulators, and ARGs for antibiotic-modifying enzymes can be beneficial for bacteria [26].

The proteins that are responsible for the mechanisms of AMR are translated from the ARGs. The mechanisms of AMR can be classified in four categories: (1) limiting uptake of a pharmaceutical agent; (2) modifying a drug target; (3) inactivating a drug; and (4) active drug efflux. Intrinsic AMR involves limiting uptake, drug inactivation, and drug efflux. Acquired AMR mechanisms may include drug target modification, drug inactivation, and drug efflux [25]. Examples for the above mentioned mechanisms are as follows. As for the uptake limitation processes, the cell wall of mycobacteria is a lipid-rich and hydrophobic, thus hydrophilic drugs have limited access to these bacteria [38]. Bacteria with no cell wall, such as *Mycoplasma spp.* are intrinsically resistant to drugs that target the cell wall (e.g., β-lactams, glycopeptides) [39]. Drug target modification can occur by drugs that, for instance, target nucleic acid synthesis, such as fluoroquinolones. By these compounds, AMR is expressed due to modifications in DNA gyrase or topoisomerase IV. These mutations induce alterations in the enzyme structure, thereby impeding its capacity to bind to the drugs [40]. Drug inactivation can be caused by the actual degradation of the drug or by the transfer of a chemical group to the drug. As for the first type, the β-lactamases hydrolyzing β-lactams, or the *tetX* mediated hydrolyzation of tetracyclines can be mentioned [41]. By the latter type, the transfer of acetyl, phosphoryl, and adenyl groups is executed by transferases. For instance, the acetylation of aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones, or the phosphorylation and adenylation of aminoglycosides commonly occurs [41–43]. Drug efflux, that is induced by efflux pumps can be found in most bacteria. The five main families of efflux pumps in bacteria are classified based on their structure and energy source: the ATP-binding cassette (ABC) family, the multidrug and toxic compound extrusion (MATE) family, the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), and the resistance-nodulation-cell division (RND) family. These families can be single-component or multi-component that influences the number of ARGs associated to their presence [25]. Initially, the importance of acquired AMR was argued since the mutation rate of bacteria could not keep up with the pace of the discovery of new antibiotics.

However, in reality, HGT plays a very significant and excess role in the quick emergence and spread of AMR [44].
It is therefore of key importance to map the sources that can contribute to the spread of resistant strains, which are the vectors for ARGs, and to determine the extent to which these genetic elements are present in different bacteria. Furthermore, the study of the MGE associations of these ARGs in media that can come into physical contact with humans and animals is also of major public and animal health importance by the assessment of the interspecies spread routes of AMR.

3.2 Genomics in microbial surveillance

3.2.1 Nucleic acid sequencing: importance and methods

Nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), carry genetic information which is read in cells to produce proteins which are responsible for the functions appearing in cells and organisms [45]. To study the genetic information, several nucleic acid sequencing methods have been developed that can help describe the sequence of nucleotides that constitute the RNA or DNA molecules [46, 47]. These sequencing platforms and the associated bioinformatic data analysis can significantly contribute to research in health science, genetics, evolutionary biology, and microbial ecology, among others [47]. At the same time, due to the newer, more efficient sequencing methods and decreasing sequencing prices data generated by sequencing devices is moving beyond the scientific arena into the clinical arena, as an important component of precision medicine [48]. Sequencing platforms are divided into three categories: first-, second- (or next-), and third-generation sequencing. The description and the evolution of the sequencing technologies is described below.

3.2.2 First-generation sequencing

The advent of DNA sequencing can be traced back to 1975, when it was first introduced by Frederick Sanger and Alan Coulson [49]. The pioneering work was subsequently refined and advanced by Sanger in 1977, leading to the development of the first DNA sequencing platform, which came to be known as 'Sanger sequencing' [50]. Sanger sequencing involves the use of DNA polymerase to replicate the DNA fragments of interest in four reaction pools. Each pool contains a mixture of one certain type of 2,3-dideoxy nucleotide triphosphates and the rest of the bases. The incorporation of the 2,3-dideoxy nucleotide triphosphates terminates replication at random positions. The length-based categorization of the freshly replicated fragments (e.g., by electrophoresis) enables the identification of the bases at each position of the sequenced nucleic acid [47, 50]. Nowadays, instead of gel electrophoresis, DNA fragments are separated by size through a long, thin, acrylic-fibre capillary in capillary sequencing machines [51]. The development of this technology has opened up new possibilities for the study of genetic material. At the same time, Sanger sequencing, commonly referred to as first-generation sequencing, has its pros and cons. Despite the high accuracy of the sequencing process and generated sequence length of up to 1000 nucleotides, the high costs and limited capacity of the technology

have led to the development of new methods for the more efficient and cost-effective analysis of genetic information [46, 47].

3.2.3 Next-generation sequencing

At the beginning of the 2000s, new technologies emerged that overcame the limitations of Sanger sequencing. These time- and cost-effective methods constitute the next-, or second-generation sequencing platforms [46, 47]. Based on the below described length of the sequences (reads) that are generated throughout the sequencing process, next generation sequencing (NGS) platforms belong to the group of short-read sequencing technologies. While there are several minor differences among the various subcategories of NGS methods, three common laboratory steps after the DNA extraction provide the basis of each variation. These three basic steps are template generation, and the associated sequencing library preparation, the sequencing, and the base detection followed by base calling. These three wet lab steps are completed with a fourth, dry lab step, namely, the bioinformatic analysis of the sequencing data [52].

By the library preparation, DNA sequences are prepared for the sequencing. At the beginning of this set of processes, the DNA undergoes mechanic, enzymatic or ultrasound-assisted fragmentation. Thus, sequences of a length of thousands of nucleobases are created. Sequencing adapters are then ligated to both ends of these fragments. These adapters are constituted of various parts. These can include the barcode, which gives the possibility to sequence different samples together and other sections, which facilitate the following step, the cluster generation or clonal amplification of the fragments. The clonal amplification is necessitated for stronger signals and consequently, more accurate base detection. Clonal templates can be generated with bead-based, solid phase and DNA nanoball-based amplification methods. By the bead-based process, the adapters are the complements of a bead-ligated oligonucleotide sequence. After being physically attached to the complements, the templates are emulsion polymerase chain reaction (PCR) amplified. Thus millions of clonal templates can be generated on each bead. The beads are evenly distributed on a solid surface and emulsions maintain template isolation during amplification [53]. By solid phase DNA amplification the thermocycling step is performed with DNA primers that are 5'-end covalently attached to the solid support. The single-stranded templates are then attached to these primers. Setting the template concentration facilitates the formation of precisely localized, non-overlapping clusters [53, 54]. Two methods, template walking [53] and bridge amplification belongs to this category [53]. Bridge amplification is utilized by Illumina, the most widely used NGS platform [46, 53]. During the DNA nanoball generation, no solid surface attachment is required. DNA templates are circularized, adapter ligated and amplified by the rolling circle method. Afterwards, DNA nanoballs are evenly spread on a special surface during hybridization [53]. The template amplification generates a set of cloned sequences that are gathered in separate clusters. Each cluster acts like an individual sequencing center point. By the bead-based and the solid phase amplification the isolation of the clusters is granted. In high-throughput sequencing devices this step is performed on flow cells, where the proper isolation of the clusters can be performed in high cluster density [53].

The next phase of all NGS methods, sequencing, can be ligation-based (SBL) or synthesis-based (SBS). By SBL, a fluorescently labelled probe hybridizes to its complementary sequence, which is located adjacent to the primed template. The dye-labelled probe attaches to the primer following the addition of DNA ligases. Non-ligated probes are then washed away. Ligated probes are identified using fluorescence imaging. This cycle is normally repeated using cleavable probes or by removing and hybridizing a new primer to the template [52]. By SBS, DNA-polymerases are used to build a complement strand. Since the nucleobases are fluorescently marked, their addition to the sequencing can be detected. The two major types of SBS are cyclic reversible termination (CRT) and single-nucleotid addition (SNA). Similarly to Sanger sequencing, fluorescently-marked chain terminating 2,3-dideoxy nucleotide triphosphates are joined to the building strand. The linking is reversible, thus the cycle can be restarted after every base. CRT platforms are of an outstanding accuracy level of 99,5% [53]. The CRT method is utilized in Illumina platforms [46]. Another popular sequencing technology branch, pyrosequencing is based on SNA. SNA approaches rely on the detection of the incorporation of dye-labelled normal deoxynucleotides into an elongating strand. Thus, each of the four nucleotides are added to the reaction iteratively without blocking the chain elongation [53].
Base calling at each position of the sequence occurs after a process where millions of fluorescent signals are detected simultaneously. Thus, unlike Sanger sequencers, NGS platforms perform sequencing in a massively parallel manner. During either of the sequencing processes, after the base detection, single-end or paired-end reads are generated. Single-end reads are sequenced from one direction, while by paired-end reads, the template strand is sequenced from the direction of both ends, thus a forward and a reverse read are generated with or without an overlap [53]. Although, read length is normally between 35-700 base pairs (bp), with an length of 100-300 bp on Illumina platforms and the a sequencing error rates are very low [46]. Within the doctoral dissertation, the presented datasets were generated using Illumina sequencers.

3.2.4 Third-generation sequencing

Third generation sequencing platforms also include high-throughput, massively parallel systems that require library preparation (DNA fragment end-preparation, adapter and barcode ligation) steps after the DNA extraction and before the sequencing. However, these technologies do not require template amplification steps [46, 55]. Two major long-read sequencing device families dominate the area: nanopore sequencers from Oxford Nanopore Technologies and the single-molecule real-time (SMRT) sequencers from Pacific Biosciences [56]. Nanopore sequencers measure the ionic current fluctuations and associate the base sequences according to the fluctuations of the electric current when single-stranded nucleic acids pass through biological nanopores that are integrated in an electrically resistant membrane [57]. SMRT sequencers detect fluorescence marks that represent the addition of nucleobases by a polymerase that is tethered to the bottom of a tiny well [58]. Thousands of bps long reads can be generated on these platforms, with the longest ever read sequenced on the nanopore platform reaching the length of 2.3 Mb [59]. Basecalling accuracy of reads produced by these technologies has initially underperformed the second-generation sequencers, but have increased

recently, and the raw base call error rate is claimed to have been reduced owing to advances in the library preparation chemistries and basecalling algorithms to <5% for nanopore sequences [60] and <1% for SMRT sequencers [61].

3.2.5 Metagenomics

The study of all genomes and genome fragments in a sample is called a metagenomic analysis. A metagenome contains both eukaryotic and prokaryotic sequences that can derive from the sampled host organism, the microorganisms colonizing the host, or from the environment. The metagenome analysis of a sample requires high-throughput sequencing methods that are able to generate sufficient sequencing depths and data volumes to provide an overview of the studied sample [62]. Metagenomic studies can be performed after 16S or 18S ribosomal RNA (rRNA) sequencing or after shotgun sequencing. The first sequencing method, 16S or 18S rRNA sequencing is targeted for a specific region of the genomes. It is used to identify and classify microorganisms by analyzing their rRNA genes. The 16S rRNA gene is specific to prokaryotes (bacteria and archaea), while the 18S rRNA gene is used for eukaryotic microorganisms, such as fungi and protists [62–65]. By the shotgun sequencing approach the genomes are fragmented at random positions, and each fragment is sequenced with a high-throughput method. Afterwards, the sequences can be reassembled during the bioinformatic analysis steps. Shotgun sequencing is not a targeted method, thus all the genetic material that is present can be processed. Metagenomes can be further classified in subcategories, such as the bacteriome, virome, resistome, virulome or mobilome. These contain all bacterial or viral genomic particles, ARGs or MGEs of a sample, respectively [62]. Metagenomic analyses are of particular importance in case of microorganisms that are difficult to culture by traditional methods [66, 67].

3.2.6 Major bioinformatic analysis steps

After sequencing, the base sequence of the reads is stored in special text files, called fastq files [46]. Besides the sequences, fastq files contain records regarding the sequencing quality of each position predicted by the sequencing platforms [68]. If multiple samples were sequenced together, the reads have to be regrouped based on the barcodes (demultiplexing) [69]. This is followed by the specific bioinformatic analysis steps that correspond to the aims of the studies [70]. Within our studies these steps always included the quality analysis and the taxon classification of the reads and the assessment of the bacteriome, the resistome and the mobilome.

3.3 The One Health approach

According to the WHO definition, One Health is a multidisciplinary approach that recognizes the interconnectedness of human, animal, and environmental health and aims to sustainably balance and optimize the health of people, animals and ecosystems [71]. The term was first used in 2003-2004, due to the emergence of severe acute respiratory disease (SARS) and subsequent spread of avian influenza H5N1 that were both linked between human and animal health [72]. Nevertheless, the application of the One Health paradigm extends beyond the

scope of interspecies or inter-environmental transfer of complete microorganisms. The overuse of antimicrobials in various sectors, including human, animal, and agricultural sectors, as well as the dissemination of microbial genomic fragments, such as ARGs, are also encompassed by the One Health approach [12].

3.4 Interfaces with One Health significance associated to the doctoral study

Any media can be of One Health significance if it can come in direct physical contact with any two of the following microbial habitats: humans, animals or environmental sources. In the followings, only interfaces that can interconnect humans and animals are presented as the studies included in the doctoral work focused on these platforms.

3.4.1 Foods and dietary supplements

3.4.1.1 Raw milk

The microbiota of livestock products may come into direct contact with the human bacteriota, either during the processing steps or during the consumption of these products. The antibiotics employed for the control of disease in farm animals frequently possess chemical structures that are identical or similar to those of human pharmaceuticals. Consequently, there is a risk that antibiotic resistance genes (ARGs) accumulated as a response to the high amount of antibiotics used in livestock farming may be transmitted to the human microbiota through animal products. The dissemination of ARGs may further diminish the efficacy of antibiotic therapies and facilitate the emergence of novel multidrug resistant strains. Fortunately, food processing frequently incorporates heat treatment steps that effectively eliminate the majority of bacteria. Consequently, the role of active DNA-export mechanisms between the intestinal and the nutriment's bacteriome is diminished [73].
Raw milk is a product sold unprocessed; thus the presence or the grade of heat-treatment steps are upon the decision of consumers. In addition to this, the consumption of non-heat-treated raw milk justified by its favorable health effects is nowadays commonly set as a trend in the developed countries [74, 75].
To the best of our knowledge, no previous study has investigated the possible presence of ARGs in raw milk. Furthermore, we have found no data on the raw milk resistome at the time of the study's execution. Only studies relying on traditional methods and the antimicrobial susceptibility testing of isolates were available [76–80].

3.4.1.2 Probiotic dairy products

Bacteria appear in the newborn body right from birth [81], and later on, their invasion continues from the environment, from other humans and animals, or raw or processed food [82, 83]. Bacteria reaching our gut through alimentation may share functional ARGs either with saprophytes or with pathogens in their physical proximity due to HGT. Therefore, popular probiotic products (such as yoghurt and kefir), have the potential to allow encounters between their bacterial

strains and those in the consumer. Yoghurt and kefir are probiotic foods with minor differences in their processing steps. Yoghurt is fermented with bacteria, whereas the production of kefir requires fungi in addition. They have both been present in the human diet for a long time and still stand their ground in today's demanding, health-conscious society. Nevertheless, besides the health benefits, consumption of probiotic food may have an adverse effect. Along with the multiplication of bacteria during the fermentation process, the bacterial resistome can also grow. If the intake of probiotic products occurs alongside the right triggers, a higher possibility of HGT is provided in the human gut. Thus, the examination of the diversity of the ARG content of kefir and yoghurt products, their grains and bacterial strains is essential.

3.4.1.3 Other probiotic products

Probiotics and probiotic products have gained a worldwide reputation and popularity in our everyday lives irrespective of cultural background, geographic location or social standards. Beneficial health effects assigned to probiotics have been reported in several studies [84]. What these studies have in common is that they state that microbes carried in probiotics must remain present in the intestinal tract for a shorter or longer period of time to exert the expected beneficial effects. Nevertheless, the success of colonization depends on several factors, thus the certainty of its realization varies from individual to individual [85]. Recently, however, the possibility of some unfavorable or sometimes even adverse effects of probiotic consumption have also been raised [86]. Recognizing that ARGs from probiotics may enter into the human body by food (e.g., probiotic products), studies on the genetic characteristics of microorganisms (including bacteria) used in the food chain have been recommended by European Food Safety Authority (EFSA) in recent years [87, 88].
According to numerous studies, [89–93], non-fermented and fermented foods, or probiotic dietary supplements contain a considerable number of ARGs, some of which are mobile. By probiotics with supposedly mobile ARGs, the likelihood of gene transmission to other bacteria in the intestinal tract increases, as these strains can even colonize the digestive system. Thus, gaining insight into the frequency and mobility of ARGs in probiotics for human or animal consumption is needed. Currently, the few accessible data on probiotic ARG mobility originate from studies with diverse methodologies [92, 94–98]. Therefore, the analysis of NGS data sets of different probiotics and probiotic-isolated and probiotic-associated bacterial strains is described in two subsequent studies in the thesis. The first study (**A**) focuses on probiotic products (metagenomes) and strains for human consumption, while the second study (**B**) highlights the AMR properties of a rich set of commonly-used probiotic strains from the Bifidobacteriales and Lactobacillales order.

3.4.2 Other animal source interfaces

3.4.2.1 Canine saliva

Human health antimicrobial use has been overshadowed for years by farm animal mass medication, although, this tendency has recently changed in some parts of the world [99]. While the appearance and advance of AMR and, as an underlying cause, the enrichment and transmis-

sion of ARGs in antibiotic-dense environments such as intensive animal production farms, is a well-examined phenomenon [100], the spread of AMR may also derive from other animal-borne routes.

Over the past decades, the number of companion animals has been tendentiously and steadily rising [101]. Between 2000 and 2017, the number of dogs in the United States escalated from 68 million to 89.7 million [102]. In total, 67.9% of all households in the U.S. were associated with the ownership of various pet species and 48% of all with dogs in 2016 [101]. In the years 2019-2020, 50% of the U.S. population owned a dog [103]. The coronavirus disease (COVID-19) pandemic outbreak has resulted in elevated companion animal acquisition rates, albeit often followed by retention or replacement [104, 105]. In addition to the popularity of keeping small animals, the quality of human–pet bonds has also changed. According to the survey of the American Veterinary Medical Association, 70% of pet owners consider their pets as family members, 17% as companions and 3% as property [106]. The role of pets can principally be defined as social companionship. Nowadays, having physical proximity is very common for pet-owner co-existences; pets often sleep together with their owners and lick their face or wounds [107]. Unfortunately and unsurprisingly, with such high dog numbers, the occurrence of dog bites is also common. Between 2001 and 2003, approximately 4.5 million dog bites were registered yearly in the United States, 19% of which necessitated medical intervention [108]. In the years 2005-2013, an average of 337,103 dog bite injuries were treated at U.S. emergency departments [109], although dog bites in general are under-reported [110]. In English hospitals, the number of dog bite admissions rose from 6.34 (95% CI 6.12–6.56) in 1998 to 14.99 (95% CI 14.67–15.31) admissions per 100,000 inhabitants in 2018 [111]. In parallel, a study on the database of the International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10) reveals that dog attack fatalities (excluding deaths caused by post-attack infectious complications) have been constantly rising between 1995 and 2016. Incidence rates reached 0.009 per 100,000 inhabitants in Europe, 0.011 in the U.S., 0.007 in Canada and 0.004 in Australia [112]. Interestingly, 3 of 5 bites are executed by family dogs, which is more common than attacks by strays [113].

The modern mindset of providing regular veterinary healthcare services to our pets and keeping them in our closest surroundings may contribute to the interspecies transmission of AMR. Several studies have already turned their attention to the role of companion animals in the headway of AMR [114–119]. Nevertheless, the significance of the direct pet-borne AMR spread route has been given less attention when compared with the rather indirect, mostly food-transmitted farm-animal-associated route. After dog bites or close encounters with saliva from dogs that often even undergo veterinary treatments, and thus may carry bacteria with a possibly enriched ARG content, resistant bacteria may be introduced to the human body, and later the HGT of AMR determinants may be exchanged with the host bacteriota. Therefore it is profitable to reveal the ARG content of canine saliva samples, attach the ARGs with the bacterial species that they derive from and report the ARGs' spreading capabilities to weigh the above-mentioned phenomenon.

3.4.2.2 Pig feces

Antibiotics have been used in pig farms for decades as treatment options for bacteria and as growth promoter in sub-therapeutic doses. The latter means of use has been banned in the European Union, along with prophylaxis and metaphylaxis [120]. Nevertheless, the amount of antibiotics used in the animal production sector correlates with the abundance of ARGs in bacteria [121]. Higher levels of ARGs may be associated with a higher potential for HGT through direct physical contact with animals and through indirect contact with animal-borne materials in the food chain. The study of AMR in food-producing animals and their environment commonly employs the targeted testing of specific bacterial genes (e.g., MRSA, *Escherichia coli*) and the determination of phenotypic resistance of cultivable species through disc diffusion or broth dilution methods. However, shotgun NGS provides a more comprehensive understanding of the overall microbial profile. Insights into the microbial genomic properties of the feces of sows from a Hungarian domestic pig farm, with a particular focus on ARGs and the diversity of bacteria present can help assess the current situation as a means of antimicrobial surveillance.

4. Materials and methods

The methods are divided in 'Wet lab processes' and 'Dry lab processes'. By the 'Wet lab processes', the presentation of the sample collection methods is followed by a description of the DNA extraction, library preparation and sequencing for each sample type. By 'Dry lab processes', the genomic data collection, bioinformatic analysis and statistical testing steps are described.

4.1 Wet lab processes

This section describes the sample collection methods and wet lab steps by the studies where they were necessitated. The selection process for the samples that were involved in the studies and were downloaded from sequence repositories and thus, did not require any wet lab steps are described by the 'Dry lab processes'.

4.1.1 Foods and dietary supplements

4.1.1.1 Raw milk

Two samples of raw milk were procured from public markets in Budapest and Szeged. The Budapest sample (Sample A) was obtained from a dairy farm with over 250 dairy cattle, while the Szeged sample (Sample B) was sourced from a farm with less than 50 dairy cattle. Before the laboratory procedures, the milk samples were stored frozen. 120 mL of raw milk was centrifuged at 10.000 g for 10 min. Total DNA was extracted from the pellet using the ZR Fecal DNA Kit from Zymo Research. Paired-end fragment reads (2 × 150 nucleotides) were generated using the TG NextSeq 500/550 Mid Output Kits v2 sequencing kit with an Illumina NextSeq sequencer.

4.1.1.2 Probiotic dairy products

Besides the presequenced datasets that were included in the study, one kefir and one yoghurt starter culture were shotgun sequenced (PRJNA644779) within the study. Total metagenome DNA of kefir (k_g_04) and yoghurt (y_g_01) samples were extracted using the UltraClean Microbial DNA Isolation kit from MoBio Laboratories. The quality of the isolated total metagenomic DNA was checked using an Agilent Tapestation 2200 instrument. The DNA samples were used for *in vitro* fragment library preparation. In vitro fragment libraries were prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). Read numbers were the following: 22 044 496 and 20 895 112 for kefir and yoghurt, respectively.

4.1.1.3 Other probiotic products

The methods for the two different studies on probiotic products and probiotic strains (referred to as **study A**) and probiotic strains (referred to as **study B**) are described separately below. By each study, the FAO/WHO definition of probiotics was followed by the sample selection,

that is, living microorganisms, that confer a health benefit to the host when administered in adequate amounts [122]. One probiotic capsule was shotgun sequenced (PRJNA644361) for **study A**. Total metagenome DNA of the probiotic capsule sample was extracted using the UltraClean Microbial DNA Isolation kit from MoBio Laboratories. The quality of the isolated total metagenome DNA was checked using an Agilent Tapestation 2200 instrument. The DNA sample was used for in vitro fragment library preparation. In vitro fragment library way prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina. Paired-end fragment reads were generated on an Illumina NextSeq sequencer using TG NextSeq 500/550 High Output Kit v2 (300 cycles). In **study B** no wet lab processes were performed by the authors. The study was performed based on datasets acquired from NCBI.

4.1.2 Other animal source interfaces

4.1.2.1 Canine saliva

No wet lab processes were performed by the authors. The study was performed based on datasets acquired from NCBI.

4.1.2.2 Pig feces

The freshly defecated feces was collected from the compartment of four suckling sows born within five days of sampling. The pool of these DNA contents was extracted using a Zymo Research ZR Fecal DNA Kit. Paired-end reads were generated using an Illumina NextSeq sequencer.

4.2 Dry lab processes

4.2.1 Foods and dietary supplements

4.2.1.1 Raw milk

Primary data analysis (base-calling) was carried out with bcl2fastq software (v.2.17.1.14, Illumina). Quality based filtering and trimming was performed by Adapterremoval [123], using 15 as a quality threshold. Only reads longer than 50 bp were retained. *Bos taurus* genome (ARS-UCD1.2) sequences as host contaminants were filtered out by Bowtie2 [124] with *very-sensitive-local* setting minimizing the false positive match level [125]. The remaining reads were taxonomically classified using Kraken2 ($k = 35$) [126] with the NCBI non-redundant nucleotide database [127]. The taxon classification data was managed in R [128] using functions of package phyloseq [129] and microbiome [130]. For further analysis, the reads assigned to Bacteria was used only [131]. Core bacteria were defined as the relative abundance of agglomerated counts at class level above 0.1% at least one of the samples. By metaSPAdes [132] the preprocessed reads were assembled to contigs, with the automatically estimated maximal $k = 55$. From these contigs having a shorter length than the shortest resistance gene of the Comprehensive Antibiotic Resistance Database (CARD) were discarded [133, 134]. The ARG content of filtered contigs was analyzed with Resistance Gene Identifier (RGI) v5.1.0 and CARD v.3.0.6

[134, 135]. Contigs harbouring ARG identified by RGI with perfect or strict cut-off were preserved and classified by Kraken2 on the same way as was described above. The plasmid origin probability of the contigs was estimated by PlasFlow v.1.1 [136]. To identify possible further MGE homologs the predicted protein sequences of contigs were scanned by HMMER [137] against data of PFAM v32 [138] and TnpPred [139]. Following Sáenz et al. [131] from the hits with lower than E 10^{-5} the best was assigned to each predicted protein within the distance of 10 ORFs. The MGE domains coexisting with ARGs were categorized as phage integrase, resolvase, transposase or transposon.
All data are publicly available and can be accessed through the PRJNA591315 from the NCBI Sequence Read Archive (SRA).

4.2.1.2 Probiotic dairy products

Primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina). The details of analyzed samples are listed in Table 1. The rest of the short read datasets were obtained from NCBI SRA repository. A query was performed in SRA to find kefir or yoghurt related shotgun sequenced samples. As a result of this search further 33 datasets originating from 8 BioProjects were selected for the study. Except for the samples of BioProjects PRJEB15432 all others came from paired-end runs. The downloaded short reads originated from BioSamples of kefir grains (n=4), kefir products (n=15), kefir strains (n=7), a yoghurt grain (n=1) and yoghurt products (n=5). Of the collected projects, a peer-reviewed publication is available for the PRJNA222257 [140], PRJEB15432 [141] and PRJEB30083 [142]. For all other samples, the only accessible metadata were the attributes in SRA. In PRJEB15432 Walsh et al.[141] followed the microbial changes during the fermentation process of kefir. They used full-fat pasteurized milk inoculated by three different grains (Fr1, Ick, and UK3 from France, Ireland and United Kingdom, respectively). The pasteurized milk (with three replications) and grains (without replication) were sampled at hour 0. In the fermentation from kefir at hour 8 (without replication) and hour 24 (with three replications), further specimens were taken.
Quality based filtering and trimming was performed by Trimmomatic [143], using 15 as a quality threshold. Only reads longer than 50 bp were retained. The remaining reads were taxonomically classified using Kraken2 ($k = 35$) [126] with the NCBI non-redundant nucleotide database [127] with two different confidence setting. The first run was performed with the default settings to select all possible bacterial reads. The following taxon classification was performed with the `--confidence 0.5` parameter to get more precise species level hits. The taxon classification data was managed in R [128] using functions of the package phyloseq [129] and microbiome [130]. For further analysis, only reads assigned to Bacteria Kingdom was used [131]. The preprocessed bacterial reads were assembled to contigs by MEGAHIT (v1.2.9) [144] using default settings. From the contigs, all possible open reading frames (ORFs) were gathered by Prodigal [145]. The protein translated ORFs were aligned to the ARGs of database CARD v.3.0.9 [134, 135] by Resistance Gene Identifier (RGI, v5.1.0) with Diamond [146]. The ORFs classified as perfect or strict were further filtered with 90% identity and 60% coverage. The RGI uses the nudged notation for ARG annotation hits where identity reached or exceeded the 95% threshold. However, the length of the genes was below the predetermined cut-off value. It is

Table 1.: The list of analyzed samples obtained from NCBI SRA. In the unified names of the samples the first character corresponds to the type of the sample (k and y, kefir and yoghurt, respectively), the second character comes from the first letter of the source (g, p and s for grain, product and strain, respectively), while the last tag is a sequence number. The last column shows the available attribute data of the biosamples.

Sample ID	BioProject	Run	Type	Source	Sample
k_g_01	PRJEB15432	ERR1653138	kefir	grain	Fr1 grain
k_g_02	PRJEB15432	ERR1653139	kefir	grain	Ick grain
k_g_03	PRJEB15432	ERR1653140	kefir	grain	UK3 grain
k_g_04	PRJNA644779	SRR12171332	kefir	grain	kefir seed culture
k_p_01	PRJEB15432	ERR1653129	kefir	product	UK3, 8 hours
k_p_02	PRJEB15432	ERR1653130	kefir	product	Fr1, 24 hours (replicate 2)
k_p_03	PRJEB15432	ERR1653131	kefir	product	Ick, 24 hours (replicate 2)
k_p_04	PRJEB15432	ERR1653132	kefir	product	UK3, 24 hours (replicate 2)
k_p_05	PRJEB15432	ERR1653135	kefir	product	Fr1, 24 hours (replicate 3)
k_p_06	PRJEB15432	ERR1653136	kefir	product	Ick, 24 hours (replicate 3)
k_p_07	PRJEB15432	ERR1653137	kefir	product	UK3, 24 hours (replicate 3)
k_p_08	PRJEB15432	ERR1653141	kefir	product	Fr1, 24 hours (replicate 1)
k_p_09	PRJEB15432	ERR1653142	kefir	product	Ick, 24 hours (replicate 1)
k_p_10	PRJEB15432	ERR1653143	kefir	product	UK3, 24 hours (replicate 1)
k_p_11	PRJEB15432	ERR1653145	kefir	product	Fr1, 8 hours
k_p_12	PRJEB15432	ERR1653146	kefir	product	Ick, 8 hours
k_p_13	PRJNA288044	SRR2082409	kefir	product	KEFIR.shotgun
k_p_14	PRJNA388572	SRR7287342	kefir	product	Metagenome from probiotic beverage K03
k_p_15	PRJNA388572	SRR8282406	kefir	product	Metagenome from probiotic beverage K02
k_s_01	PRJDB4955	DRR064132	kefir	strain	*Lactobacillus parakefiri JCM 8573*
k_s_02	PRJNA222257	SRR1151211	kefir	strain	*Lactobacillus kefiranofaciens subsp. kefiranofaciens DSM 5016*
k_s_03	PRJNA222257	SRR1151212	kefir	strain	*Lactobacillus kefiranofaciens subsp. kefirgranum DSM 10550*
k_s_04	PRJNA222257	SRR1151213	kefir	strain	*Lactobacillus kefiri DSM 20587*
k_s_05	PRJNA222257	SRR1151226	kefir	strain	*Lactobacillus parakefiri DSM 10551*
k_s_06	PRJNA635855	SRR11965732	kefir	strain	*Acetobacter syzygii str. K03D05*
k_s_07	PRJNA635872	SRR11966381	kefir	strain	*Lactobacillus plantarum K03D08*
m_01	PRJEB15432	ERR1653133	milk	milk	0 hours (replicate 1)
m_02	PRJEB15432	ERR1653134	milk	milk	0 hours (replicate 2)
m_03	PRJEB15432	ERR1653144	milk	milk	0 hours (replicate 3)
y_g_01	PRJNA644779	SRR12171305	yoghurt	grain	yoghurt seed culture
y_p_01	PRJEB30083	ERR2982980	yoghurt	product	Yoghurt-A
y_p_02	PRJEB30083	ERR2982981	yoghurt	product	Yoghurt-B
y_p_03	PRJEB30083	ERR2982982	yoghurt	product	Yoghurt-C
y_p_04	PRJEB30083	ERR2982983	yoghurt	product	Yoghurt-D
y_p_05	PRJEB30083	ERR2982984	yoghurt	product	Yoghurt-E

important to indicate if a predicted resistance gene is a nudged hit as they are more prone to false positive results. Thus, the findings were presented including and excluding the nudged hits. For the analysis of ARG abundance changes during kefir fermentation, only ARGs with maximal coverage greater than 75% in samples taken at different time points were included. Contigs harbouring ARGs were classified by Kraken2 using the NCBI RefSeq [147] complete bacterial genomes database. In keeping with Hendriksen at al. [148] the ARG abundance was

expressed as fragments per kilobase per million fragments (FPKM) [149] of contigs containing ARGs. For the ith contig $FPKM_i = q_i/(l_i \times Q) \times 10^6$, where q_i is the number of reads that mapped to the contig, l_i is the length of contig and Q is the total number of mapped reads. To calculate q values, all bacterial reads were aligned to the contigs by Bowtie2 [124] with the parameter of `-very-sensitive-local`. To identify possible further MGE homologs the predicted protein sequences of contigs were scanned by HMMER [137] against data of PFAM v32 [138] and TnpPred [139]. Similar to Sáenz et al. [131] from the hits with lower than E 10^{-5} the best ones were assigned to each predicted protein within the distance of 10 ORFs. The MGE domains coexisting with ARGs were categorized as phage integrase, resolvase, transposase or transposon. The plasmid origin probability of the contigs was estimated by PlasFlow v.1.1 [136]. According to the ARG abundance of the samples, a dissimilarity matrix was calculated using the Bray-Curtis index [150] with package vegan [151]. With the same library and the same matrix, a permutational multivariate analysis of variance was applied to quantify the associations between the dissimilarity and independent variables (type, source, BioProject). For the visualization of the sample distances based on this matrix, a principal coordinate analysis (PCoA) was performed with package ape [152]. The relationship between the detected ORF length and the sequencing depth was explored using a linear model. All analyses and plotting were done in R-environment [128].

4.2.1.3 Other probiotic products

The methods for two different studies (**A** and **B**) on probiotic products and/or probiotic strains are described separately below.

By **study A**, primary data analysis (base-calling) was carried out with Bbcl2fastq software (v2.17.1.14, Illumina). Furthermore, we selected freely available samples from the sequencing of probiotic products for human consumption or from bacterial strains isolated from such products from NCBI SRA repository. The details of analysed samples are listed in Table 2 (see page 26).

The quality based filtering and trimming of the raw short reads was performed by TrimGalore (v.0.6.6, `https://github.com/FelixKrueger/TrimGalore`, accessed on 22/03/2021), setting 20 as a quality threshold. Only reads longer than 50 bp were retained and taxonomically classified using Kraken2 (v2.1.1) [126] and a database created (24 March 2021) from the NCBI RefSeq complete archaeal, bacterial and viral genomes. For this taxon assignment, the `-confidence 0.5` parameter was used to obtain more precise species level hits. The taxon classification data was managed in R [128] using functions of the packages phyloseq [129] and microbiome [130]. The preprocessed reads were assembled to contigs by MEGAHIT (v1.2.9) [144] using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. From the contigs having more than 500 bp, all possible open reading frames (ORFs) were gathered by Prodigal (v2.6.3) [145]. The protein translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v.3.1.1) [134, 135] by Resistance Gene Identifier (RGI, v5.1.1) with Diamond [146] The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. All nudged hits were excluded. The iMGE content of the ARG harbouring contigs was analysed by

MobileElementFinder (v1.0.3) [153]. Following the distance concept of Johansson et al. [153] for each bacterial species, those with a distance threshold defined within iMGEs and ARGs were considered associated. In the MobileElementFinder database (v1.0.2) for *Escherichia coli*, the longest composite transposon (cTn) was the Tn1681. In the case of this species, its length (24,488 bp) was taken as the cut-off value. For *Lactococcus lactis*, this threshold was the length of the Tn5721 transposon, 11,256 bp. For enterococci, the database contained cTn, the Tn6246 (5147 bp) transposon, in *E. faecium* only. The same threshold was used for *E. faecalis* contigs. As the database neither contains species-level, nor genus-level cTn data for *Bacillus*, *Bifidobacterium* and *Streptomyces* species, a general cut-off value was chosen for the contigs of these species. This value was declared as the median of the longest cTns per species in the database (10,098 bp). The average nucleotide identity (ANI) was calculated for the region of iMGE and associated ARGs by FastANI (v1.32) [154]. The plasmid origin probability of the contigs was estimated by PlasFlow (v.1.1) [136]. The phage content of the assembled contigs was predicted by VirSorter2 (v2.2.1) [155]. The findings were filtered for dsDNAphages and ssDNAs. All data management procedures, analyses and plottings were performed in R environment (v4.0.4) [128].

Study B is based on NGS data from isolates of 12 commonly used probiotic bacterial species that have been isolated in other studies. The bacterial species were selected based on a non-systematic review. This involved a search of the PubMed database (`https://pubmed.ncbi.nlm.nih.gov/`) using the keywords 'kefir', 'yoghurt', 'probiotic', and 'bacteria', for papers published in English after 2000. From the hits, we selected those that reported data on a relevant number of probiotic bacterial species [156–161]. Data that met the following criteria: having genomic library source, being whole-genome sequenced (WGS), and Illumina platform based were downloaded from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository. The selected species (and sample download dates) were: *Bifidobacterium animalis* (4/12/2022), *Lacticaseibacillus casei* (4/12/2022), *Lacticaseibacillus paracasei* (4/12/2022), *Lactiplantibacillus plantarum* (3/12/2022), *Lactobacillus delbrueckii* (25/-12/2022), *Lactobacillus helveticus* (4/12/2022), *Lactobacillus kefiranofaciens* (25/12/2022), *Lactobacillus kefiri* (25/12/2022), *Lactococcus lactis* (6/12/2022), *Leucoconstoc mesenteroides* (25/12/2022) *Levilactobacillus brevis* (2/12/2022), *Streptococcus thermophilus* (3/12/2022). The source data collected on samples were grouped into three categories: FFP (nonfermented Food or Fermented food or Probiotic), intestinal, and others. The FFP group included samples with the following origins: fermented beverage (n=12), fermented food (n=69), fermented food (dairy) (n=124), fermented food (non-dairy) (n=141), milk (n=10), milk (farm animal) (n=14), milk (human) (n=34), milk/dairy product (n=26), probiotic dietary product (n=1), probiotic dietary supplement (n=146), starter culture (n=2). The results of the analyses for FFP samples are presented below. The detailed metadata for each sample is available at the link: `https://doi.org/10.6084/m9.figshare.21877134.v1`.
The quality based filtering and trimming of the raw short reads was performed with TrimGalore (v.0.6.6, `https://github.com/FelixKrueger/TrimGalore`), setting 20 as a quality threshold. Only reads longer than 50 bp were retained. Cleaned reads from each bacterial species were aligned to the representative reference genome of the corresponding bacterium (*B. an-*

imalis: NC_017216.2, *L. casei*: NZ_AP012544.1, *L. paracasei*: NC_022112.1, *L. plantarum*: NZ_CP028221.1, *L. delbrueckii*: NZ_CP018218.1, *L. helveticus*: ASM2283254v1, *L. kefiranofaciens*: NZ_CP061341.1, *L. kefiri*: NZ_CP029971.1, *L. lactis*: NZ_CP059048.1, *L. mesenteroides*: NZ_CP028251.1, *L. brevis*: NZ_CP015398.1, *S. thermophilus*: NZ_LR822015.1.) for each sample by Bowtie2 [124]. Reads from samples that covered at least 80% of their own reference genome were de novo assembled with MEGAHIT (v1.2.9) [144] using default settings. From the contigs all possible open reading frames (ORFs) were gathered with Prodigal (v2.6.3) [145]. The protein translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v.3.2.5) [134, 135] by Resistance Gene Identifier (RGI, v6.0.0) with Diamond [146]. The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. All nudged hits were excluded. The iMGE content of contigs harbouring ARG was analyzed with MobileElementFinder (v1.0.3) and its database (MGEdb v1.0.2) [153]. Following the distance concept of Johansson and colleaues (2021), an ARG was considered to be associated with an iMGE if it was within a given distance. In the MGEdb we found data only for *L. lactis*, the longest composite transposon (cTn) for that species was the *Tn5721*, its length (11,256 bp) was taken as the cut-off value. For the rest of the species, a general threshold value was declared as the median of the longest cTns per species in the database (10,098 bp). The plasmid origin probability of the contigs was estimated by PlasFlow (v.1.1) [136]. The phage content of the assembled contigs was prediced by VirSorter2 (v2.2.1) [155]. The findings were filtered for dsDNAphages and ssDNAs. The 95% CI was estimated using the exact method for prevalence [162]. All data management procedures, analyses and plottings were performed in R environment (v4.2.1) [128].

4.2.2 Other animal source interfaces

4.2.2.1 Canine saliva

Deep-sequenced canine saliva datasets were searched in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository. In May 2021, two shotgun metagenomic BioProjects (PRJNA648123 [163]—The 10,000 Dog Genome Consortium and PRJNA683923 [164]—Broad Institute, Darwin's Ark project) with more than 100,000,000 paired-end reads per sample were identified (Table 3.). Both projects collected and sequenced dog saliva samples to investigate polymorphisms in the dog genome from which the samples were derived. The median read count (interquartile range, IQR) of the samples was 177.7×10^6 (26.6×10^6) and 417.7×10^6 (90.1×10^6) in datasets PRJNA648123 and PRJNA683923, respectively.

Table 3.: The list of analyzed samples was obtained from the National Center for Biotechnology Information Sequence Read Archive. Column Run contains the NCBI SRA run identifiers. Bacterial read count represents the number of reads that were classified taxonomically to any bacteria.

ID	BioProject	Run	Bacterial Read Count
1	PRJNA648123	SRR12330029	2,900,387
2		SRR12330041	16,153,172
3		SRR12330042	13,072,781
4		SRR12330043	13,774,332
5		SRR12330044	6,123,646
6		SRR12330045	16,707,766
7		SRR12330098	18,826,266
8		SRR12330104	27,598,592
9		SRR12330220	9,938,948
10		SRR12330260	17,642,933
11		SRR12330298	17,277,697
12		SRR12330356	13,988,719
13		SRR12330364	17,378,513
14		SRR12330377	12,155,726
15		SRR12330378	34,183,357
16		SRR12330382	22,353,314
17		SRR12330383	22,886,951
18		SRR12330384	18,328,656
19		SRR12330385	6,631,504
20	PRJNA683923	SRR13340534	0
21		SRR13340535	6,752,169
22		SRR13340537	8,245,374
23		SRR13340538	41,212,470
24		SRR13340539	13,028,655
25		SRR13340540	6,964,460
26		SRR13340541	6,279,921

Quality-based filtering and trimming of the raw short reads was performed by TrimGalore (v.0.6.6, `https://github.com/FelixKrueger/TrimGalore`, accessed on 24 September 2022), setting 20 as a quality threshold. Only reads longer than 50 bp were retained and taxonomically classified using Kraken2 (v2.1.1) [126] and a database created (24 March 2021) from the NCBI RefSeq complete archaeal, bacterial, viral and plant genomes. For this taxon assignment, the `--confidence 0.5` parameter was used to obtain more precise species-level hits. The taxon classification data were managed in R [128] using functions of the packages phyloseq (v1.36.0) [129] and microbiome (v1.14.0) [130]. Reads classified as origin of bacteria were assembled to contigs by MEGAHIT (v1.2.9) [144] using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. All possible open reading frames (ORFs) were gathered by Prodigal (v2.6.3) [145] from the contigs. The protein-translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v.3.1.3) [134, 135] by Resistance Gene Identifier (RGI, v5.2.0) with Diamond [146]. The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. All nudged hits were excluded. The iMGE content of the ARG-harboring

contigs was analyzed by MobileElementFinder (v1.0.3) and its database (v1.0.2) [153]. Following the distance concept of Johansson et al. [153] for each bacterial species, only those with a distance threshold defined within iMGEs and ARGs were considered associated. In the MobileElementFinder database (v1.0.2) for *Bacteroides*, the longest composite transposon (cTn) was the Tn*6186*. In the case of this genus, its length (8505 bp) was taken as the cut-off value. For the genera *Enterococcus* and *Klebsiella*, *Tn6246* (5147 bp) and *Tn125* (10,098 bp) provided the thresholds, respectively. In the case of *Escherichia coli*, this limit was the length of the *Tn1681* transposon, namely 24,488 bp, while for *Pseudomonas aeruginosa Tn6060* (25,440 bp). As the database neither contains species-level, nor genus-level cTn data for the rest of the species, a general cut-off value was chosen for the contigs of these species. This value was declared as the median of the longest cTns per species in the database (10,098 bp). The plasmid origin probability of the contigs was estimated by PlasFlow (v.1.1) [136]. The prophage content of the assembled contigs was prediced by VirSorter2 (v2.2.3) [155]. The findings were filtered for dsDNAphages and ssDNAs. All data management procedures, analyses and plottings were performed in R environment (v4.1.0) [128].

4.2.2.2 Pig feces

Within the study, quality based filtering and trimming was performed by Adapterremoval [123], using 15 as a quality threshold. Only reads longer than 50 bp were retained. *Sus scrofa* genome sequences as host contaminants were filtered out based on the NCBI *Sus scrofa* reference genome (Sscrofa11.1) by Bowtie2 [124] with *very-sensitive-local* setting minimizing the false positive match level [125]. The remaining reads were taxonomically classified using Kraken2 ($k = 35$) [126] with the NCBI non-redundant nucleotide database [127]. The taxon classification data was managed in R [128] using functions of package phyloseq [129] and microbiome [130]. For further analysis, the reads assigned to bacteria were used only [131]. Core bacteria was defined as the relative abundance of agglomerated counts at class level above 0.1% at least one of the samples. By metaSPAdes [132] the preprocessed reads were assembled to contigs. From these contigs having a shorter length than the shortest resistance gene of the Comprehensive Antibiotic Resistance Database (CARD) were discarded [134, 135]. The ARG content of filtered contigs was analyzed with Resistance Gene Identifier (RGI) v5.1.0 and CARD v.3.0.6 [134, 135]. Contigs harboring ARGs identified with perfect or strict cut-off were preserved and classified by Kraken2 on the same way as was described above. The plasmid origin probability of the contigs was estimated by PlasFlow v.1.1 [136]. To identify further MGE homologs the predicted protein sequences of contigs were scanned by HMMER [137] against data of PFAM v32 [138] and TnpPred [139]. Following Sáenz et al. [131] from the hits with lower than E 10^{-5} the best was assigned to each predicted protein within the distance of 10 ORFs. The MGE domains coexisting with ARGs were categorized as phage integrase, resolvase, transposase or transposon.

Table 2.: The list of analysed samples obtained from NCBI SRA. In the unified names of the samples the first character corresponds to the type of the sample (s and m, isolate and metagenome, respectively), the second tag is a sequence number. Except the signed (*) all samples were paired end sequenced. The last column shows the available information about the biosamples.

Sample ID	BioProject	Run	Description
Isolates			
s01	PRJEB14693	ERR1554589	*Lactiplantibacillus plantarum*
s02	PRJEB14693	ERR1554590	*Lactiplantibacillus plantarum*
s03	PRJEB14693	ERR1554591	*Lactiplantibacillus plantarum*
s04	PRJEB38007	ERR4421718	*Pseudomonas* sp. RGM2144
s05	PRJNA312743	SRR3205957	*Limosilactobacillus fermentum*
s06	PRJNA347617	SRR4417252	*Limosilactobacillus fermentum*
s07	PRJNA635872	SRR11966381	*Lactiplantibacillus plantarum*
s08	PRJNA639653	SRR12037315	*Lactobacillus delbrueckii* subsp. *bulgaricus*
s09	PRJNA639653	SRR12037316	*Lactobacillus delbrueckii* subsp. *bulgaricus*
s10	PRJNA639653	SRR12037890	*Streptococcus thermophilus*
s11	PRJNA649814	SRR12375795	*Enterococcus faecalis*
s12	PRJNA649814	SRR12375796	*Enterococcus faecalis*
s13	PRJNA649814	SRR12375797	*Enterococcus faecalis*
s14	PRJNA650131	SRR12376423	*Escherichia coli*
s15	PRJNA650131	SRR12376425	*Escherichia coli*
s16	PRJNA650131	SRR12376427	*Escherichia coli*
s17	PRJNA650131	SRR12376429	*Escherichia coli*
s18	PRJNA650131	SRR12376431	*Escherichia coli*
s19	PRJNA650131	SRR12376433	*Escherichia coli*
s20	PRJNA639653	SRR12412204	*Lacticaseibacillus rhamnosus*
Microbiota			
m01	PRJNA474998	SRR8132838	probiotic powder (FC13678)
m02	PRJNA475000	SRR8138827	probiotic powder (FC13669)
m03	PRJNA474989	SRR8140233	probiotic powder (FC13655)
m04	PRJNA474995	SRR8140386	probiotic powder (FC13628)
* m05	PRJNA508569	SRR8289759	probiotic product (2)
m06	PRJNA508569	SRR8289760	probiotic product (1)
* m07	PRJNA508569	SRR8289761	probiotic product (4)
* m08	PRJNA508569	SRR8289762	probiotic product (3)
* m09	PRJNA508569	SRR8289763	probiotic product (6)
* m10	PRJNA508569	SRR8289764	probiotic product (5)
m11	PRJNA542229	SRR9040978	dietary supplement (PB4)
m12	PRJNA542229	SRR9040979	dietary supplement (PB10)
m13	PRJNA542229	SRR9040980	dietary supplement (PB11)
m14	PRJNA542229	SRR9040981	dietary supplement (PB2)
m15	PRJNA542229	SRR9040982	dietary supplement (PB14)
m16	PRJNA542229	SRR9040983	dietary supplement (PB13)
m17	PRJNA542229	SRR9040984	dietary supplement (PB16)
m18	PRJNA542229	SRR9040986	dietary supplement (PB18)
m19	PRJNA542229	SRR9040987	dietary supplement (PB17)
m20	PRJNA542229	SRR9040988	dietary supplement (PB8)
m21	PRJNA542229	SRR9040989	dietary supplement (PB19)
m22	PRJNA542229	SRR9040990	dietary supplement (PB12)
m23	PRJNA542229	SRR9040991	dietary supplement (PB9)
m24	PRJNA542229	SRR9040992	dietary supplement (PB6)
m25	PRJNA542229	SRR9040993	dietary supplement (PB5)
m26	PRJNA542229	SRR9040994	dietary supplement (PB7)
m27	PRJNA644361	SRR12153424	probiotic capsule

5. Results

5.1 Food and dietary supplements

5.1.1 Raw milk

5.1.1.1 Bacteriome

After DNA extraction and sequencing, from sample A 17,773,004 while from sample B 8,425,326 paired-end reads were recovered. By the quality filtering, 0.20% and 0.80% of the reads were discarded from sample A and B, respectively. The reads were aligned to the host (*Bos taurus*) genome. As expected, most of the genetic material originated from the milking cow, from sample A 96.41% and from sample B 97.01% of the cleaned reads were filtered out due to host origin.
Of the reads, not aligning to the cow genome, we were able to classify 42.11% in sample A and 52.96% in sample B to known taxa. 185,982 reads of sample A and 11,437 reads of sample B were identified to belong to the kingdom of Bacteria. In sample A 93.54% of the reads were classified as Gram-positive bacteria, while in sample B this proportion was only 40.54%. The detailed composition of the core bacteriomes at class level is shown in Fig. 1.

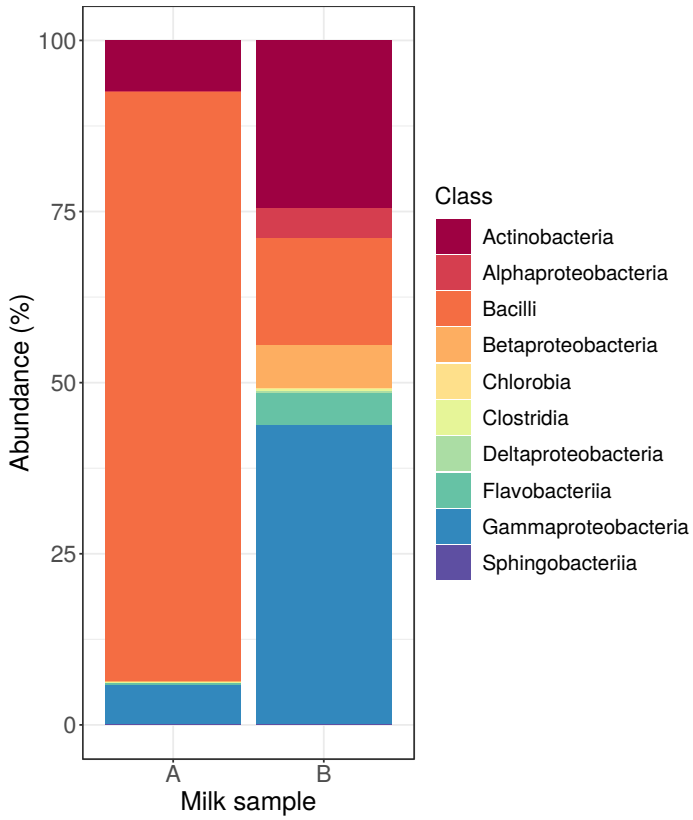

Figure 1.: Core bacteriome composition. Relative abundance of bacterial classes by milk samples.

5.1.1.2 Resistome and mobilome

Reads with overlapping pieces were assembled into longer DNA contigs by the metaSPAdes tool. The assembled contigs having a shorter length than 162bp (sample A: 0.68%, sample B: 0.33% of all contigs) were excluded. The remaining contig's median length was 268 (IQR: 126.5) and 244 (IQR: 42) in sample A and B, respectively. Then, the contigs having any open reading frame (ORF) matched with an ARG in the Comprehensive Antibiotic Resistance Database (CARD) were collected. The detected ARGs and particular properties are presented in Table 4.

The identified ARGs were classified with the Resistance Gene Identifier (RGI) tool according to the ratio of their coverage in the samples and to the identity between the contigs assembled from the sequenced reads and the CARD ARG reference sequences. In Table 4 we list each perfect or strict ARG hit predicted by RGI. We were able to identify three perfect ARG matches in sample A, *mepR, mgrA* and *Staphylococcus aureus norA*. According to the taxonomical classification of the contigs harbouring these ORFs their most likely origin is bacteria from *Staphylococcus* genus. The MGE analysis showed that none of these ORFs is mobile.

The sequence coverages of the strict matches in sample A ranged between 2.12% and 100%, with a mean of 36.61%. The identity of ORFs and CARD ARG reference sequences ranged between 95.02% and 100%, with a mean of 99.59%. Contigs containing ARG were classified on genus level and *Acinetobacter* (2.86%), *Carnobacterium* (11.43%), *Chryseobacterium* (2.86%), *Corynebacterium* (2.86%), *Kocuria* (11.43%), *Lactococcus* (8.57%), *Leuconostoc* (2.86%), *Micrococcus* (2.86%), *Moraxella* (5.71%), *Staphylococcus* (37.14%) and *Streptococcus* (11.43%) genera were identified.

In the bacterial genome, ARGs may be located on chromosomes or on plasmids, the latter ones being more likely to translocate between bacteria. With the PlasFlow tool, we identified contigs harbouring chloramphenicol acetyltransferase, PC1 beta-lactamase (*blaZ*) and *tet(38)* ARGs that may be encoded on plasmids. The results of MGE domain coexisting analysis showed that PC1 beta-lactamase (*blaZ*) ARG might be mobile since the contig had a phage integrase ORF within the distance of 10 ORFs.

There were no perfect matches in sample B that is not surprising since its overall bacterial nucleic acid content was less than 10% of that of sample A. The sequence coverages of the strict matches in this sample ranged between 5.71% and 24.83%, with the mean of 13.31%. The identity between ORFs and CARD ARG reference sequences was 100.00% in each detected ARG. Contigs containing ARGs were classified and genera *Acinetobacter* (50%) and *Delftia* (50%) were identified. None of the identified ARGs could be related to any MGEs.

The detected ARGs in both samples were matched to their corresponding antibiotics. Since one antibiotic compound may be related to more than one ORFs, we decided to select those to which we could link the ORFs with the broadest coverage and the highest identity to the reference ARG sequence. The maximal coverage and identity of detected ORFs are shown in Figure 2. In sample A ARGs known to be decreasing the effectiveness of acridine dye, cephalosporin, fluoroquinolone, penam and peptide antibiotics were found in full length and with 100% identity. There were two other ARGs identified in sample A in full length and with identity above 99% that encoded resistance against further antibiotics (cephamycin and tetracycline).

Table 4.: ARGs identified in milk samples. The coverage column shows the fraction of CARD ARG reference sequence covered by the most similar ORF sequence. Identity represents the proportion of the identical nucleotides between the detected ORF and CARD ARG reference sequence. Species column shows the most likely species related to the ARG harbouring contig classified by Kraken2. For some contigs, the species level classification was ambiguous, genus reported only. The localization of contigs with ARG and longer than 1000 bp predicted by PlasFlow. Mobile genetic element domains coexisting with ARG are listed in column MGE.

ARG	Coverage %	Identity %	Genus	Species	Localization	MGE
Perfect RGI match in sample A						
mepR	100.00	100.00	*Staphylococcus*	*aureus*	chromosome	
mgrA	100.00	100.00	*Staphylococcus*	*aureus*	unclassified	
Staphylococcus aureus norA	100.00	100.00	*Staphylococcus*	*aureus*	chromosome	
Strict RGI match in sample A						
AAC(6')-Iic	30.05	100.00	*Carnobacterium*	*maltaromaticum*		
Acinetobacter baumannii AbaQ	17.97	100.00	*Leuconostoc*	*mesenteroides*		
APH(2")-Ig	29.74	100.00	*Chryseobacterium*			
APH(3")-Ia	7.35	100.00	*Acinetobacter*	sp. TTH0-4		
APH(3')-Ia	8.12	100.00				
APH(7")-Ia	13.25	100.00	*Lactococcus*	*raffinolactis*		
arlR	74.89	95.12	*Staphylococcus*	*aureus*		
arlR	30.14	98.48	*Staphylococcus*	*aureus*		
arlS	29.93	100.00	*Staphylococcus*	*aureus*		
arlS	70.95	99.69	*Staphylococcus*	*aureus*	chromosome	
baeS	4.71	100.00				
BUT-1	11.59	100.00	*Moraxella*	*osloensis*		
Campylobacter coli chloramphenicol acetyltransferase	52.17	100.00	*Lactococcus*	*raffinolactis*	plasmid	
CatU	11.98	100.00	*Streptococcus*	*thermophilus*		
cfr(B)	24.36	100.00	*Streptococcus*	*urinalis*		
DHA-1	99.75	99.75	*Staphylococcus*	*aureus*	chromosome	
ErmW	10.61	100.00				
ICR-Mo	28.32	98.10	*Moraxella*	*osloensis*		
Klebsiella pneumoniae KpnF	68.81	100.00	*Corynebacterium*	*provencense*		
MCR-3.2	12.75	100.00	*Kocuria*	sp. BT304		
mecD	11.80	100.00	*Macrococcus*	*caseolyticus*		
mepA	100.00	99.78	*Staphylococcus*	*aureus*	chromosome	
mphM	27.09	100.00	*Carnobacterium*	*maltaromaticum*		
mphO	11.36	100.00	*Kocuria*			
MuxC	2.12	100.00				
norB	9.23	100.00				
OCH-2	10.26	100.00		*Brevibacterium* phage Cantare		
PC1 beta-lactamase (blaZ)	100.00	95.02	*Staphylococcus*		plasmid	phage integrase
PEDO-1	20.98	100.00	*Lactococcus*	*lactis*		
PEDO-3	51.90	100.00	*Carnobacterium*	*maltaromaticum*		
QnrB42	20.09	100.00				
srmB	14.18	100.00	*Carnobacterium*	*maltaromaticum*		
Staphylococcys aureus LmrS	12.71	100.00	*Staphylococcus*	*aureus*	chromosome	
Staphylococcys aureus LmrS	86.25	99.27	*Staphylococcus*	*aureus*	unclassified	
tet(38)	100.44	99.33	*Staphylococcus*	*aureus*	plasmid	
tetS	13.42	100.00				
tetS	11.23	97.22				
vanJ	18.48	100.00	*Streptococcus*	*thermophilus*	unclassified	
vanRG	19.57	100.00	*Streptococcus*	*thermophilus*		
vanTN	6.43	100.00	*Kocuria*			
ykkC	25.89	100.00	*Kocuria*			
Strict RGI match in sample B						
mefE	5.71	100.00	*Delftia*	*tsuruhatensis*		
OXA-269	13.55	100.00				
OXA-442	9.12	100.00				
PEDO-1	24.83	100.00	*Acinetobacter*	sp. TTH0-4		

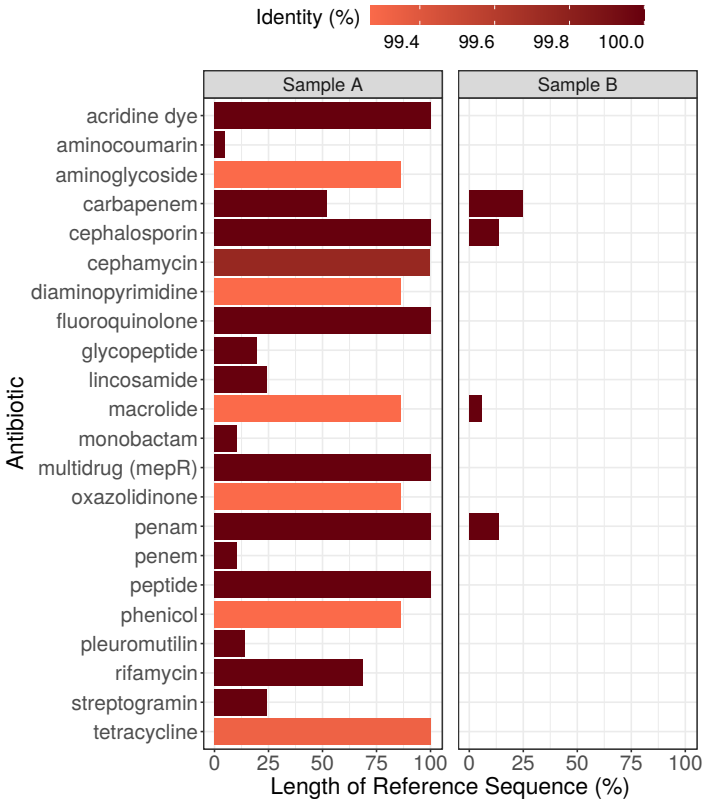

Figure 2.: Maximal coverage and identity of detected ORFs by antibiotics. The ORF covered proportion of the reference ARG sequence (X axis) and the identity % of predicted protein (color).

5.1.2 Probiotic dairy products

5.1.2.1 Bacteriome

When classified by taxon, the number of reads aligning to bacterial genomes differed in the various samples (Fig 3/a). Two samples (k_g_04 and y_g_01 from bioproject PRJNA644779) contained $\sim$ 20 million reads of bacterial origin. From bioproject PRJNA388572, sample k_p_15 had $\sim$ 50 million bacterial reads, while k_p_14 contained more than 63 million. Excluding these four extremities, the average bacterial read count of the metagenomic samples was 6.7×10^5 (ranging between 7.3×10^4 and 1.4×10^6). The median sequencing depth of the strain k_s_01, k_s_02, k_s_03, k_s_04, k_s_05, k_s_06, k_s_07 were 46, 119, 115, 111, 6, 54, 108, respectively. Figure 3/b. demonstrates the relative abundances of the dominant bacterial species identified in the samples. 99% of all bacteria identified were related to these species. In kefir grains the dominant species were a *Lactobacillus kefiranofaciens* ($57.7\% \pm 40.5\%$), *Lactobacillus kefiri* ($15.7\% \pm 17\%$), *Streptococcus thermophilus* ($15.4\% \pm 30.8\%$), *Lactococcus lactis* ($6.8\% \pm 13.5\%$), *Leuconostoc mesenteroides* ($1.7\% \pm 3.4\%$), *Leuconostoc pseudomesenteroides* ($1\% \pm 2\%$) and *Lactobacillus helveticus* ($1\% \pm 0.7\%$) in descending order of abundance. The most significant species in the products overlapped with those in the kefir grains, although they had differences in their relative abundance (*L. kefiranofaciens* ($55.4\% \pm 29\%$), *L. mesenteroides* ($35.7\% \pm 30\%$), *Acetobacter ghanensis* ($2.1\% \pm 4.4\%$), *L. helveticus* ($2.1\% \pm 1\%$), *L. kefiri* ($1.8\% \pm 2\%$), *Acetobacter orientalis* ($0.6\% \pm 2\%$), *Acetobacter oryzoeni* ($0.2\% \pm 0.5\%$)). The one yoghurt grain

examined was dominated by *Streptococcus thermophilus* (92.8%), *Bifidobacterium animalis* (3.6%) and *Lactobacillus delbrueckii* (3.5%) while the core bacteriome of the yoghurt product consisted of *S. thermophilus* ($83.9\% \pm 13.8\%$), *L. delbrueckii* ($10.1\% \pm 16.2\%$), *Lactobacillus acidophilus* ($4.6\% \pm 3.3\%$), and *B. animalis* ($1.2\% \pm 2.1\%$).

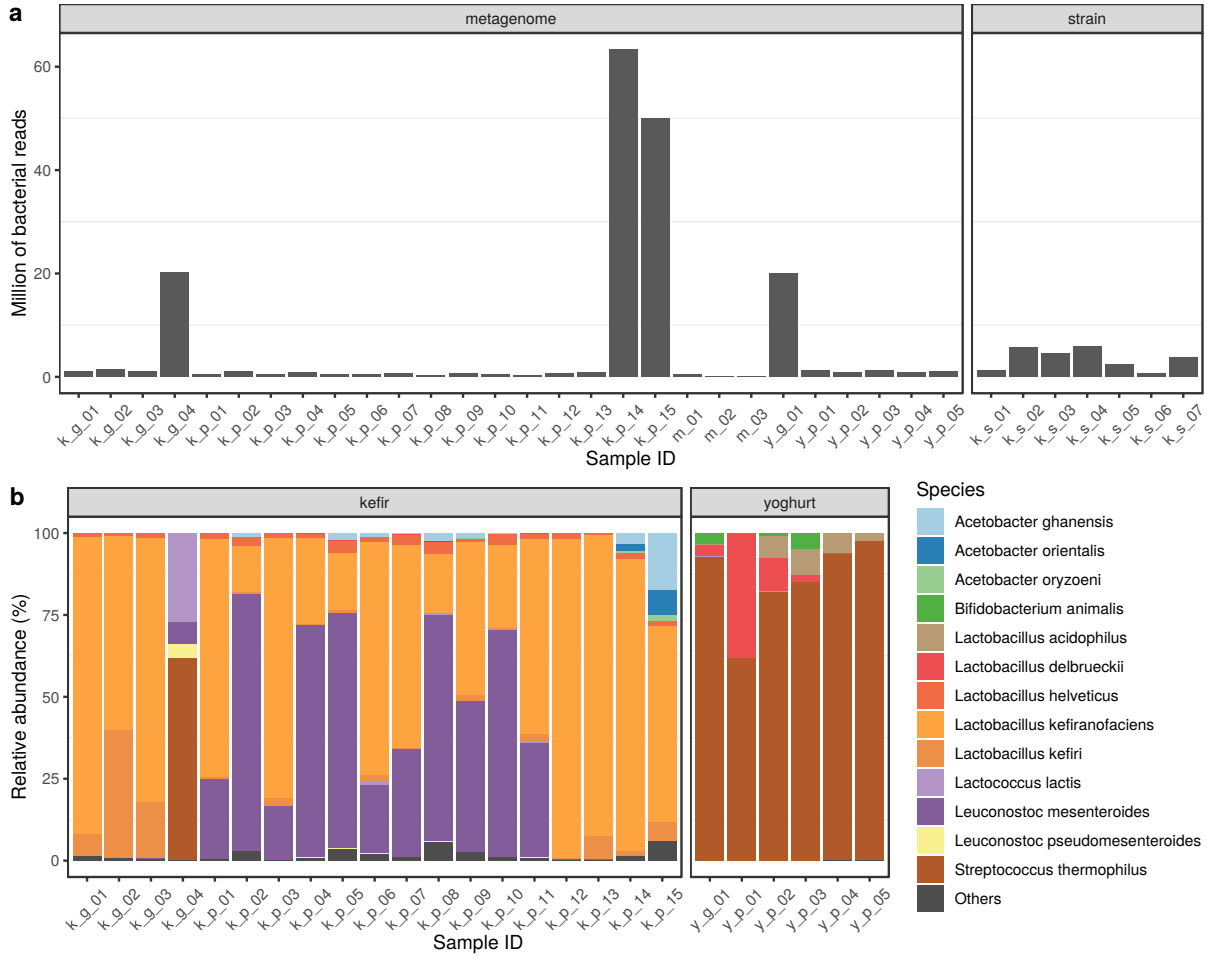

Figure 3.: Bacterial content of the samples. **a** The number of reads classified bacterial by Kraken2 on the NCBI NT database. Metagenome includes the samples deriving from grains, milk or products. **b** Relative abundances of the most common bacterial species in the grain and product samples.

5.1.2.2 Resistome and mobilome

According to our findings based on perfect and strict matches, AMR gene abundances show a great diversity in various types and sources of samples (Fig. 4/a). Samples (k_s_01, k_s_04, k_s_05, k_s_06, k_p_08) that did not meet the ORF filtering criteria were not plotted on Fig. 4 and 5. The highest ARG abundance was observed in the kefir strain samples (average: 282 FPKM, sd: 51.1) followed by the kefir product (240±78.6) and the kefir grains (209±106). The

yoghurt samples had lower abundances, in the only one grain, FPKM was 17.9, while in the products we found 45.7±32.2.

A Bray-Curtis distance-based principal coordinate analysis (PCoA) was performed to gain insight into the dissimilarity of the sample ARG abundances (Fig. 4/b). With a permutational multivariate analysis of variance on the same distance matrix, we found that the type of the sample explains the 22.17% ($p<0.001$) of dissimilarity among the sample resistomes. For the source grouping, the same measure was 18.92% ($p<0.001$). Based on Fig. 4/b one might conclude that the strongest effect on the dissimilarity is the bioproject of origin, as the analysis showed that it explains 35.56% ($p<0.001$) of the dissimilarity variances.

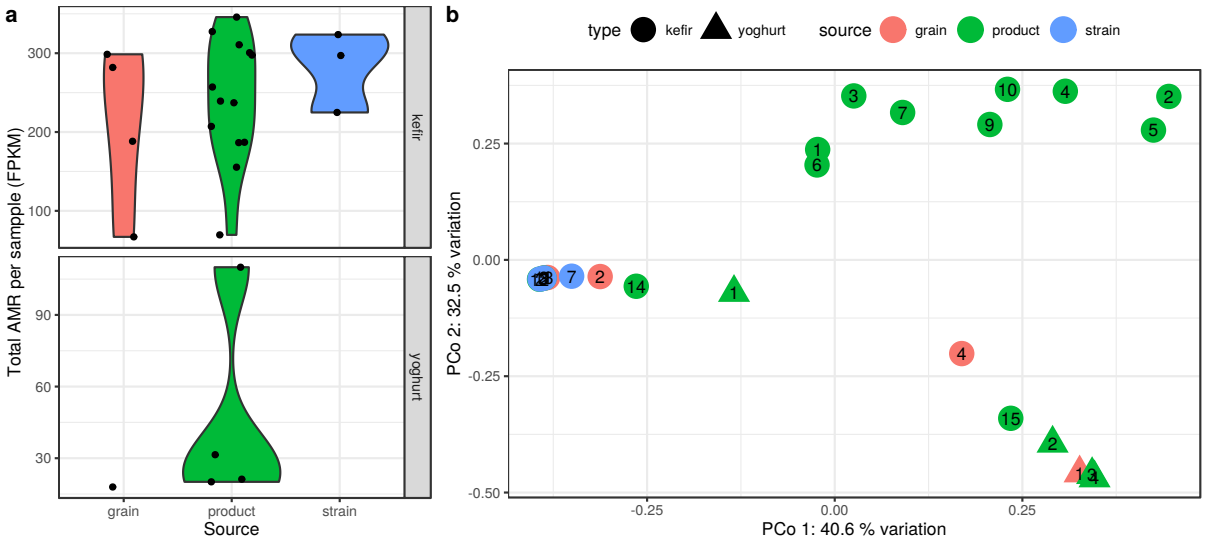

Figure 4.: Antimicrobial resistance (AMR) abundance of the samples. **a** Violin plot representing the distribution of the total AMR FPKM per sample, grouped by type and source. The horizontally jittered dots represent the FPKM of the samples. **b** The AMR abundance diversity (β-diversity) of the samples. It is plotted on the first two axes of principal coordinate analysis (PCoA) performed on Bray-Curtis distance which was calculated using the relative abundances of contigs harbouring ARGs. The symbols show the type, the colours the source, while the numbers correspond to the sequence number in the Sample ID. Some samples (k_s_01, k_s_04, k_s_05, k_s_06, k_p_08) are not shown as their ORFs did not meet filtering criteria.

In our kefir samples, we identified 22 ARGs in the product, 2 in the grain and 1 in the strain. In yoghurt, there was 1 ARG in the product and 2 in the grain (Fig. 5/a). The relative abundances of antibiotic classes affected are shown in Fig. 5/b for each sample. The detected ARGs and their most probable bacteria of origin are summarized on Fig. 6.

The kefir ARGs identified in the product may help bacteria in the defence against aminocoumarins, aminoglycosides, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, elfamycins, fluoroquinolones, fosfomycins, glycylcyclines, lincosamides, macrolides, monobactams, nitrofurans, nitroimidazoles, oxazolidinones, penams, penems, peptides, phenicols, pleuromutilins, rifamycins, streptogramins, tetracyclines and triclosan. Contigs containing these ARGs belonged to the genomes of *Enterobacter hormaechei* (genes: *acrB*; *acrD*; *ACT-36*; *bacA*; *baeR*; *CRP*; *emrB*; *emrR*; *Escherichia coli marR* mutant conferring antibiotic resistance; *E. coli soxS* with mutation conferring antibiotic resistance; *E. coli UhpT* with mutation conferring resistance to fosfomycin; *FosA2*; *marA*; *mdtB*; *mdtC*; *msbA*; *oqxA*; *oqxB*; *ramA*), *L. helveti-*

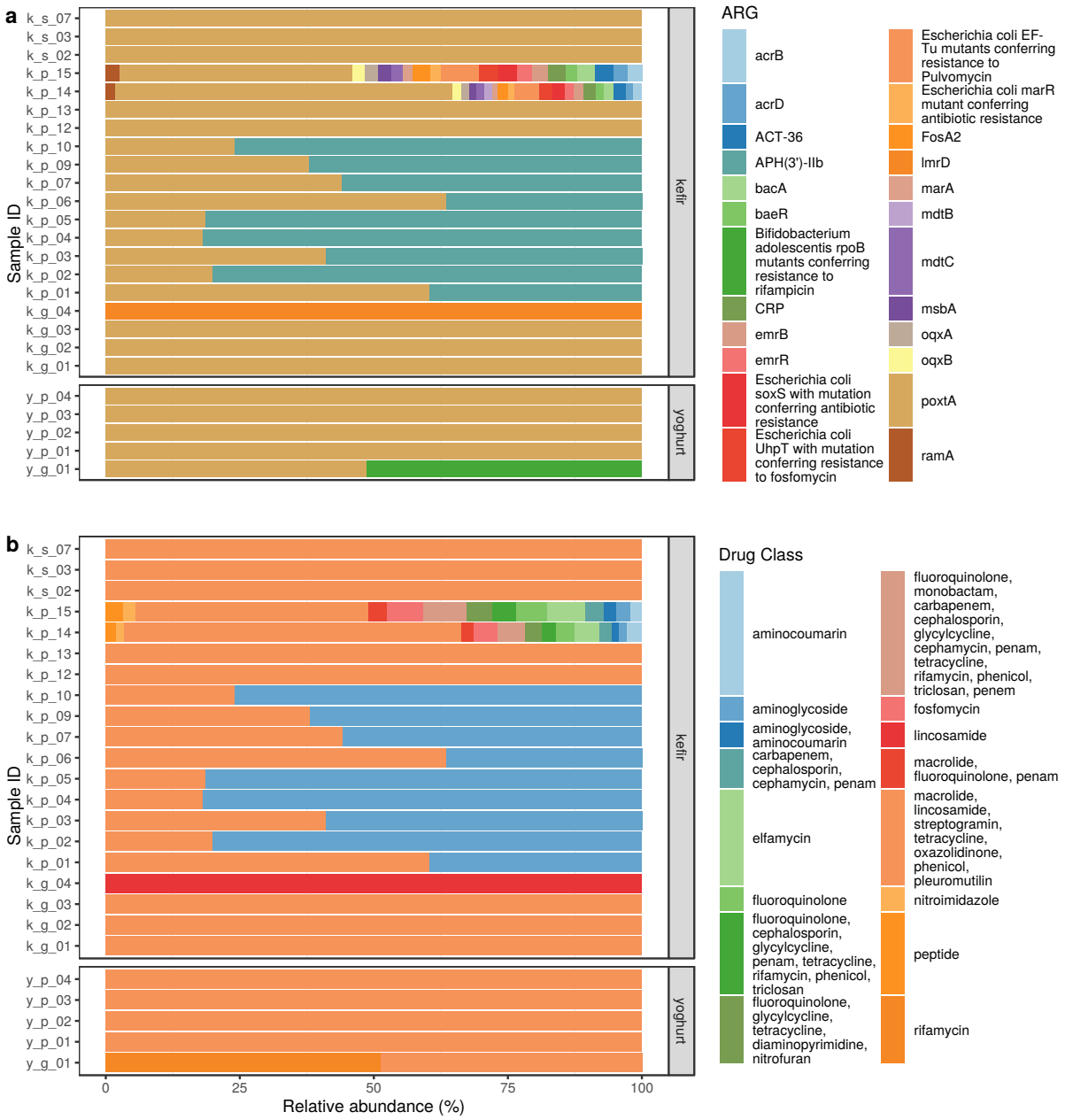

Figure 5.: Antimicrobial resistance (AMR) abundance in kefir and yoghurt samples. **a** Relative abundance of AMR genes. ORFs having at least 60% length and 90% base sequence identity with the reference ARG sequence are shown. Some samples (k_s_01, k_s_04, k_s_05, k_s_06, k_p_08) are not shown as their ORFs did not meet filtering criteria. **b** Relative abundance of drug classes related to the ARGs identified in the samples.

cus (gene *poxtA*), *L. kefiranofaciens* (gene *poxtA*) and *L. mesenteroides* (gene *APH(3')-IIb*). ARGs originating from the kefir grain may play a role in the appearance of resistance against lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins and tetracyclines and were found in the genomes of *L. kefiranofaciens* (gene *poxtA*) and *L. lactis* (gene *lmrD*). Gene *poxtA* deriving from kefir strains (*L. kefiranofaciens* and *L. plantarum*) confers resistance against lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins and tetracyclines. Genes found in yoghurt grains encoded resistance against lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, rifamycins, streptogramins

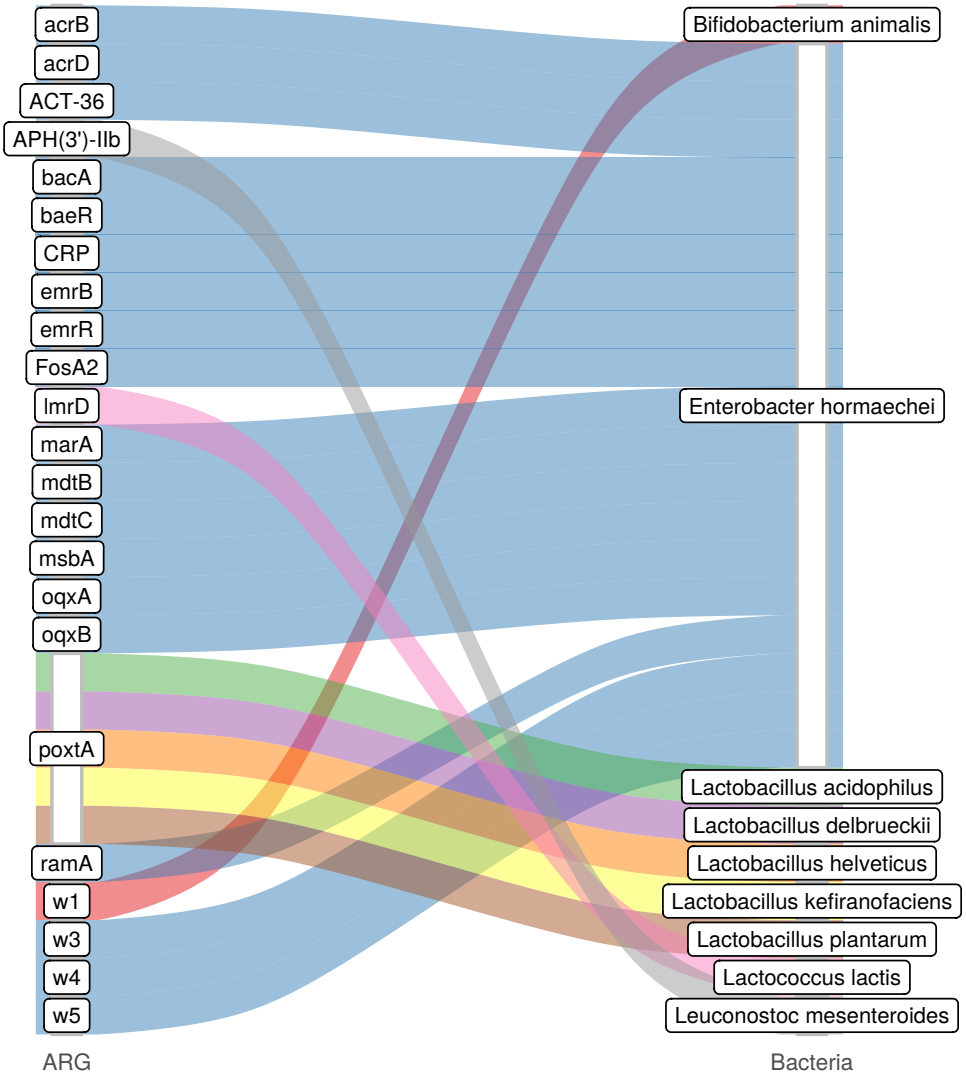

Figure 6.: Identified ARGs and their most probable bacteria of origin. The gene names that are too long have been abbreviated (w1: *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin; w3: *Escherichia coli marR* mutant conferring antibiotic resistance; w4: *E. coli soxS* with mutation conferring antibiotic resistance; w5: *E. coli UhpT* with mutation conferring resistance to fosfomycin).

and tetracyclines, while the ARGs of the product itself may weaken the efficacy of lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins and tetracyclines. Contigs involving ARGs of the yoghurt product could have been connected to *L. acidophilus* (gene *poxtA*) and *L. delbrueckii* (gene *poxtA*). However, the ARGs of the grains aligned to the reference sequence of *B. animalis* (gene *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin) and *L. delbrueckii* (gene *poxtA*).

Based on the ARG abundances the proportion of resistance mechanisms was calculated for each sample. In the kefir product samples the dominant mechanism of identified ARGs was the antibiotic target protection (50.73%), followed by antibiotic inactivation (45.45%), antibiotic efflux (2.03%), antibiotic target alteration (1.07%), antibiotic efflux; reduced permeability to antibiotic (0.32%), antibiotic target alteration; antibiotic efflux; reduced permeability to antibiotic (0.27%), antibiotic target alteration and antibiotic efflux (0.13%). In the kefir grain samples, the

main mechanisms were antibiotic target protection (91.98%) and antibiotic efflux (8.02%). In the kefir strains and yoghurt products, the antibiotic target protection was the only mechanism detected. In the one yoghurt grain sample, antibiotic target alteration; antibiotic target replacement (51.28%) and antibiotic target protection (48.72%) are the possible resistance mechanisms.
The results of MGE domain coexisting analysis showed that the ARG *lmrD* in sample k_g_04 might be mobile since the contig containing the gene had a transposase ORF within the distance of 10 ORFs. According to the analysis executed with PlasFlow [136] there were not any identifiable contigs with plasmid origins harboring ARGs.

5.1.2.3 ARG abundance changes during kefir fermentation

According to the metagenomic analysis published by Walsh et al. [141], ARG abundances change during the fermentation process (Fig. 7/a). In the case of all three grains (Fr1, Ick, UK3) *APH(3')-IIb* is present in the kefirs fermented for 24 hours, while it is absent in all other time phases except for sample UK3 after 8 hours. *PoxtA* was detectable in all samples except the 8 hour Fr1 kefir sample. The abundance fold change of 24 hours with respect to grain samples was 0.10, 0.59 and 0.26 for the starter culture Fr1, Ick and Uk3, respectively. Between the hour 8 and 24 samples, *poxtA* abundance showed a 0.34-fold change in the Ick kefir, while in the case of the UK3 kefir sequence this value reached 0.62.
Contigs harbouring ARGs were classified taxonomically (Fig. 7/c). All contigs containing the gene *APH(3')-IIb* were assigned to *L. mesenteroides*. Contigs with *poxtA* were assigned to the reference genome of *L. helveticus* and *L. kefiranofaciens*.
All bacterial reads were then aligned to the reference genomes of bacteria mentioned above, and the hits are expressed proportionally (Fig. 7/b). In contrast to *L. kefiranofaciens* that showed a decreasing tendency, an increase in time is observable by the relative abundances of *L. mesenteroides*. The proportion of reads assigned to *L. helveticus* shows no tendential increase or decrease in time. The increase of abundance of *APH(3')-IIb* shows a positive association with the relative abundance of *L. mesenteroides*. Similarly, *poxtA* abundance is decreasing with the relative abundance of *L. kefiranofaciens*.

5.1.2.4 Excluding nudged hits

In order to set the alignment restrictions of ORFs to reference ARGs even stricter, we selected for a subgroup of reference ARGs that fit the ORFs from the starting base position on. Thus, nudging on the reference sequence by the alignment was avoided. With such a shrinkage, we reduced the number of detectable ARGs to four samples (Fig. 8). Sample k_g_04 from bioproject PRJNA644779 contained an ARG against lincosamides while the gene found in sample y_g_01 is responsible for resistance against rifamycin. Contigs harbouring these ARGs had the best alignment to *L. lactis* and *B. animalis*, respectively. Bioproject PRJNA388572 had two samples with similar matches, except for gene *mdtB* which appeared in full length in sample k_p_14 and was absent in k_p_15, this gene is responsible for aminocoumarin resistance. As some other ARGs of the sample also have the potential to confer resistance against aminocoumarin, the AMR profiles of the samples appeared to be the same, including aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimi-

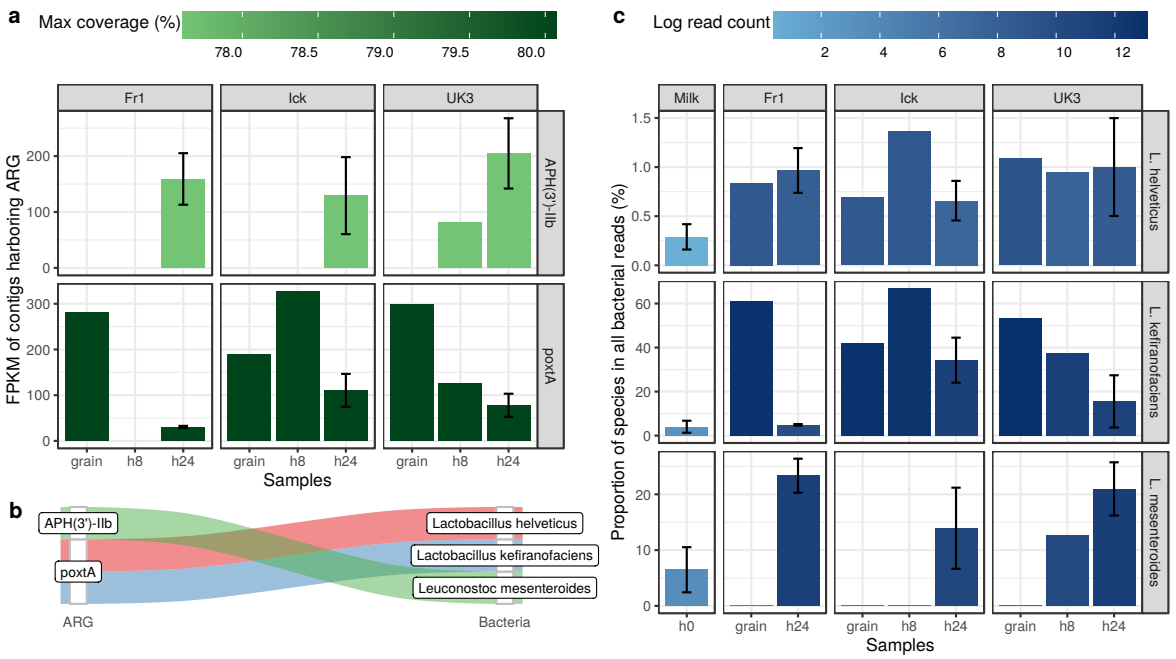

Figure 7.: Changes during kefir fermentation. **a** ARG abundance expressed as FPKM based on the alignment of bacterial reads to the ARG harbouring contigs. **b** ARGs and their most likely origins. **c** Relative abundances of bacteria with a probable ARG content.

dine, elfamycin, fluoroquinolone, fosfomycin, glycylcycline, macrolide, monobactam, nitrofuran, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline and triclosan. Comparing this list to the nudged results oxazolidinone, pleuromutilin and streptogramin resistance genes were absent. Contigs containing ARGs had the best alignment to the genome of *E. hormaechei* in both cases [165].

All four samples of both bioprojects included at least 20 million bacterial reads in the assembly of the contigs. The other samples consisted of significantly fewer reads. Consequently, as Sims and colleagues (2014) found it is not possible to distinguish whether the absence of protein-coding genes or the disruption of open reading frames (ORFs) represent a deficiency of the assembly or real evolutionary gene loss [166]. Examining the relationship between the number of bacterial reads and length of identified ARGs (including nudges) with a linear model we found that after each extra 100,000 reads the coverage of reference gene raises by 7% by the ARG coding ORFs ($p<0.0001$). In samples k_g_04, k_p_14, k_p_15 és a y_g_01, we randomly chose the average read number of the other samples (677 340) to reanalyze how much these results differ from the original ones executed on the full database. Contigs assembled contained one gene that was identified previously (excluding nudges), namely *lmrD* from sample k_g_04. ORFs predicted to be ARGs had a median coverage of 16.10% on the reference ARG sequences. In contrast, ORFs aligning to ARGs composed of the full read content of the four samples had a median coverage of 99.21%.

5.1.3 Other probiotic products

In the following section the two probiotic product-related studies included in the doctoral work are referred to as **study A** and **study B**.

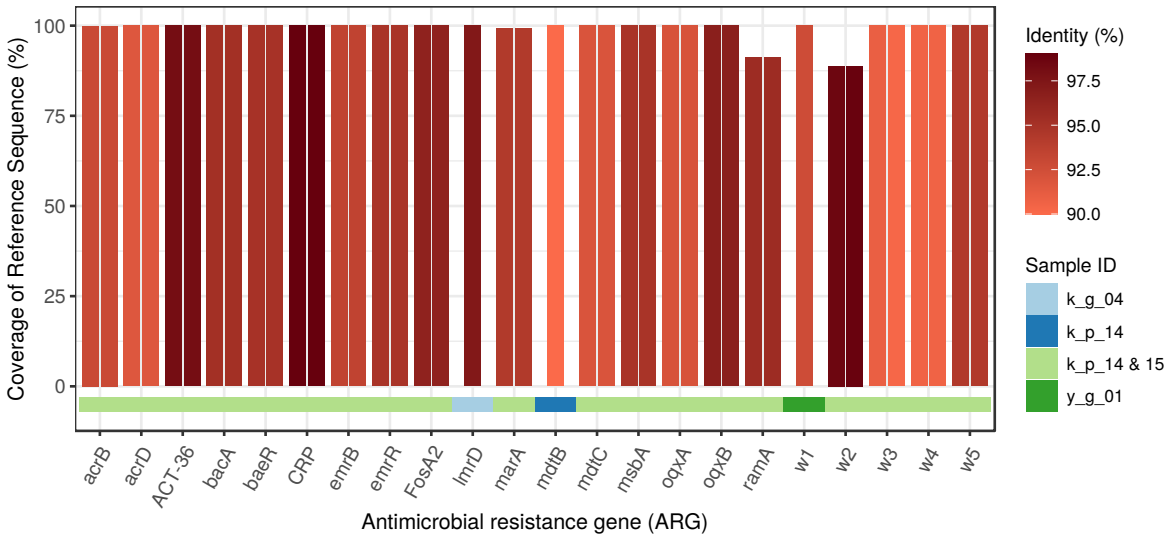

Figure 8.: Identified ARGs excluding nudged findings. The coverage and identity of detected open reading frames (ORFs) by ARGs. The ORF covered proportion of the reference ARG sequence and the identity % of predicted protein (colour). The gene names which are too long have been abbreviated (w1: *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin; w2: *Escherichia coli EF-Tu* mutants conferring resistance to Pulvomycin; w3: *E. coli marR* mutant conferring antibiotic resistance; w4: *E. coli soxS* with mutation conferring antibiotic resistance; w5: *E. coli UhpT* with mutation conferring resistance to fosfomycin).

5.1.3.1 Bacteriome (A)

By taxon classification, the number of reads aligning to bacterial genomes differed in the various samples. The median bacterial read count of the metagenomic samples was 8.2×10^6 (IQR: 4.4×10^6). The median sequencing depth of the isolated strains was 220 (IQR: 94.8). The taxonomic origin of the short reads generated from isolates is shown in Table 2. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the metagenomic samples is shown in Figure 9. These dominant genera (with mean prevalence) in descending order were *Lactobacillus* (40%), *Enterococcus* (35%), *Bifidobacterium* (34%), *Limosilactobacillus* (34%), *Lactococcus* (32%), *Lacticaseibacillus* (31%), *Bacillus* (26%), *Weizmannia* (22%), *Ligilactobacillus* (19%), *Streptococcus* (18%), *Lactiplantibacillus* (12%) and *Sphingobacterium* (2%).

5.1.3.2 Resistome and mobilome (A)

The median length of the filtered contigs harbouring ARGs constructed by de novo assembly was 102,711 bp (IQR: 105,696). The number of ARGs found on the contigs ranged from 1 to 12. Besides 182 perfect ARG matches, a further 225 hits were classified strict (RGI) and met the criteria of having 90% coverage and 90% sequential identity. ARGs were detected in all metagenomic samples and in few isolates (Figure 18 (see page 52.)). The majority of isolates (s01, s02, s03, s04, s05, s06, s07, s08, s09, s10, s20) contained no ARG. The highest number of ARGs was found in samples s14–s19, obtained from sequencing six *Escherichia coli* strains isolated from the same probiotic product. It is important to highlight that we also found the *H-NS* gene in these samples which is not indicated in Figure 18 (see page 52.), as its effect is

anti-AMR. The most common ARGs were the *rpoB* mutants conferring resistance to rifampicin, *TEM-116* and *tet(W/N/W)* genes, detected in 18, 15 and 13 samples, respectively.

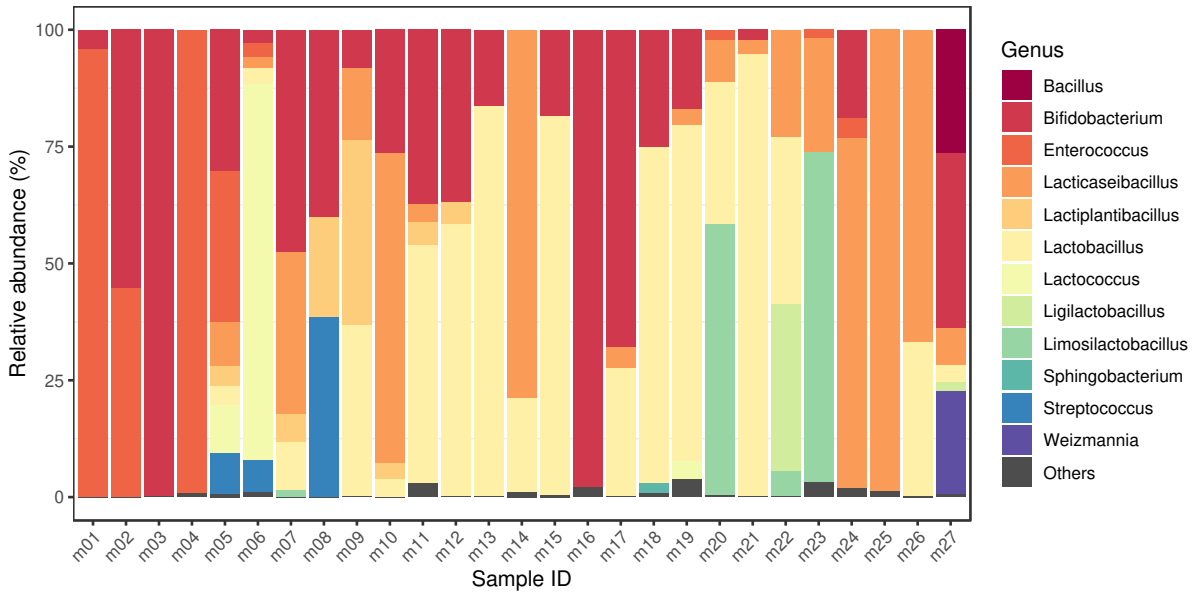

Figure 9.: Bacteriome of samples. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the metagenomic samples. The dominant genera (with mean prevalence) in descending order were *Lactobacillus* (40%), *Enterococcus* (35%), *Bifidobacterium* (34%), *Limosilactobacillus* (34%), *Lactococcus* (32%), *Lacticaseibacillus* (31%), *Bacillus* (26%), *Weizmannia* (22%), *Ligilactobacillus* (19%), *Streptococcus* (18%), *Lactiplantibacillus* (12%) and *Sphingobacterium* (2%). Sample accession numbers for the Sample IDs are listed in Table 2.

The proportion of resistance mechanisms was calculated based on the ARG diversity. The dominant mechanism of identified ARGs was the antibiotic efflux (58.33%), antibiotic inactivation (11.11%), antibiotic target alteration (11.11%), antibiotic target protection (9.72%), antibiotic target alteration and antibiotic efflux (4.17%), antibiotic efflux and reduced permeability to antibiotic (1.39%), antibiotic target alteration and antibiotic efflux and reduced permeability to antibiotic (1.39%), antibiotic target alteration and antibiotic target replacement (1.39%) and antibiotic target replacement (1.39%).

There was no detectable ARG in the studied samples originating from *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii subsp. bulgaricus*, *Limosilactobacillus fermentum*, *Pseudomonas sp. RGM2144* or *Streptococcus thermophilus* species.

The identified ARGs associated with bacteria by species are as follows. *Bacillus subtilis*: *aadK*, *B. subtilis mprF*, *B. subtilis pgsA* with mutation conferring resistance to daptomycin, *bmr*, *lmrB*, *mphK*, *vmlR*, *ykkC*, *ykkD*. *Bifidobacterium animalis*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *tet(W/N/W)*. *B. bifidum*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *B. bifidum ileS* conferring resistance to mupirocin, *tet(W/N/W)*. *B. breve*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *tetW*. *B. longum*: *B. adolescentis rpoB* mutants conferring resistance to rifampicin, *tet(W/N/W)*. *Enterococcus faecalis*: *dfrE*, *efrA*, *efrB*, *emeA*, *lsaA*, *tetM*. *E. faecium*: *AAC(6')-Ii*, *eatAv*, *msrC*. *Escherichia coli*: *acrB*, *acrD*, *acrE*, *acrF*, *acrS*, *bacA*, *baeR*, *baeS*, *cpxA*, *CRP*, *emrA*, *emrB*, *emrK*, *emrR*, *emrY*, *eptA*, *E.*

coli acrA, *E. coli acrR* with mutation conferring multidrug antibiotic resistance, *E. coli ampC* beta-lactamase, *E. coli ampC1* beta-lactamase, *E. coli ampH* beta-lactamase, *E. coli emrE*, *E. coli GlpT* with mutation conferring resistance to fosfomycin, *E. coli marR* mutant conferring antibiotic resistance, *E. coli mdfA*, *E. coli soxR* with mutation conferring antibiotic resistance, *E. coli soxS* with mutation conferring antibiotic resistance, *evgA*, *evgS*, *gadW*, *gadX*, *kdpE*, *marA*, *mdtA*, *mdtB*, *mdtC*, *mdtE*, *mdtF*, *mdtG*, *mdtH*, *mdtM*, *mdtN*, *mdtO*, *mdtP*, *msbA*, *PmrF*, *TEM-116*, *TolC*, *ugd*, *YojI*. *Lactococcus lactis*: *lmrD*. *Streptomyces albulus*: *AAC(3)-IV*.
The ARGs belonging to the genome of *Bacillus subtilis* may play a role in the appearance of resistance against aminoglycosides, lincosamides, macrolides, oxazolidinones, peptides, phenicols, pleuromutilins, streptogramins, tetracyclines; *Bifidobacterium animalis*: rifamycins, tetracyclines; *Bifidobacterium bifidum*: mupirocins, rifamycins, tetracyclines; *Bifidobacterium breve*: rifamycins, tetracyclines; *Bifidobacterium longum*: rifamycins, tetracyclines; *Enterococcus faecalis*: acridine dye, diaminopyrimidines, fluoroquinolones, lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, rifamycins, streptogramins, tetracyclines; *Enterococcus faecium*: aminoglycosides, lincosamides, macrolides, oxazolidinones, phenicols, pleuromutilins, streptogramins, tetracyclines; *Escherichia coli*: acridine dye, aminocoumarins, aminoglycosides, benzalkonium chlorides, carbapenems, cephalosporins, cephamycins, fluoroquinolones, fosfomycins, glycylcyclines, lincosamides, macrolides, monobactams, nitroimidazoles, nucleosides, penams, penems, peptides, phenicols, rhodamines, rifamycins, tetracyclines, triclosans; *Lactococcus lactis*: lincosamides; *Streptomyces albulus*: aminoglycosides.
The frequencies of iMGEs, phages and plasmids associated with ARGs by bacteria of origin are summarised in Figure 10.

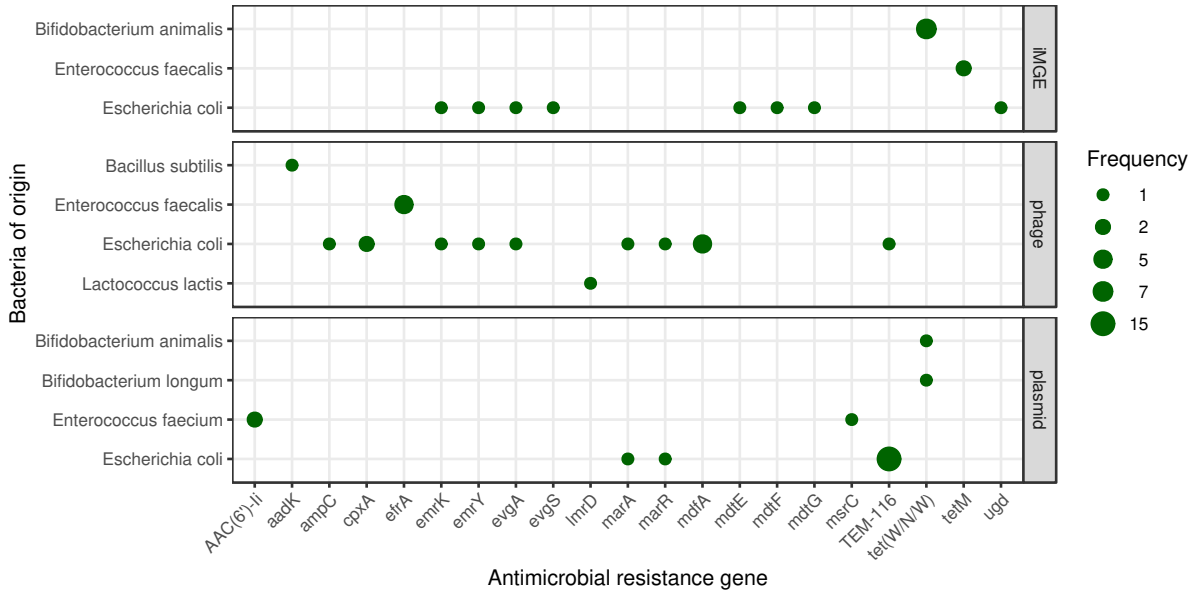

Figure 10.: Mobile ARG frequency by bacteria of origin. The size of the dots indicates the occurrence frequency of the given gene flanked by iMGE, positioned in plasmid or phage.

Based on the distance method proposed by Johansson and colleagues (2021) iMGE associated ARGs were detected in three species (*Bifidobacterium animalis*, *Enterococcus faecalis* and *Escherichia coli*) [153]. In seven metagenomic samples (m01, m02, m03, m07, m16, m17,

m24) we found tet(W/N/W) associated with ISBian1 insertion sequence on contigs classified as *B. animalis*. In two further samples (m02, m06) on *E. faecalis* contigs, *tetM* is linked to the transposon Tn6009. The ARG *mdtG* in the *E. coli* sample s14 and the ARG *ugd* in s15 are associated with IS3 and IS100, respectively. On two different contigs in the sample s17, multiple ARGs were detected with iMGE. One of them has the ISKpn24 associated with *mdtE* and *mdtF*. The other one has the IS102 linked to *emrY*, *emrK*, *evgA* and *evgS* genes. According to the average nucleotide identity (ANI) analysis most of the contig region of iMGE and associated ARGs had a high level of conservation (ANI > 97%). Nevertheless, both contigs classified as *E. faecalis* showed ANIs below 80%.

In samples m08 and m21, we identified one-one plasmid associated contig with *tet(W/N/W)* classified as *Bifidobacterium longum* and *Bifidobacterium animalis*, respectively. In the samples m20 and m23 associated with *Enterococcus faecium*, contigs from *AAC(6')-Ii* that were predicted to originate from plasmids were detected. Further, contigs of *E. faecium* from sample m23 contained the ARG *msrC*. In the samples m11, m12, m13, m14, m15, m16, m17, m19, m20, m21, m22, m23, m24, m25 and m26, *Escherichia coli* contigs from plasmids harboured the gene *TEM-116*. In the *E. coli* isolate sample s15, one contig of plasmid had the *marA* and *marR* genes.

By phage prediction, only dsDNAphages were detected. One contig, classified as *Bacillus subtilis* from the m05 metagenomic sample, contained prophage harbouring gene *aadK*. One prophage in predicted *Enterococcus faecalis* originated contig was found in sample m04 having gene *efrA*. The same content was detected in sample m01 on contigs classified as *E. faecalis*. All three *E. faecalis* isolates (s11, s12, s13) contained contigs harbouring the gene *efrA* associated to a prophage. In sample m17, one *E. coli* classified contig had the gene *TEM-116*, while a *Lactococcus lactis* classified one carried the gene *lmrD* associated to a prophage. All the *E. coli* isolates contained contigs with prophages harbouring ARG. In sample s17 and s19 the *mdfA* gene is presented associated to a prophage. Sample s15 contains contigs harbouring prophage with the gene *marA*, *marR*. Sample s16 includes contigs with prophages carrying the gene *emrK*, *emrY*, and *evgA*. The gene *ampC* was found in sample s15, while the gene *cpxA* in samples s14 and s18 was associated to prophages.

5.1.3.3 Bacteriome (B)

For the 12 species, a total of 2244 samples were downloaded. After fitting to the reference genomes, 1453 of these samples were retained for covering at least 80% of the reference genome. Of these, 579 samples derived from FFP sequencing isolates, 559 had intestinal origins, and 314 originated from other sources. The 579 FFP samples were collected between 1901 and 2022, while the corresponding release date was between 10/2/2014 and 15/11/2022. By 8 of the 579 samples, the year of sample collection could not have been determined. For the release date, no missing data was observable. For 562 of the FFP samples, the country of origin could have been determined (n=31), which is presented with the sample number per country in Fig. 19/a (see page 53.). Sample element numbers for each species: *B. animalis* (n=65), *L. casei* (n=1), *L. paracasei* (n=33), *L. plantarum* (n=212), *L. delbrueckii* (n=41), *L. helveticus*

(n=66), *L. lactis* (n=79), *L. mesenteroides* (n=12), *L. brevis* (n=47), *S. thermophilus* (n=23). By *L. kefiranofaciens* and *L. kefiri*, there were no samples of FFP origin. Among samples with other origins, there were representatives 5 of *L. kefiranofaciens* and 2 of *L. kefiri*.

5.1.3.4 Resistome and mobilome (B)

Out of 579 FFP samples, 169 were ARG-positive (29.19%, 95% CI: 25.51-33.08), prevalence by country is shown in Fig. 19/b (see page 53.) and by species in Fig. 11. The identified ARGs and their number per species are summarised in Table 5. These results show that there was no identified ARG in the species of *L. casei* and *L. paracasei*.

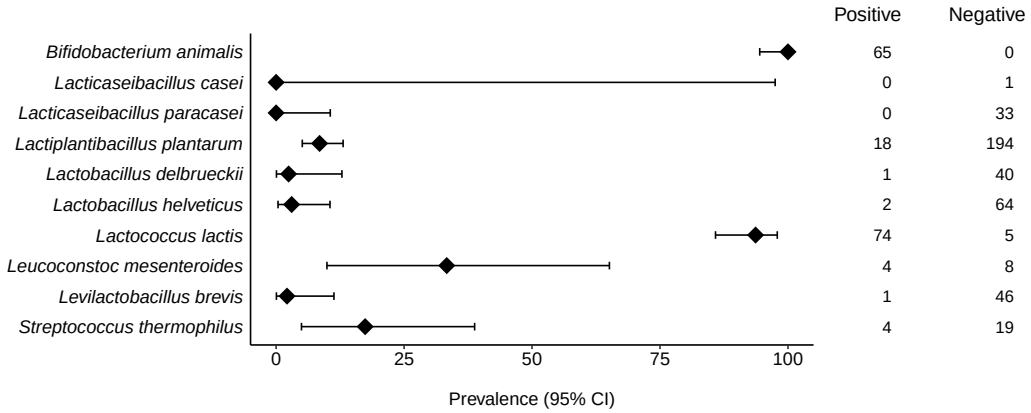

Figure 11.: Prevalence of the ARG positive samples by species. The number of ARG positive and negative samples is listed besides the prevalence (black square) and its 95% CI (horizontal lines). Samples were collected between 1901 and 2022 (n=579 isolates).

Table 5.: Identified ARGs by species with the number of samples harboring the gene. Samples were collected between 1901 and 2022 (n=579 isolates).

Bacteria n: number of samples	Antimicrobial resistance gene n: number of samples harboring the gene
Bifidobacterium animalis (n=65)	*Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin (n=65); *Limosilactobacillus reuteri cat-TC* (n=1); *tet(W)* (n=65)
Lactiplantibacillus plantarum (n=212)	*AAC(6')-Ii* (n=2); *ANT(3")-IIa* (n=6); *ANT(6)-Ia* (n=1); *catA8* (n=1); *eatAv* (n=2); *ErmB* (n=1); *lnuA* (n=2); *msrC* (n=2); *TEM-1* (n=1); *TEM-181* (n=1); *tet(C)* (n=1); *tet(M)* (n=5); *tet(S)* (n=1)
Lactobacillus delbrueckii (n=41)	*TEM-116* (n=1)
Lactobacillus helveticus (n=66)	*lnuA* (n=2)
Lactococcus lactis (n=79)	*ErmB* (n=1); *lmrD* (n=71); *tet(M)* (n=1); *tet(S)* (n=4)
Leucoconstoc mesenteroides (n=12)	*ANT(3")-IIa* (n=3); *lmrD* (n=1)
Levilactobacillus brevis (n=47)	*lnuA* (n=1)
Streptococcus thermophilus (n=23)	*ErmB* (n=2); *tet(S)* (n=2)

The *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin ARGs found in *B. animalis* were variants of the RIF pocket [167] with amino acid variants (A443V, V516E, E525V, D532E, A533V, E543K, K552E, Q554E, A557V, V559D, G560A, E561A, E562G, V565E, S570E, S571M). We found that all positions but A443V had the amino acid encoding the resistance in all 65 samples.

The ARGs belonging to the genome of *B. animalis* may play a role in the appearance of resistance against phenicols, rifamycins, tetracyclines; *L. plantarum*: aminoglycosides, cephalosporins, lincosamides, macrolides, monobactams, penams, penems, phenicols, pleuromutilins, streptogramins, streptogramin A, streptogramin B, tetracyclines; *L. delbrueckii*: cephalosporins, monobactams, penams, penems; *L. helveticus*: lincosamides; *L. lactis*: lincosamides, macrolides, streptogramins, streptogramin A, streptogramin B, tetracyclines; *L. mesenteroides*: aminoglycosides, lincosamides; *L. brevis*: lincosamides; *S. thermophilus*: lincosamides, macrolides, streptogramins, streptogramin A, streptogramin B, tetracyclines.

The resistance mechanism proportions linked to the ARGs identified by species were as follows. *B. animalis*: antibiotic inactivation (1.54%; 1/65), antibiotic target alteration and antibiotic target replacement (100.00%; 65/65), antibiotic target protection (100.00%; 65/65). *L. plantarum*: antibiotic efflux (5.56%; 1/18), antibiotic inactivation (77.78%; 14/18), antibiotic target alteration (5.56%; 1/18), antibiotic target protection (55.56%; 10/18). *L. delbrueckii*: antibiotic inactivation (100.00%; 1/1). *L. helveticus*: antibiotic inactivation (100.00%; 2/2). *L. lactis*: antibiotic efflux (95.95%; 71/74), antibiotic target alteration (1.35%; 1/74), antibiotic target protection (6.76%; 5/74). *L. mesenteroides*: antibiotic efflux (25.00%; 1/4), antibiotic inactivation (75.00%; 3/4). *L. brevis*: antibiotic inactivation (100.00%; 1/1). *S. thermophilus*: antibiotic target alteration (50.00%; 2/4), antibiotic target protection (50.00%; 2/4).

No ARGs were found in any samples that could be linked to bacteriophages. In 66% (112/169) of the ARG-containing samples, at least one gene could be linked to a plasmid or integrative mobile element. In 62 samples of *B. animalis*, *tet(W)* was linked to iMGE, while *tet(M)* and *tet(S)* were associated with iMGEs in one *L. plantarum* sample each. Plasmid-related ARGs per bacterium with sample numbers (n) are as follows. *B. animalis*: *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin (n=30), *Limosilactobacillus reuteri cat-TC* (n=1), tet(W) (n=40). *L. plantarum*: *ANT(3")-IIa* (n=2), *ANT(6)-Ia* (n=1), *catA8* (n=1), *ErmB* (n=1), *lnuA* (n=2), *msrC* (n=1), *TEM-1* (n=1), *TEM-181* (n=1), *tet(C)* (n=1), *tet(M)* (n=5), *tet(S)* (n=1). *L. delbrueckii*: *TEM-116* (n=1). *L. helveticus*: *lnuA* (n=2). *L. lactis*: *ErmB* (n=1), *lmrD* (n=20), *tet(M)* (n=1), *tet(S)* (n=4). *L. brevis*: *lnuA* (n=1). *S. thermophilus*: *ErmB* (n=2), *tet(S)* (n=2). The proportion of iMGE- and plasmid-associated mobile ARGs identified in a given bacterial species is shown in Fig. 12.

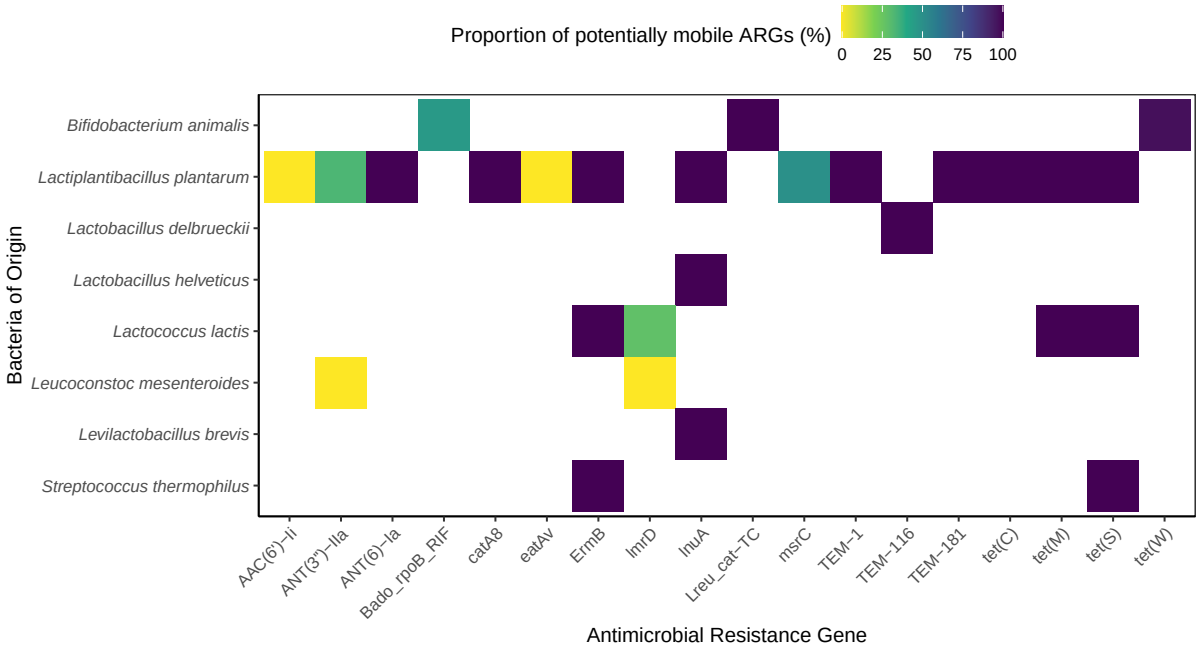

Figure 12.: Proportion of potentially mobile ARGs. The proportion is the number of samples containing potentially mobile (linked to iMGE or plasmid) ARGs divided by the total number of samples containing ARGs. The gene *Limosilactobacillus reuteri cat-TC* and *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin are abbreviated as Lreu_cat-TC and Bado_rpoB_RIF, respectively. Samples were collected between 1901 and 2022 (n=169 isolates).

5.2 Other animal source interfaces

5.2.1 Canine saliva

5.2.1.1 Bacteriome

By taxon classification, the number of reads aligning to bacterial genomes differed in the samples. In the saliva, median bacterial read count of the samples was 4.3×10^6 (IQR: 3.4×10^6). A total of 16 major bacterial genera were detected within the saliva samples, out of which several aerobic and anaerobic genera often become isolated from infected dog bite wounds. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the saliva samples is shown in Figure 13.

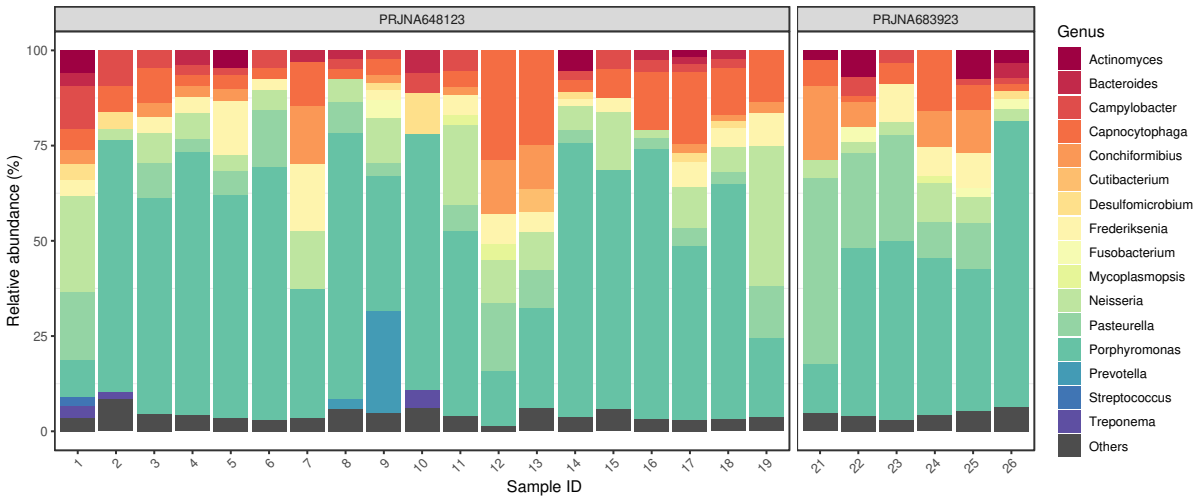

Figure 13.: Saliva core bacteriome. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the samples. In the sample No. 20, no reads were classified to bacteria.

In the saliva samples, the dominant genera (with mean prevalence) in descending order were *Porphyromonas* (49%), *Prevotella* (15%), *Pasteurella* (12%), *Neisseria* (10%), *Capnocytophaga* (9%), *Conchiformibius* (7%), *Frederiksenia* (7%), *Cutibacterium* (6%), *Actinomyces* (5%), *Campylobacter* (4%), *Desulfomicrobium* (4%), *Bacteroides* (3%), *Fusobacterium* (3%), *Mycoplasmopsis* (3%), *Treponema* (3%) and *Streptococcus* (2%). In the sample No. 20, no reads were classified to bacteria.

5.2.1.2 Resistome and mobilome

Applying the above-mentioned filtering conditions, we identified 69 ARGs that are presented together with their prevalence rates within the samples and the drug classes that these ARGs affect adversely in Table 6. These ARGs per sample with their coverage and the sequence identity rate are shown in Figure 20 (see page 54.). As a result of the taxon classification on the contigs harboring the ARGs, it was possible to predict the bacterial species of putative origin for all but ten genes (Table 7).

The dominant mechanisms of identified ARGs were antibiotic inactivation (47.69%), antibiotic target protection (23.41%), antibiotic target alteration (15.90%), antibiotic efflux (7.80%) and antibiotic target replacement (5.20%).

Table 6.: Identified ARGs and the drug classes affected by them. The frequency columns show how many samples the genes occurred in.

ARG(s)	Frequency n	Frequency %	Drug Class
aac(6')-Im	2	7.7	aminoglycoside
aad(6)	2	7.7	aminoglycoside
aadA2	1	3.8	aminoglycoside
aadA3	1	3.8	aminoglycoside
aadA5	1	3.8	aminoglycoside
aadA15	1	3.8	aminoglycoside
aadS	12	46.2	aminoglycoside
acrA	1	3.8	cephalosporin, fluoroquinolone, glycylcycline, penam, phenicol, rifamycin, tetracycline, triclosan
ant(2")-Ia	1	3.8	aminoglycoside
ant(3")-IIa	1	3.8	aminoglycoside
ant(6)-Ib	1	3.8	aminoglycoside
aph(2")-IIa	3	11.5	aminoglycoside
aph(3")-Ib	10	38.5	aminoglycoside
aph(3')-Ia	5	19.2	aminoglycoside
aph(3')-IIa	1	3.8	aminoglycoside
aph(3')-IIIa	4	15.4	aminoglycoside
aph(6)-Id	10	38.5	aminoglycoside
bacA	1	3.8	peptide
blaACT-12	1	3.8	carbapenem, cephalosporin, cephamycin, penam
blaOXA-2	12	46.2	carbapenem, cephalosporin, penam
blaOXA-85	1	3.8	carbapenem, cephalosporin, penam
blaOXA-119	1	3.8	carbapenem, cephalosporin, penam
blaOXA-347	16	61.5	carbapenem, cephalosporin, penam
blaROB-1	21	80.8	cephalosporin, penam
blaROB-9	1	3.8	cephalosporin, penam
blaROB-10	3	11.5	cephalosporin, penam
blaTEM-116	2	7.7	cephalosporin, monobactam, penam, penem
catIII	2	7.7	phenicol
cfxA2	20	76.9	cephamycin
cmlA9	1	3.8	phenicol
dfrA14	3	11.5	diaminopyrimidine
emrE	1	3.8	macrolide
emrK	1	3.8	tetracycline
ereA	1	3.8	macrolide
ermB	9	34.6	lincosamide, macrolide, streptogramin
ermF	18	69.2	lincosamide, macrolide, streptogramin
ermG	2	7.7	lincosamide, macrolide, streptogramin
ermX	1	3.8	lincosamide, macrolide, streptogramin
fosA2	1	3.8	fosfomycin
gadW	1	3.8	fluoroquinolone, macrolide, penam
gadX	1	3.8	fluoroquinolone, macrolide, penam
lnuB	2	7.7	lincosamide
lnuC	2	7.7	lincosamide
lsaE	2	7.7	lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
mdtN	1	3.8	acridine dye, disinfecting agents and intercalating dyes, nucleoside
mef(En2)	12	46.2	macrolide
mel	5	19.2	lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline
oqxA	1	3.8	diaminopyrimidine, fluoroquinolone, glycylcycline, nitrofuran, tetracycline
pgpB	23	88.5	peptide
qacL	2	7.7	disinfecting agents and intercalating dyes
SAT-4	1	3.8	nucleoside
sul1	7	26.9	sulfonamide
sul2	8	30.8	sulfonamide
tet32	19	73.1	tetracycline
tet44	1	3.8	tetracycline
tetH	3	11.5	tetracycline
tetM	5	19.2	tetracycline
tetO	18	69.2	tetracycline
tetQ	20	76.9	tetracycline
tetS	1	3.8	tetracycline
tetW	5	19.2	tetracycline
tetWNW	5	19.2	tetracycline
tetX	10	38.5	glycylcycline, tetracycline
tetX1	1	3.8	tetracycline
tetX4	1	3.8	glycylcycline, tetracycline
tetX5	10	38.5	tetracycline
tetY	1	3.8	tetracycline
tetZ	1	3.8	tetracycline
ugd	1	3.8	peptide

Table 7.: Identified ARGs and the predicted bacterial species of origin. For ten genes (*aadA5*, *aadA15*, *ant(2")-Ia*, *bacA*, *blaACT-12*, *cmlA9*, *fosA2*, *oqxA*, *tetX1* and *tetY*), no species-level prediction was obtained as to which bacterium the contig carrying the gene might have originated from.

ARG(s)	Bacteria of Origin
aac(6')-Im	*Clostridioides difficile*
aad(6)	*Enterococcus faecium*, *Staphylococcus aureus*
aadA2	*Acinetobacter baumannii*
aadA3	*Neisseria animaloris*
aadS	*Bacteroides fragilis*, *Capnocytophaga* sp. H2931, *Capnocytophaga* sp. H4358, *Capnocytophaga stomatis*, *Chryseobacterium indologenes*, *Riemerella anatipestifer*
acrA	*E. coli*
ant(3")-IIa	*Aeromonas hydrophila*
ant(6)-Ib	*Amedibacterium intestinale*
aph(2")-IIa	*C. difficile*
aph(3")-Ib	*Corynebacterium* sp. 1959, *Haemophilus parahaemolyticus*, *Klebsiella michiganensis*, *Moraxella bovis*, *Variovorax* sp. SRS16
aph(3')-Ia	*Corynebacterium* sp. 1959, *E. coli*, *Klebsiella quasipneumoniae*, *Variovorax* sp. PAMC28562
aph(3')-IIa	*E. coli*
aph(3')-IIIa	*E. faecium*, *S. aureus*, *Streptococcus agalactiae*
aph(6)-Id	*Corynebacterium* sp. 1959, *K. michiganensis*, *Neisseria shayeganii*, *Providencia rettgeri*, *Variovorax* sp. SRS16
blaOXA-2	*A. baumannii*, *A. hydrophila*, *P. aeruginosa*
blaOXA-85	*Fusobacterium ulcerans*
blaOXA-119	*Geobacter sulfurreducens*
blaOXA-347	*Alistipes shahii*, *B. fragilis*, *Bacteroides heparinolyticus*, *Capnocytophaga* sp. H2931, *Capnocytophaga* sp. H4358, *C. stomatis*, *Chryseobacterium* sp. POL2, *Elizabethkingia anophelis*, *Empedobacter brevis*, *Myroides odoratimimus*, *R. anatipestifer*
blaROB-1	*Actinobacillus pleuropneumoniae*, *Conchiformibius steedae*, *Glaesserella parasuis*, *Haemophilus haemolyticus*
blaROB-9	*G. parasuis*
blaROB-10	*Bibersteinia trehalosi*
blaTEM-116	*E. coli*
catIII	*K. michiganensis*
cfxA2	*Capnocytophaga cynodegmi*, *Parabacteroides distasonis*, *Porphyromonas cangingivalis*, *Porphyromonas crevioricanis*, *Porphyromonas gingivalis*, *Tannerella forsythia*
dfrA14	*K. michiganensis*, *Ochrobactrum anthropi*
emrE	*E. coli*
emrK	*E. coli*
ereA	*Geobacter daltonii*
ermB	*Enterococcus gilvus*, *Enterococcus* sp. FDAARGOS_375, *S. agalactiae*, *Streptococcus suis*
ermF	*A. shahii*, *B. fragilis*, *C. stomatis*, *C. indologenes*, *P. distasonis*, *P. cangingivalis*, *Prevotella intermedia*, *R. anatipestifer*
ermG	*C. difficile*
ermX	*Trueperella pyogenes*
gadW	*E. coli*
gadX	*E. coli*
lnuB	*S. suis*
lnuC	*Streptococcus equi*, *Streptococcus gwangjuense*
lsaE	*S. suis*
mdtN	*E. coli*
mef(En2)	*B. fragilis*, *P. cangingivalis*, *P. gingivalis*, *P. intermedia*
mel	*Streptococcus pluranimalium*
pgpB	*P. gingivalis*
qacL	*P. aeruginosa*
SAT-4	*S. aureus*
sul1	*A. hydrophila*, *P. aeruginosa*
sul2	*Corynebacterium* sp. 1959, *H. parahaemolyticus*, *K. michiganensis*, *M. bovis*, *Pasteurella multocida*, *P. rettgeri*
tet32	*Blautia hansenii*, *Bulleidia* sp. zg-1006, *C. difficile*, *Clostridium cellulovorans*, *Eubacterium maltosivorans*, *Eubacterium* sp. NSJ-61, *Faecalibacterium prausnitzii*, *Lachnoanaerobaculum umeaense*, *Peptoclostridium acidaminophilum*, *Roseburia intestinalis*, *Streptococcus anginosus*, *Streptococcus constellatus*, *S. equi*
tet44	*A. intestinale*
tetH	*Proteus vulgaris*, *Pseudomonas putida*
tetM	*C. difficile*, *Enterococcus faecalis*, *Mogibacterium pumilum*, *Streptococcus* sp. FDAARGOS_521
tetO	*C. difficile*, *Enterococcus hirae*, *Murdochiella vaginalis*, *Streptococcus acidominimus*, *S. anginosus*, *S. constellatus*, *S. equi*, *S. suis*
tetQ	*Alistipes indistinctus*, *Bacteroides dorei*, *B. heparinolyticus*, *Bacteroides ovatus*, *Bacteroides* sp. HF-5287, *Phocaeicola coprophilus*, *P. crevioricanis*, *Prevotella fusca*, *P. intermedia*
tetS	*Streptococcus parauberis*
tetW	*Enterocloster bolteae*, *F. prausnitzii*, *Megasphaera stantonii*, *S. suis*
tetWNW	*Filifactor alocis*, *M. pumilum*
tetX	*B. fragilis*, *P. distasonis*, *P. intermedia*, *R. anatipestifer*
tetX4	*R. anatipestifer*
tetX5	*B. fragilis*, *C. stomatis*, *R. anatipestifer*
tetZ	*Rothia nasimurium*
ugd	*E. coli*

Many of the identified ARGs are harbored by iMGEs, prophages or plasmids. The frequencies of iMGEs, prophages and plasmids associated with ARGs by bacteria of origin are summarized in Figure 14. Some genes could have been attached to two of the above-mentioned mobility groups in the genome of one species, including the iMGE and prophage co-appearance

of aminoglycoside resistance encoding *aad(6)* and *aph(3')-IIIa* in *E. faecium*; the iMGE and plasmid co-appearance of *aph(3')-Ia* in *Corynebacterium* sp. 1959 and *K. quasipneumoniae*; *aph(3')-Ia* and *aph(6)-Id* in *Variovorax* sp. SRS16; *aph(3")-Ib* in *Variovorax* sp. PAMC28562; tetracycline resistance encoding *tetM* in *E. faecalis*; and prophage and plasmid co-appearance of macrolide, lincosamide and streptogramin resistance encoding *ermB* in *Enterococcus* sp. FDAARGOS_375. The *blaOXA-2*, *blaOXA-347* and *blaTEM-116* genes associated with amoxicillin–clavulanate resistance all appeared in plasmids in various species; moreover, *blaOXA-2* was associated with both an iMGE and a plasmid in the genome of *P. aeruginosa*.

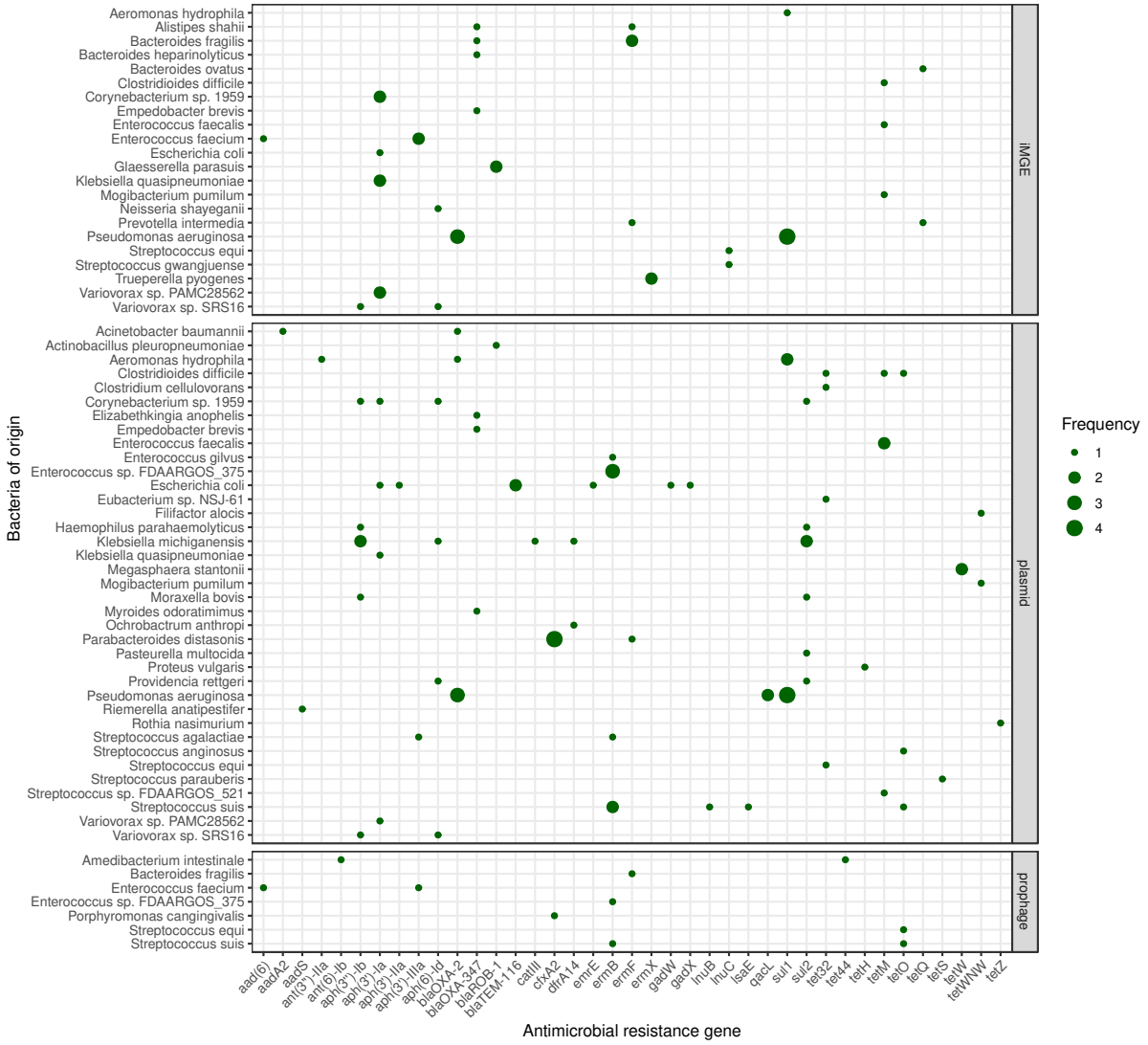

Figure 14.: Mobile ARG frequency by bacteria of origin. The size of the dots indicates the occurrence frequency of the given gene flanked by iMGE, positioned in plasmid or prophage.

5.2.2 Pig feces

5.2.2.1 Bacteriome

After the DNA extraction, library preparation and sequencing, 5,519,047 paired-end reads were generated. 1,026,974 reads belonged to the kingdom of Bacteria. The relative abundance rates of the bacterial genera constituting the core bacteriome in descending order was the following:

Escherichia (25,4%), *Prevotella* (11,7%), *Bacteroides* (9,4%), *Megasphaera* (4,35%), *Faecalibacterium* (4,33%), *Desulfovibrio* (3,91%), *Ruminococcus* (3,5%), *Dysosmobacter* (3,44%), *Blautia* (2,8%), *Lactobacillus* (2,5%), *Streptomyces* (2,43%), *Acidaminococcus* (2,41%), *Pseudomonas*(2,21%), *Muribaculum* (1,99%), *Clostridium* (1,88%), *Lachnoclostridium* (1,85%), *Streptococcus* (1,82%), *Paenibacillus* (1,75%), *Oscillibacter* (1,68%), *Bacillus* (1,48%), *Clostridioides* (1,41%), *Ruthenibacterium* (1,34%), *Flavonifractor* (1,29%), *Roseburia* (1,18%), *Collinsella* (1,11%), *Alistipes* (1,05%), *Corynebacterium* (0,9%), and *Selenomonas* (0,89%). *Escherichia spp.*, *Prevotella spp.* and *Bacteroides spp.* were relatively the most abundant in the sample. The detailed composition of the core bacteriome at genus level is shown in Fig. 15.

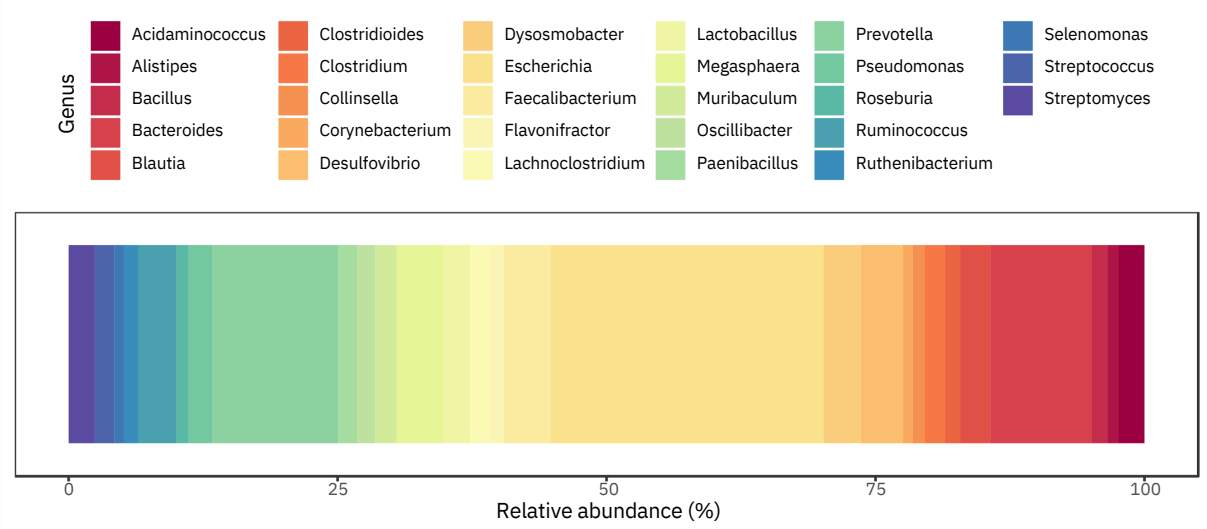

Figure 15.: Core bacteriome composition. Relative abundance of bacterial genera.

5.2.2.2 Resistome and mobilome

In addition to the taxonomic classification of the bacteria, the ARG content of the bacteriome was also assessed. By the identification of ARGs, hits were retained if the open reading frame (ORF) spanned a minimum of 60% of the length of the reference gene and exhibited a minimum of 90% base sequence identity (see Table 8). For the antibiotic classes affected by ARGs, the ORFs identified with the highest coverage and sequence identity were extracted (Figure 16). In total, 54 different ARG types were identified, with the potential to affect the efficacy of at least 25 classes of antibiotics. Antibiotic classes and compound classes with antibiotic activity against which the predicted ARGs appeared with perfect coverage and base sequence identity between the identified ORFs and the reference sequences were the following: acridine dye, aminocoumarines, aminoglycosides, benzalkonium chloride, carbapenems, cephalosporins, cefamycins, elfamycins, fluoroquinolones, phosphomycin, glycylcyclines, lincosamides, macrolides, monobactams, nitroimidazoles, nucleosides, penams, penems, polypeptides, phenicols, phenols, rhodamines, rifamycins, streptogramines, tetracyclines and triclosans.

Table 8.: The set of antmicrobial resistance genes detected.

Antimicrobial resistance gene (ARG)	Coverage %	Identitiy %	Drug Class
AAC(6)-Im	100	97.74	aminoglycoside
ACI-1	100	100	cephalosporin, penam, penem
acrB	81.03	100	cephalosporin, phenicol, fluoroquinolone, glycylcycline, penam, rifamycin, tetracyclin, triclosan
acrD	100	100	aminoglycoside
AcrF	100	100	cephalosporin, cephamycin, fluoroquinolon, penam
AcrS	100	99.71	cephalosporin, cefamycin, fluoroquinolone, penam
APH(2")-IIa	100	95.65	aminoglycoside
baeR	100	99.17	aminoglycoside, aminocoumarin
CfxA6	67.37	99.1	cefamycin
cpxA	100	100	aminoglycoside, aminocoumarin
CRP	62.38	98.47	fluoroquinolone, macrolide, penam
emrA	100	99.74	fluoroquinolone
emrB	100	100	fluoroquinolone
emrK	100	100	tetracycline
emrY	100	99.41	tetracycline
eptA	100	99.82	polypeptide
ErmG	65.98	98.14	lincosamide, macrolide, streptogramin
E. coli acrA	92.7	100	cephalosporin, phenicol, fluoroquinolone, glycylcycline, penam, rifamycin, tetracycline, triclosan
E. coli acrR multidrug AMR coding variant	100	100	cephalosporin, phenicol, fluoroquinolone, glycylcycline, penam, rifamycin, tetracycline, triclosan
E. coli ampC -lactamase	100	97.08	cephalosporin, penam
E. coli ampH -lactamase	100	99.74	cephalosporin, penam
E. coli EF-Tu puromycin resistance coding variant	94.87	99.74	elfamycin
E. coli ermE	100	97.27	macrolide
E. coli GipT fosfomycin resistance coding variant	100	99.78	fosfomycin
E. coli marA AMR coding variant	100	98.61	cephalosporin, phenicol, fluoroquinolone, glycylcycline, penam, rifamycin, tetracycline, triclosan
E. coli mdfA	100	97.07	benzalkonium chloride, rhodamine, tetracycline
E. coli soxR AMR coding variant	100	100	cephalosporin, phenicol, fluoroquinolone, glycylcycline, penam, rifamycin, tetracycline, triclosan
E. coli soxS AMR coding variant	100	100	cephalosporin, cefamycin, phenicol, fluoroquinolone, glycylcycline, carbapenem, monobactam, penam, penem, rifamycin, tetracycline, triclosan
evgA	100	100	macrolide, fluoroquinolone, penam, tetracycline
evgS	100	99.75	macrolide, fluoroquinolone, penam, tetracycline
gadX	100	100	macrolide, fluoroquinolone, penam
H-NS	70.07	100	cephalosporin, cefamycin, fluoroquinolone, macrolide, penam, tetracycline
kdpE	100	99.56	aminoglycoside
marA	100	96.55	aminocoumarin
mdtA	100	98.55	aminocoumarin
mdtB	98.85	99.61	aminocoumarin
mdtE	100	100	fluoroquinolone, macrolide, penam
mdtF	100	100	fluoroquinolone, macrolide, penam
mdtG	100	100	fosfomycin
mdtH	100	99.75	fluoroquinolone
mdtM	100	97.07	acridine, phenicol, fluoroquinolone, lincosamide, nucleoside
mdtN	100	100	acridine, nucleoside
mdtO	100	99.85	acridine, nucleoside
mdtP	100	99.8	acridine, nucleoside
mphB	86.71	98.53	macrolide
msbA	100	100	nitroimidazole
PmrF	100	100	polypeptide
tet(A)	100	97.54	tetracycline
tet(Q)	94.98	96.79	tetracycline
TolC	99.6	100	aminoglycoside, aminocoumarin, cephalosporin, cefamycin, phenicol, fluoroquinolone, glycylcycline, carbapenem, macrolide, penam, penem, polypeptide, rifamycin, tetracycline, triclosan
YojI	100	100	polypeptide

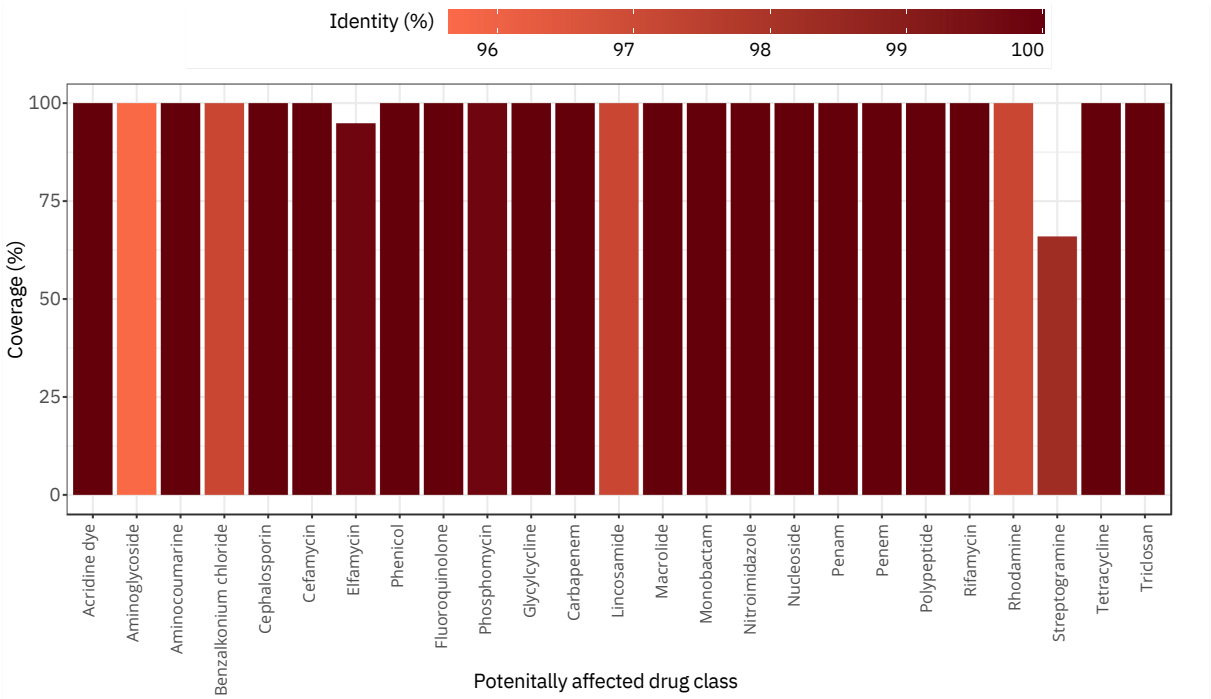

Figure 16.: Maximal ORF coverage and identity by antibiotics. The ORF covered proportion of the reference sequence is indicated on axis Y in percentage. The darkening of the column colours is in parallel with the growth in the identity of the reference sequences and the detected ARGs. As antibiotic groups may be linked to more than one ORFs, only those antibiotics are indicated on this figure that are linked to an ORF having the most outstanding coverage and identity to the reference ARG sequence.

Antibiotic groups with clinical significance against which ARGs were predicted to be present with perfect coverage and base sequence identity based on the ORFs identified in the feces were aminoglycosides, penicillins and cephalosporins, monobactams, carbapenems, macrolides, lincosamides, phenicols, polypeptides (polymixins, bacitracin), rifamycins, streptogramins and tetracyclines. Of particular importance is the emergence of the *eptA* and *pmrF* genes, which, if phenotipically expressed, confer resistance to polymyxins and colistin, respectively. Furthermore, of similar clinical importance, ARGs were detected that confer resistance to carbapenems. Although several gene families conferring resistance to fluoroquinolones have been detected, the two most important gene families related to this drug class, *gyr* and *par* were not included in our sample. The majority of contigs containing ARGs yielded a classification at the class level. In light of these findings, it can be stated that 81% of the identified ARGs were classified as Gammaproteobacteria, 3.8% as Clostridia and 1.9% as Bacteroidia. The origins of seven ARGs were identified at the species level, indicating the probable source of the gene (Figure 17). Four of these ARGs originated from *Escherichia coli*, two from *Clostridioides difficile* and one from *Bacteroides ovatus*.

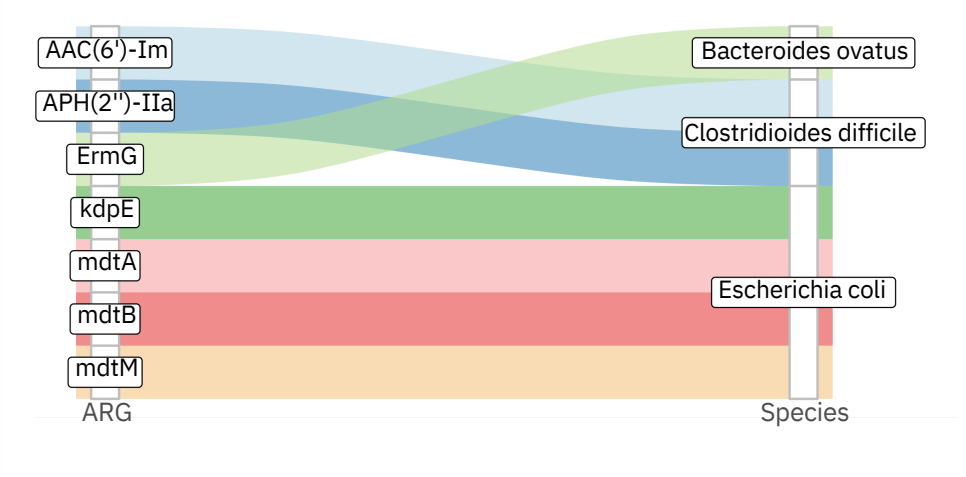

Figure 17.: The most probable origin of ARGs. ARG harbouring contigs that could be linked to bacterial species. By other ARGs than these, the species-level origins were not decidable.

The MGE analysis did not identify any ORFs that could facilitate the mobility of any of the ARGs within 10 adjacent ORFs. However, based on other MGE analysis steps, contigs harbouring *AAC(6')-Im, APH(2'')-IIa, baeR, CfxA6, Escherichia coli marR* variant encoding AMR and *marA* are very likely to have plasmid origins.

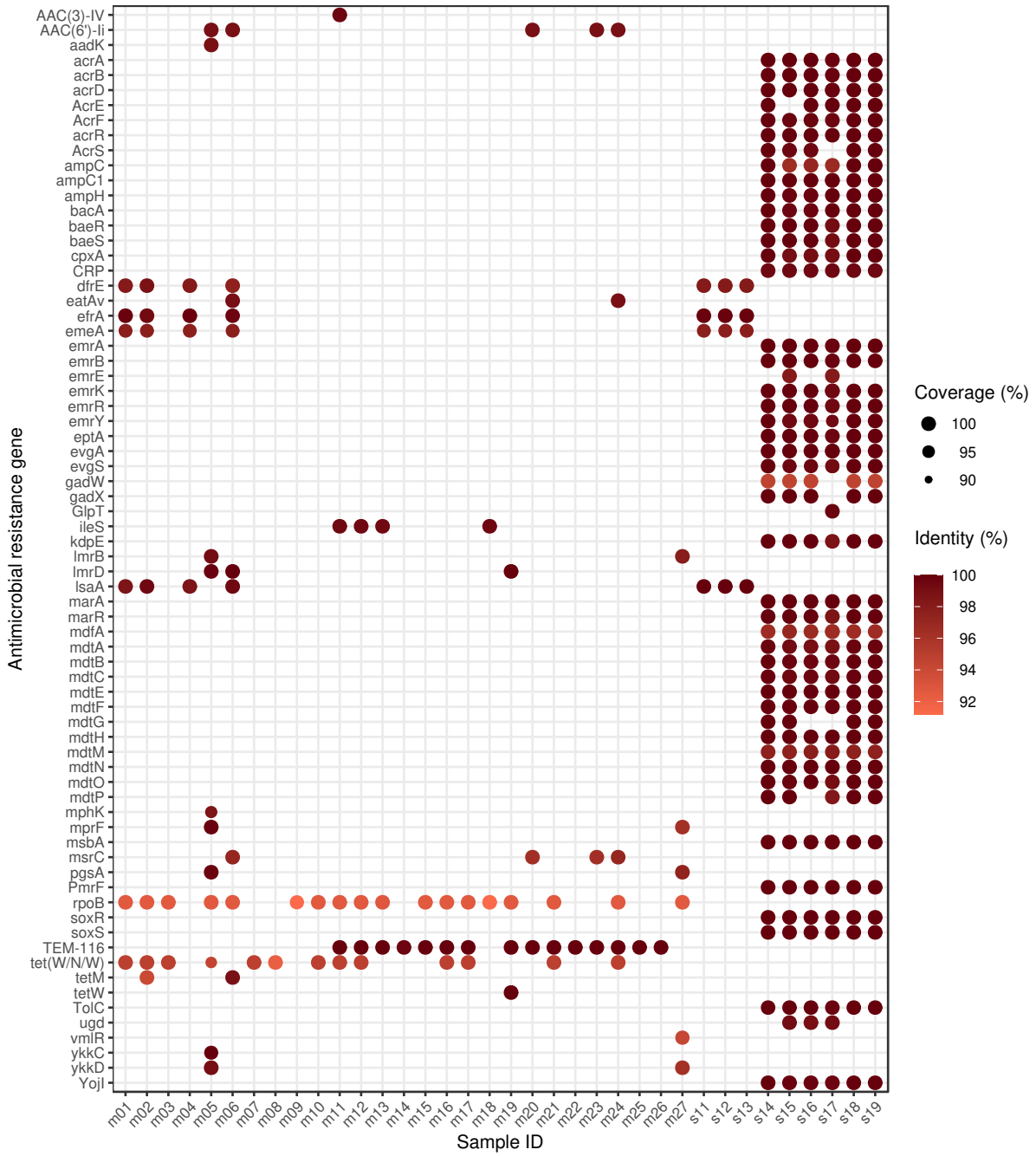

Figure 18.: Identified ARGs by samples. For each sample–ARG combination, only the best finding is plotted. The size and the colour of the dots correspond to the coverage and the sequence identity of hits on reference genes, respectively. In samples s01–s10 and s20, there was no identifiable ARG. The gene names that are too long have been abbreviated (*acrA*: *Escherichia coli acrA*; *acrR*: *E. coli acrR* with mutation conferring multidrug antibiotic resistance; *ampC*: *E. coli ampC* beta-lactamase; *ampC1*: *E. coli ampC1* beta-lactamase; *ampH*: *E. coli ampH* beta-lactamase; *emrE*: *E. coli emrE*; *GlpT*: *E. coli GlpT* with mutation conferring resistance to fosfomycin; *ileS*: *Bifidobacterium bifidum ileS* conferring resistance to mupirocin; *marR*: *E. coli marR* mutant conferring antibiotic resistance; *mdfA*: *E. coli mdfA*; *mprF*: *Bacillus subtilis mprF*; *pgsA*: *B. subtilis pgsA* with mutation conferring resistance to daptomycin; *rpoB*: *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin; *soxR*: *E. coli soxR* with mutation conferring antibiotic resistance; *soxS*: *E. coli soxS* with mutation conferring antibiotic resistance).

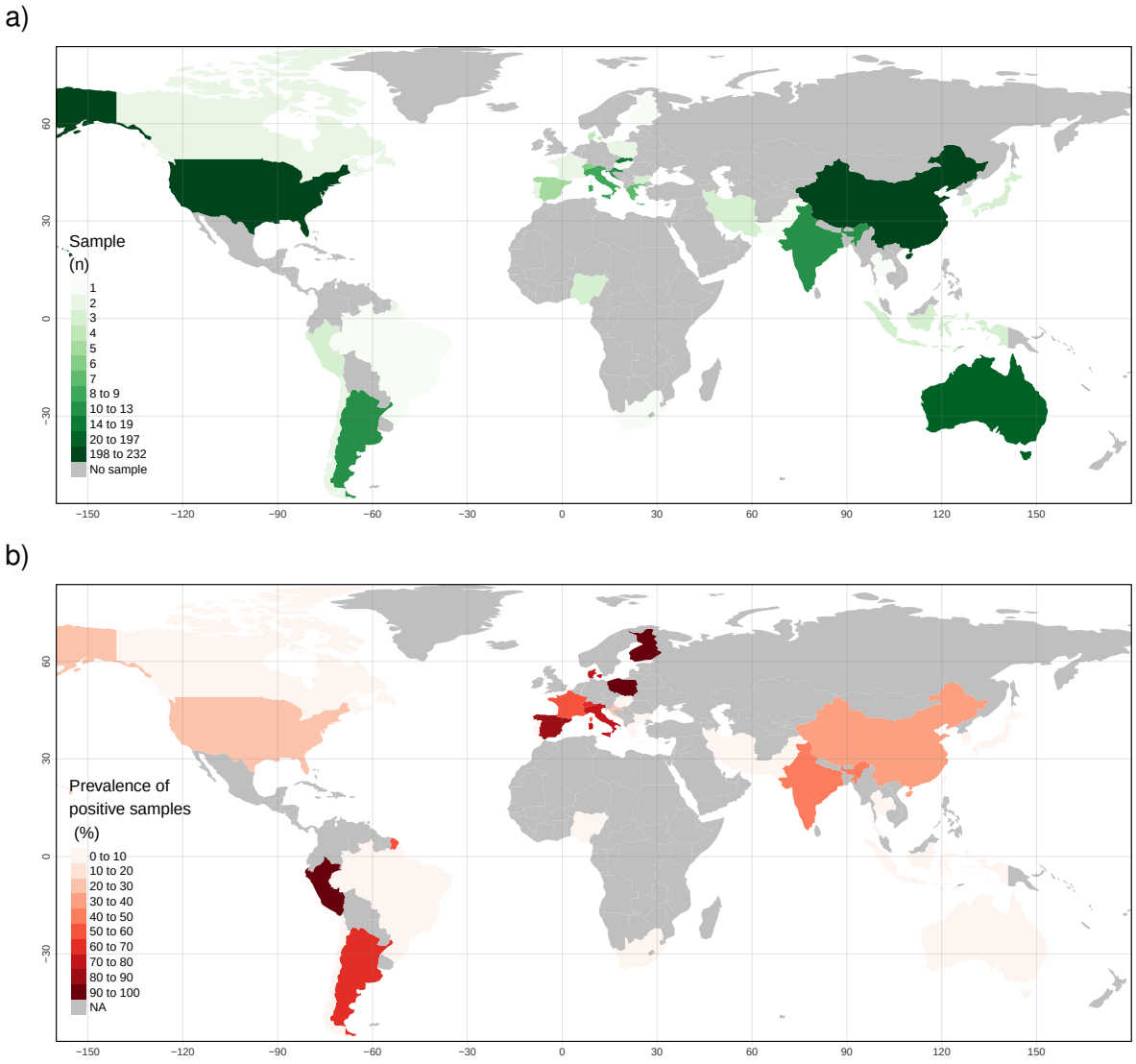

Figure 19.: Geographic distribution of sample number and prevalence of ARG positive samples. Map of the number (a) of FFP samples from 31 countries and the prevalence (b) of ARG-containing samples. In Argentina (number of positives: 6 / number of all samples: 10), Australia (1/20), Brazil (0/1), Bulgaria (0/3), Canada (0/2), Chile (0/2), China (60/198), Croatia (2/10), Denmark (3/4), Finland (1/1), France (1/2), Greece (0/7), Hungary (0/1), India (4/10), Indonesia (0/3), Iran (0/3), Italy (6/8), Japan (0/3), Korea (0/2), Nigeria (0/3), Pakistan (0/1), Peru (3/3), Poland (2/2), Portugal (0/2), Slovakia (0/14), South Africa (0/1), Spain (4/5), Switzerland (4/6), Taiwan (0/1), Thailand (0/1), United States (67/232). We could not identify the country of origin for 18 samples, 5 of which were ARG-positive. Samples were collected between 1901 and 2022 (n=579 isolates).

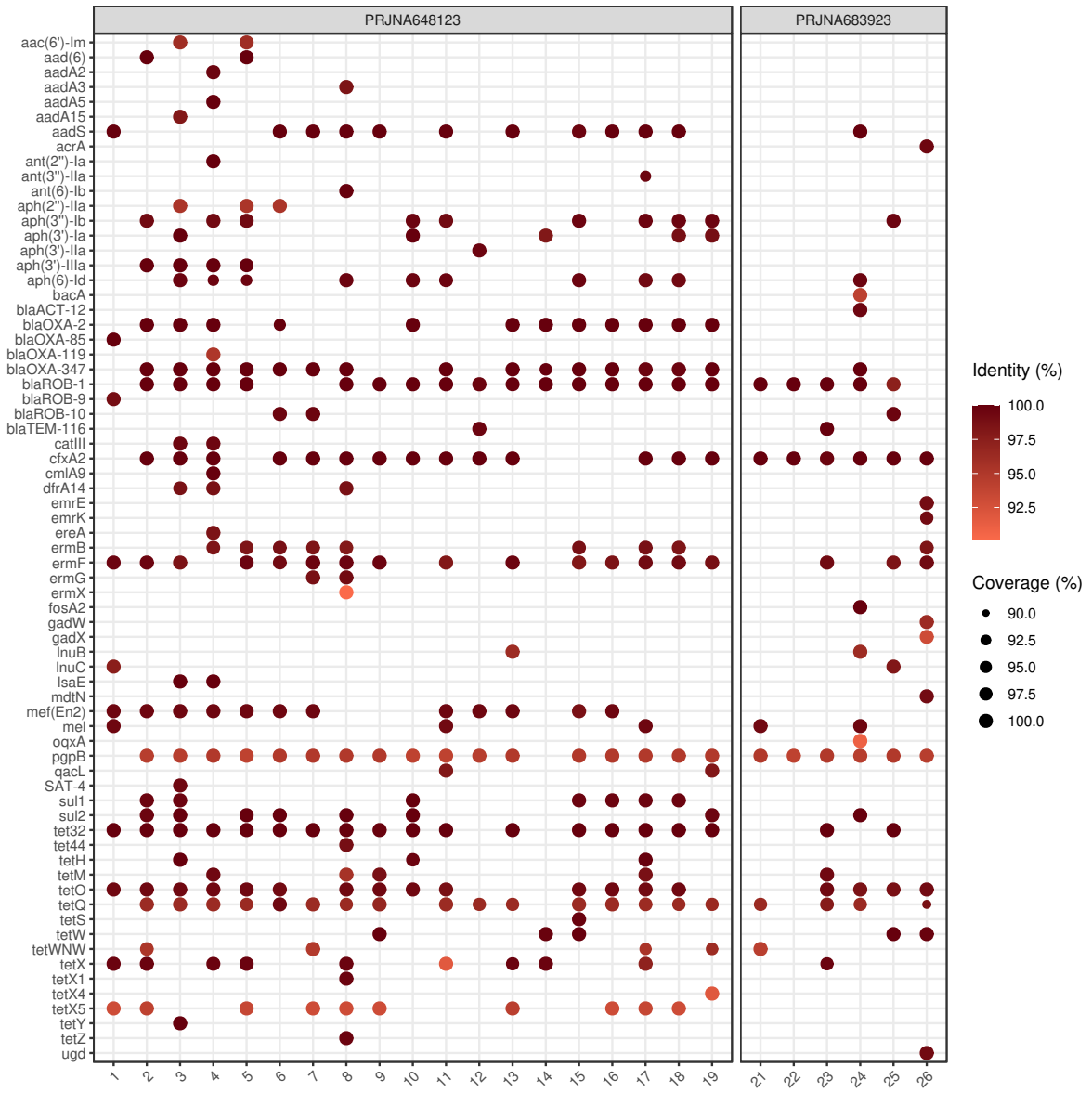

Figure 20.: Identifed ARGs by samples. For each sample–ARG combination, only the best finding is plotted. The size and the color of the dots correspond to the coverage and the sequence identity of hits on reference genes, respectively. In sample No. 20, there was no identifiable ARG. The gene names that are too long are abbreviated (*acrA*: *E. coli acrA*; *emrE*: *E. coli emrE*).

6. Discussion

6.1 Foods and dietary supplements

6.1.1 Raw milk

AMR is a natural feature of microorganisms that have originally occurred as a means of defence in the rivalry amongst the members of the microbiotas [3]. The ubiquity of ARGs is beyond question. Genes against antibiotics are present both in non-pathogenic and pathogenic bacteria. With the extended agricultural and clinical use of antibiotics, the number of ARGs are on the rise, and the growing number and spread of multi-resistant bacteria strains pose a global threat to global health. According to the CDC's Antibiotic Resistance Threats in the United States, 2019 report [168], more than 2.8 million antibiotic-resistant infections occur in the U.S. each year, and more than 35,000 people die as a result. In addition, 223,900 cases of *Clostridioides difficile* occurred in 2017 and at least 12,800 people died. Dedicated prevention and infection control efforts in the U.S. and around the world are working to reduce the number of infections and deaths caused by antibiotic-resistant germs. However, the number of people facing antibiotic resistance is still too high. The AMR Threats Report warns that not only people but also animals carry bacteria in their guts which may include antibiotic-resistant bacteria either with intrinsic or with acquired ARGs [28]. Beyond disease control, animals may be given antibiotics for growth promotion or increased feed efficiency. Since bacteria are exposed to low doses of the drugs over a long period, this inappropriate antibiotic use can lead to the development of resistant bacteria [168]. The CDC report notes that when animals are slaughtered and processed for food, resistant germs in the animal gut can contaminate meat or other animal products, but do not mention the possible contamination of milk.

Detected ARGs in raw milk can be transferred from non-pathogens to pathogens via HGT. The over-expression of such genes, e.g. *norA* (regulated by *mgrA*) and *mepA* (regulated by *mepR*) coding multidrug efflux pumps confer resistance to fluoroquinolones (including norfloxacin or ciprofloxacin) and even disinfectants [169–172]. Ciprofloxacin is a broad-spectrum antibiotic used to treat a variety of bacterial infections, including intra-abdominal, respiratory tract, skin, urinary tract, and bone and joint infections. Norfloxacin might be used for uncomplicated urinary tract infections (including cystitis) or the prevention of spontaneous bacterial peritonitis in cirrhotic patients, among others. *MepA* was also shown to result in resistance to tigecycline [173], an antibiotic that was developed to tackle complicated infections caused by multiresistant bacteria such as *Staphylococcus aureus*, *Acinetobacter baumannii*, and *E. coli*.

The two milk samples differ both in the composition of core bacteriome and the ARG abundance, each sample contains both saprophytes and facultative pathogens. In sample A the bacteriome was dominated by Gram-positive bacteria. Furthermore, most of the contigs harbouring ARG were classified taxonomically belonging to Gram-positive bacteria. In sample B, the Gram-negative bacteria governed the bacteriome. So the lower ARG abundance in sample B might come from the lower proportion of Gram-positives. Nevertheless, in sample B, not just the number of detected ARGs was lower, but the maximal coverage of the ARGs as well. One may find the reason for this phenomenon, the lower sequencing depth of sample B. The iden-

tity of these ORFs with the reference ARGs are very high so we may assume the assembled ORFs originated from ARGs. Accordingly, the possible reason of the lower coverage of ARGs may be caused by the insufficient read counts for assembly the complete ORFs. One possible argumentation of the ARGs' difference between sample A and B may be derived from the fact that health issues (e.g. mastitis) are relatively more common in large scale farms. Since the use of antibiotics is more permissive in veterinary practice - compared to human medicine - in the treatment of bacterial infections, it places a selective pressure on the bacteria of herds, what might increase the frequency and the diversity of ARGs.

Our results show that indeed ARGs can be present in raw milk. However, it should be the subject of further research to identify how resistant bacterial DNA gets into the milk. It may either be present in the cow's udder or it may mix into the milk as contamination during or after milking.

At raw milk's environment of origin (dairy farms), the use of antimicrobial agents is widespread. Consequently, the microbiome of this product may show relatively high levels of resistance. Without heat-treatment, bacteria that are present in raw milk are not hindered from further multiplication what results in the amplification of their resistance genes either. Such a rise in the number of ARGs may increase the risk of HGT events. This risk may even be higher in case of mobile ARGs (e.g. *blaZ*, which was detected on a plasmid and near to a phage integrase ORF).

Beyond human intervention, there are natural mechanisms that limit ARG-transfer [24]. First of all, donor and recipient populations need to be present at the same physical space [174] and reach a specific critical density to ensure proper connectivity for a successful gene transfer event. Chances for a series of HGT events among two physically distant populations are relatively low except for the case when there is positive selection driven by any factors (e.g. selection by antibiotics). The second factor arises from the fact that genes encoding resistance against the same compounds may limit each other's spread. A population owning genes against a particular antibiotic is not under selective pressure to gain any other ARGs with the same effect. As a conclusion of earlier evolutionary steps, possession of resistance determinants of the same substrate profiles are possible. However, in a population where the distribution of these genes is stable, the chances of new recruitment are lower. Tertiary, acquisition of resistance genes sets metabolic costs deriving from the transfer and integration mechanisms needed. These costs vary by each ARG, and only affordable genes are spread [24].

Even though the bacterial compositions of milk is affected by the heat treatment [175, 176], the question may arise whether the ARG content of raw and pasteurized milk are different. In water, DNA degradation starts by 90 °C[177]. The HTST pasteurization (high temperature/short time) is performed at 72 °C for 15-40 seconds, while ultra-pasteurization (UHT) is at 135 °C for 1-2 seconds. Summarizing this information, one may conclude that the resistomes do not differ significantly in HTST and raw milk. On the other hand in UHT milk some DNA degradation might be suspected. Nevertheless, some aspects are broadening the picture, that are worth taking into consideration. First of all, in raw milk, the members of the bacteriota remain viable and may multiply depending on the storing temperature. The proliferation of bacterial cells increases the amount of the sample's extractable bacterial DNA content what appears in the results of the sequencing as raised bacterial read rates. Consequently, after the assembly of the reads, the

likelihood of having contigs containing ARGs is higher. Pasteurization kills 99.99% of bacteria; thus, their multiplication has a low significance. Secondly, the bacteriome of milk consumers (humans and animals) may gain the ARGs of the milk-resistome by transformation and transduction only [73], as pasteurization decreases the number of viable bacteria. In contrast, raw milk's higher viable bacterial cell count facilitates conjugation to the consumers' bacteriome while the above-mentioned HGT mechanisms [73] are also kept. Of course, this phenomenon rather has an impact on the risk of HGT than on the resistome of raw or pasteurized milk.
Nevertheless, heat-treatment of raw milk seems to be an advantageous and a more than considerable step that besides inhibiting the amplification of genes having a potential risk, makes active gene transfer mechanisms lose their significance. On the other hand, even though it reduces the number of multiplication cycles, after the lysis of cells free DNA fragments appear in the sample that may still be uptaken by newly arriving bacteria.
However, the interpretation of resistome studies is yet to be deepened. The combination of next-generation sequencing, metagenomic and computational methods provides valuable data on the presence of ARGs. Moreover, it makes it possible to find genes in full coverage and length, and to identify their taxonomical classes of origin and their exact sequential surroundings. Synteny with MGEs is a fact to be taken into consideration when examining the risks meant by an ARG. Thus, the combination of methods mentioned above serves as a core component of today's necessarily expanded AMR research.
As a means of evolutionary pressure, the use of antibiotics selects bacterial strains that have ARGs. Moreover, in the production animal sector, the application of such compounds increases not only the number of antibiotic-resistant bacterial strains but also the frequency of their appearance. After the consumption of animal products, these strains may meet the human microbiota, and the circumstances may be appropriate for the HGT derived spread of ARGs among these populations. This phenomenon unfolds a possible source of acquisition of human pathogens' AMR other than the direct presence of antibiotic residuals in animal products.
Our findings suggest the ARG content of unprocessed animal products may play a role in the development of AMR in human pathogens. Nevertheless, the generalization of these findings requires more comprehensive studies to transcend our results that are based on a limited sample size.

6.1.2 Probiotic dairy products

Studying ARGs that may enter the body with food, including fermented dairy products, can lead to critical health considerations. The characteristics of bacterial diversity and ARG abundance are well observable in both kefir and yogurt. ARG abundance is much higher in kefir than in yoghurt. One possible reason for this phenomenon could be the presence of fungi in kefir seed cultures. Since fungi may produce antibacterial toxins, bacteria having ARGs may gain a competitive advantage when coexisting with fungi.
Each bacterium (*Bifidobacterium animalis* [156], *Enterobacter hormaechei* [160, 165], *Lactobacillus acidophilus* [159, 160], *Lactobacillus delbrueckii* [159, 160], *Lactobacillus helveticus* [159, 160], *Lactobacillus kefiranofaciens* [159, 160, 178], *Lactobacillus plantarum* [159, 160, 179], *Lactococcus lactis* [157, 159, 160, 180], *Leuconostoc mesenteroides* [157, 159, 160,

180]) obtained from the taxon classification of contigs containing ARGs is widely used in the production of fermented dairy products. Li and colleagues [181] analyzed the ARG content of isolated bacteria from fermented drinks and yoghurts. According to their results, *APH(3")-III*, *APH(6')-APH(2")*, *sul1*, *tet(M)* were detectable in *Lactobacillus bulgaricus* strains, while *APH(3")-II*, *sul1*, *sul2*, *strA*, *strB*, *tet(M)* derived from *Streptococcus thermophilus*. In our study *APH(3')-IIb* gene belonging to the APH gene family, supposedly originated from *L. mesenteroides*. Similarly, Carr and colleagues [182] found a strong co-occurrence between *APH(3')-Ia* and *L. mesenteroides* in Chinese saliva samples. Further similarity with the results of Carr and colleagues [182] is that *lmrD* originated from *Lc. lactis*. Guo and colleagues [183] detected ARGs in *Lactobacillus* strains of fermented milk products. They detected *erm(B)*, *gyrA*, *rpoB*, *vanE*, *vanX* in *Lactobacillus casei*, gene *APH(3")-III*, *dfrD*, *erm(B)*, *gyrA*, *tet(W)*, *vanX* in *L. helveticus*, *erm(B)* and *vanX* in *L. plantarum*. We found the *poxtA* gene associated with *L. helveticus* and *L. plantarum*. The *emrB* gene was identified in a contig from the genome of *E. hormaechei*.

During the fermentation of milk, the bacteria in seed cultures (and in milk) multiply and dominate the beverage. If any of these bacteria harbour ARGs, the amount of these genes will be increased in the final products. Based on data generated by Walsh and colleagues (2016) *L. helveticus* and *L. kefiranofaciens* are the most probable origin of the contigs harbouring *poxtA*. Sequences containing *APH(3')-IIb* could have been stemmed from *L. mesenteroides*. According to Walsh and colleagues (2016), during fermentation the relative abundance of *L. kefiranofaciens* and *L. mesenteroides* increased [141]. Not surprisingly, in our reanalysis of the same data we found the same trends. While Marsh and colleagues (2013) presented similar changes of these species in kefir [184], Wurihan and colleagues (2019) showed opponent alterations in koumiss fermentation [185]. ARG abundances showed a positive association with the relative abundances of their most probable bacteria of origin. An increase in the relative abundance of *L. mesenteroides* was followed by the *APH(3')-IIb* abundance. In contrast, *poxtA* abundance dropped simultaneously with the decrease of the relative abundance of *L. kefiranofaciens*.

The two most abundant ARGs were *poxtA* and *APH(3')-IIb*, which were both presents in yoghurt and kefir samples. *PoxtA* (phenicol-oxazolidine-tetracycline resistance gene), an frequent ARG in Gram-positive bacteria, confers resistance to a wide range of critical antibiotics. The ABC-F class ATP binding ribosomal protection protein encoded by this gene is mainly present in *Enterococcus spp.* and *Staphylococcus spp.*. It was also identified in a methicillin-resistant *Staphylococcus aureus* (MRSA) strain that showed increased MIC to linezolid, a member of the oxazolidine class of ABs [186]. The study highlighted that *Staphylococcus spp.*, *Enterococcus spp.* and interestingly, *Pediococcus acidilactici* harbouring the gene are all of animal origin and can be spread horizontally with the help of MGEs. In line with other papers [187] the study suggests that phenicols and other antiribosomal agents used in veterinary medicine might have played a role in the selection of *poxtA*. This was also confirmed by Elghaieb and colleagues (2019), who identified the gene in cow milk and animal wastewater. As oxazolidines are prohibited in food animals, and phenicols are not permitted in dairy cattle in Europe, the source of these genes in Hungarian samples remains to be elucidated [188]. *Pseudomonas aeruginosa* harbours an array of aminoglycoside-modifying genes, altering the drug by acetylation,

adenylation or phosphorylation (APH). The presence of *APH(3')-IIb* in kefir samples is deliberately worrying as aminoglycoside 3'-phosphotransferases can mediate high-level resistance against several aminoglycosides. These genes might be plasmid-borne or chromosomally encoded; *APH(3')-IIb* is the latter, but a transposon-mediated mechanism has been suggested to be responsible for spreading the resistance genes [189, 190]. As the gene was almost exclusively described in *P. aeruginosa* previously, and the likely origin was *L. mesenteroides* in our study, the routes of resistance gene transfer related to this gene need to be further investigated. Although penicillins and cephalosporins are the most frequently used antibiotics for dairy cows, interestingly, the abundance of ARGs facilitating resistance against β-lactams was low. This phenomenon, together with the ARGs related to unused antibiotics in veterinary dairy medicine, raises the suspicion that the source of the abundant ARGs might not be a direct consequence of antibiotic use at dairy farms.

Bacteria entering the digestive tract with food, are provided with the opportunity of contacting other non-pathogenic and pathogenic bacteria. At the same time, one of the main prerequisites of HGT processes is the physical proximity of the participating bacteria. By virtue of the fulfilment of this requirement, various genes, including ARGs, can be exchanged by bacteria during HGT processes. If an ARG harbours on a mobile DNA-sequence, the probability of its HGT is higher. We found only one gene, namely *lmrD* in sample k_g_04, that is supposedly doing this. This deduction is based on the genomic environment of *lmrD*.

Antibiotic resistance caused by multidrug-resistant bacteria is a significant global public health threat [191]. Infections with drug-resistant bacteria may result in major morbidity and mortality and increase the cost of health care when compared to infections by non-resistant strains of the same species. Even with the strictest filtering restrictions, we identified ARGs undermining the efficacy of aminoglycosides, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, elfamycins, fluoroquinolones, fosfomycins, glycylcyclines, lincosamides, macrolides, monobactams, nitrofurans, nitroimidazoles, phenicols, rifamycins and triclosans. These findings raise several clinical considerations. For instance, carbapenems are broad-spectrum antibiotics used for the treatment of necrotizing pancreatitis [192] and severe intraabdominal infections. Tigecycline, a recently developed third-generation tetracycline antibiotic belonging to the glycylcycline class, is one of the few therapeutic options for carbapenem-resistant bacteria, like *Klebsiella pneumoniae* [193, 194] and carbapenem-resistant *Enterobacteriaceae* (CRE) [195]. Another group of ARGs identified in our study code resistance against fluoroquinolones. Emerging fluoroquinolone resistance in *Campylobacter* strains which are the leading cause of bacterial gastroenteritis in the world is a significant public health concern similarly to the rising incidence of fluoroquinolone-resistant cases of typhoid fever and invasive non-typhoidal Salmonella (iNTS) infections. We have also identified genes coding cephalosporin resistance in our samples. Cephalosporins belong to the most frequently used antibiotics globally. Intravenous third generation cephalosporins (e.g. ceftriaxone) are more potent against Gram-negative bacteria. They are frequently used in cholecystitis, spontaneous bacterial peritonitis or as a preventive measure in acute gastrointestinal haemorrhage [196]. ORFs harbouring ARGs that code resistance against macrolides may also raise serious public health concerns. Macrolide antibiotics absorb excellently from the gastrointestinal tract and have few side effects. Clarithromycin is still considered as a member of the first-line treatment protocol for *Helicobacter pylori* eradication in

areas with a low resistance to clarithromycin [197]. Azithromycin can contribute to the resolution of acute infections by immunomodulatory effects [198]. It is frequently used for the treatment of acute watery or febrile diarrhoea and dysentery syndrome [199]. Tetracycline resistance genes that we found predestine a potential loss in the efficacy of various tetracycline compounds. Once commonly used, nowadays rarely administered tetracycline has been recently rediscovered, as a component of *H. pylori* eradication regimen, partly due to increasing rate of resistance to other antibiotics (including the above-mentioned clarithromycin) [197].
As ARGs reaching the human body may originate from fermented dairy products, further examinations would be worthwhile to clarify the details and understand the practical medical significance. For this, it would be appropriate to analyze the samples of starter cultures and final products and register the results at set time points during the fermentation period. According to our findings, sequencing depth plays a significant role in the coverage of ORFs identified as ARGs, thus involving at least 20 million clusters is recommended by similar studies. The samples we examined and the studies we found in the literature [82, 83, 181, 183] confirm the hypothesis that foods of animal origin may contain significant amounts of diverse ARGs. The reason for the appearance of ARGs is complex, and the routes of appearance and spread are difficult to track. As sequencing techniques become cheaper, regular genetic monitoring of products of animal origin, including starter cultures, should be considered in addition to the strict control of antibiotics used in animal husbandry.

6.1.3 Other probiotic products

Similarly to the Methods and Results, this section is divided into the discussion of results obtained from **study A** and **study B**, respectively.

The results of **study A** are presented to demonstrate that the bacteria of probiotics may not only carry significant amounts of ARGs, but in numerous cases, those genes may also be mobile, thereby contributing to their spread to other bacteria and having possible consequences on the antibiotic treatment efficacy.
Bacterial genera identified in the metagenomic samples also appear in many probiotic related articles of the current international literature. Various species of bacilli, bifidobacteria, enterococci, lacticaseibacilli, lactiplantibacilli, lactobacilli, lactococci, ligilactobacilli, limosilactobacilli and streptococci are the core members of commercial probiotic bacterial communities [200–209]. Two identified bacterial genera (*Sphingobacterium*, *Weizmannia*) in the various samples are less frequent probiotic components. The possibility of exploiting *Sphingobacteria* in probiotic foods was previously mentioned based on the characterization of flour and batter samples of sorghum and pearl millet [210]. Members of the genus were detected by the high-throughput sequence analyses of fermented beverages [211]. Probiotic *Weizmannia* species (e.g., former *Bacillus coagulans*) have recently been reclassified [212], and have an unquestionable probiotic significance [213]. It is important to note that there may be notable differences in the gene pool between strains of particular species, so the results presented do not mean that all strains of a given species contain the genes identified here.

While at least one ARG was found in each metagenomic sample, less than half of the isolates contained any of them. No ARG was detected in *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Limosilactobacillus fermentum*, *Pseudomonas sp. RGM2144* or *Streptococcus thermophilus*. Contigs originating from *Bacillus subtilis*, *Bifidobacterium animalis*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Lactococcus lactis* and *Streptomyces albulus* each contained at least one ARG.

The available literature was screened to evaluate our findings and gain reliable knowledge of the ARGs that could have been attached to bacteria at the species level. All ARGs found in *Bacillus subtilis* (*aadK*, *B. subtilis mprF*, *B. subtilis pgsA* with mutation conferring resistance to daptomycin, *bmr*, *lmrB*, *mphK*, *vmlR*, *ykkC*, *ykkD*) have previously been identified in *B. subtilis* and many of them were even reported to be specific for this species or the *Bacillus* genus [214–220]. In the *Bifidobacterium* genus, ARGs were associated with four species (*B. animalis*, *B. bifidum*, *B. breve* and *B. longum*). None of the *B. animalis*, *B. bifidum*, *B. breve* and *B. longum* related *B. adolescentis rpoB* mutants conferring resistance to rifampicin and *tet(W/N/W)* are specific for the identified species but both genes have previously been described in them [92, 167, 221, 222]. *B. bifidum ileS* conferring resistance to mupirocin reported in *B. bifidum* supposedly cannot be exclusively linked to this species of the genus, but it had been identified in it before [223]. Out of the *Enterococcus faecalis* deriving genes, *dfrE* was first identified in *E. faecalis* [224], but according to a recent study it is not exclusive to this species any more [225]. The genes *efrA* and *efrB* have been described in *E. faecalis* and *E. faecium* [226, 227]. Gene *emeA* has only been identified in *E. faecalis* so far [226]. Apart from *E. faecalis*, *lsaA* has been attached to *Streptococcus agalactiae*, while *tetM* appears in a broad spectrum of bacterial species [228–232]. All three ARGs (*AAC(6')-Ii*, *eatAv*, *msrC*) associated with *E. faecium* have been previously published as appearing in this species, and the first two are even specific for it [233–236]. All ARGs originating from *Escherichia coli* (*acrB*, *acrD*, *acrE*, *acrF*, *acrS*, *bacA*, *baeR*, *baeS*, *cpxA*, *CRP*, *emrA*, *emrB*, *emrK*, *emrR*, *emrY*, *eptA*, *E. coli acrA*, *E. coli acrR* with mutation conferring multidrug antibiotic resistance, *E. coli ampC* β-lactamase, *E. coli ampC1* β-lactamase, *E. coli ampH* β-lactamase, *E. coli emrE*, *E. coli GlpT* with mutation conferring resistance to fosfomycin, *E. coli marR* mutant conferring antibiotic resistance, *E. coli mdfA*, *E. coli soxR* with mutation conferring antibiotic resistance, *E. coli soxS* with mutation conferring antibiotic resistance, *evgA*, *evgS*, *gadW*, *gadX*, *kdpE*, *marA*, *mdtA*, *mdtB*, *mdtC*, *mdtE*, *mdtF*, *mdtG*, *mdtH*, *mdtM*, *mdtN*, *mdtO*, *mdtP*, *msbA*, *PmrF*, *TEM-116*, TolC, ugd, YojI) have previously been described in this species and many of them are even specific to it, according to the Comprehensive Antibiotic Resistance Database (CARD) [134, 135]. Gene *lmrD*, the only ARG deriving from *Lactococcus lactis* has been identified in this species along with some others [237, 238]. Even though *AAC(3)-IV* has been identified in several studies [239, 240], according to our knowledge this is the first time it has been detected in *Streptomyces albulus*.

Gene *TEM-116*, which is often referred to as a clinically significant extended-spectrum β-lactamase (ESBLs), was the most frequently identified finding in our study. ESBLs are most commonly defined as the members of a ubiquitous enzyme family that is capable of conferring resistance to penicillins, first-, second- and third-generation cephalosporins and aztreonam, and of being impeded by β-lactamase inhibitors such as clavulanic acid [241]. The 400 *TEM*

variants that have been identified so far, can be disclosed in two clusters with one deriving from *TEM-1* (the first *TEM* protein to be described) and one linked to *TEM-116* as a progenitor [242]. In line with our findings, gene *TEM-116* is reported to be present worldwide harbouring in the conjugative plasmids of a wide range of Gram-negative bacteria. Despite its wide geographical dissemination, establishment on multiple plasmids and centrality in the *TEM* family network indicating it is a naturally occurring enzyme with microbiologically proven ESBL characteristics [243, 244], some concerns have arisen about its designation, after the gene was found in non-ESBL producing *Klebsiella pneumoniae* strains [245]. Moreover, commercial Taq polymerases used in PCRs may be contaminated with *bla*$_{TEM-116}$ DNA which could lead to the erroneous identification of the gene in samples that do not actually contain it [246, 247]. In our study, each sample in which this gene was detected originated from the same bioproject (PRJNA542229). As the samples come from different dietary supplements, one may interpret that this finding is an artefact or contamination as a consequence of some sample preparation steps. Nevertheless, as more detailed information on sample preparation is not available, this issue cannot be resolved.

As seen above, and as described in other publications [24], there is still a great deal of variation in details which need to be clarified by the interpretation of ARGs. Nevertheless, the suspicion that the identified ARGs may undermine the efficacy of several antibiotic classes, including acridine dye, aminocoumarins, aminoglycosides, benzalkonium chloride, carbapenems, cephalosporins, cephamycins, diaminopyrimidines, fluoroquinolones, fosfomycins, glycylcyclines, lincosamides, macrolides, monobactams, mupirocins, nitroimidazoles, nucleosides, oxazolidinones, penams, penems, peptides, phenicols, pleuromutilins, rhodamines, rifamycins, streptogramins, tetracyclines and triclosans raises some clinical considerations. According to the latest CDC report on antimicrobial use in the U.S., amoxicillin (penam), azithromycin (aminoglycoside), amoxicillin and clavunalic acid (penam, increased activity), cephalexin (cephalosporin) and doxycycline (tetracycline) are the most commonly administered compounds [248]. Moreover, based on the latest WHO report on global antimicrobial use, amoxicillin (penam), ciprofloxacin (fluoroquinolon), sulphametoxazole and trimethoprim are the most commonly prescribed oral drugs and ceftriaxone (cephalosporin), gentamicin (aminogylcoside) and benzylpenicillin (penam) are the most commonly used parenteral compounds in 4 surveyed countries of the African region. In six countries of the region of the Americas, amoxicillin (penam), cefalexin (cephalosporin) and doxycycline (tetracycline) are the antibiotics with the highest oral consumption rates and ceftriaxone (cephalosporin), oxacillin (penam) and gentamicin (aminogylcoside) are the ones with the highest parenteral use. In the European region, reports were made of 46 countries. Among orally administered antibiotics, amoxicillin (penam), amoxicillin and β-lactamase inhibitors (penam, increased activity) and doxycycline (tetracycline) are the top 3 compounds, while ceftriaxone (cephalosporin), gentamicin (amynoglycoside), and cefazoline (cephalosporin) are the most common parenteral ones. Amoxicillin (penam), azithromycin (macrolide) and amoxicillin and β-lactamase inhibitors (penam, increased activity) are the most commonly consumed oral antibiotics and ceftriaxone (cephalosporin), benzathine benzylpenicillin (penam) and procaine benzylpenicillin (penam) are the top 3 parenterally administered agents in the Eastern Mediterranean region. In the six surveyed countries of the Western Pacific region amoxicillin (penam), doxycycline (tetracycline) and amoxicillin and β-lactamase in-

hibitors (penam, increased activity) are the most commonly prescribed oral antibiotics, while cefazolin (cephalosporin), ceftriaxone (cephalosporin) and cefuroxime (cephalosporin) are the most frequently used parenteral compounds [249]. Many of the most highly prioritized antibiotics could be affected by the presence of the detected ARGs. Meanwhile, out of the 15 antibiotic groups mentioned in the latest WHO report on critically important antimicrobials (CIA) for human medicine, nine (aminoglycosides, carbapenems and other penems, cephalosporins, glycylcyclines, macrolides, monobactams, oxazolidinones, penicillins of various cathegories, quinolones) could possibly be affected by the ARGs identified in the various samples [249].
It is important to underline that all the six *E. coli* isolates contained the gene *H-NS*, which plays a crucial role in the global gene regulation of various bacteria, including this species. The expression of a wide variety of genes is repressed by *H-NS*, and its deletion increases AMR and decreases drug accumulation. Even though, this gene is stored in CARD [134, 135], its functional effect is adverse to that produced by ARGs [250].
If ARGs are transmitted from probiotic bacteria to pathogenic bacteria within the consumer's body, they may reduce the effectiveness of antibiotic therapy on the diseases participating pathogenic bacteria cause. The execution of gene transfer processes is more likely among bacteria that are in close physical proximity to each other and if the ARGs are associated to a mobile genetic environment. According to our results a considerable number of ARGs, such as those which are iMGEs-linked or have resided in plasmids or prophages.
The co-occurence of *tet(W/N/W)* and ISBian1 is in line with the findings of Rozman et al. (2020), according to which all genomes of *B. animalis* subsp. (*lactis* or *animalis*) (n = 42) available in 2019 contained this gene. Moreover, by the investigation of the mobility characteristics of *tetW*, out of the transposases belonging to the family of the insertion sequences, ISBian1 seemed to be subspecies dependent in *B. animalis* subsp. *lactis* and flanking *tetW* in the majority of the strains [92]. Our results of *tetM* linking to the transposon Tn6009 in *E. faecalis* is consistent with finding of Zangue et al. in South-African faecal samples [251].
In two samples, contigs harbouring *tet(W/N/W)* originating from *Bifidobacterium longum* and *Bifidobacterium animalis* were predicted to belong to plasmids. Several studies reported a wide prevalence of the *tetW* gene in *Bifidobacteria* [92, 94, 252, 253]. While the co-occurrence of *tetW* and its flanking transposase is a common genetic feature of *B. animalis*, previous reports lack the identification of plasmids in *B. animalis*, even though the gene was associated with plasmids in other bacterial species [254]. Despite *AAC(6')-Ii* deriving from *E. faecium* being located in the chromosome in previous studies and it being defined as a chromosome-borne ARG on CARD [134, 135, 255], our research indicates it may take place in a plasmid. An *E. faecium*-associated contig contained gene *msrC*. According to the available literature, *msrC* is a chromosomal-encoded gene that is mentioned as an intrinsic property of *E. faecium* strains [134, 135, 256]. While the expected bacterial species of origin was confirmed, our finding raises the likelihood of the gene being connected to a plasmid as well. In 15 samples, *E. coli*-originated contigs harboured the gene *TEM-116*. Plasmid origin is a common feature of ESBL genes such as *TEM-116* according to several publications and is often referred to as a feature to facilitate their quick spread [257–259]. In the *E. coli* isolate sample s15, one contig had the *marA* and *marR* genes. These widespread multiple antibiotic resistance genes had been identified on plasmids before [260]. The gene *efrA* harbouring in contigs with a predic-

tion of phage origins were identified in all publicly available *E. faecalis* genome sequences by Panthee and colleagues (2021) too, along with a large set of phages in the genomes [261].
As our results derive from in silico data analysis, it is only possible to describe the features that prove and facilitate presence and mobility of the genes. Whether or not the identified genes operate in the bacterial strains of a given probiotic cannot be determined. In order to clarify this, additional functional, e.g., gene expression studies, should be performed.
An important aspect to take into consideration by the interpretation of the ARG occurrence in probiotics is that constituent strains can often naturally be, or rendered multiresistant, so that they can be co-administered with oral antibiotics and reduce gastrointestinal side effects [262, 263]. In our study we could not distinguish whether the examined samples contained the ARGs for this purpose. Moreover, as ARGs were found in the vast majority of the samples tested, not a negligible proportion of them, it is possible that the presence of ARGs in bacteria may also play a role in their probiotic effect. ARGs play a role in defence against antibiotics and may provide general fitness against specific toxic effects for bacteria [264, 265]. One may make an analogy with earlier practice. In livestock farming, antibiotics have been widely used as feed supplements for yield enhancement on a purely empirical basis. By this practice, antibiotics have put pressure on the gut bacteria and selected for resistant strains. As a result, animal feed efficiency and production indicators have improved. When probiotics are consumed, the expectation is that the 'good' bacteria will colonise the gut. In numerous animal husbandry areas (e.g., broiler chicken production), the producers try to achieve this by continuous probiotic feeding. If these probiotics also contain bacterial strains harbouring ARGs, they achieve very similar results as before with the selective effect of antibiotic utilisation. If it is true that certain ARGs are essential for the efficacy of probiotic bacteria, then the selection of strains should be carried out with consideration of the human health consequences. That is, bacterial strains that contain ARGs having no significant influence on human antimicrobial therapy efficiency should be used. However, based on our results, it can also be suggested that bacteria that do not contain ARGs at all can be used as probiotic components. To have a more detailed insight into this topic, several further studies would be needed. For instance, they could also focus on reducing the mobility of genes whose presence may be necessary for the probiotic nature of particular bacteria. Based on the results, we consider it essential to monitor the ARG content of probiotic preparations and their mobility characteristics in the fight against AMR.

Study B confirms that numerous ARGs are present in probiotic bacterial species constituting the bacteriome of edible products and that many of them are mobile. Thus, the application and intake of certain probiotic bacterial strains have the potential to contribute to the appearance and spread of AMR. The prevalence rates of ARGs were relatively diverse among the examined bacterial species. All samples of *B. animalis* and *L. lactis* appeared to be outstandingly ARG-rich. Nevertheless, in the case of *B. animalis* the high prevalence was underlain by the 100% detection rate of two genes, namely *tet(W)* and *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin. Tetracycline resistance encoding *tet(W)* is regularly associated with probiotic *B. animalis* strains [252, 266, 267], moreover at certain subspecies, this ARG is considered to be innate, phylogenetically distinct from other bacterial species and possess a negligible risk of transfer [267]. At the same time, *tet(W)* is commonly flanked by transposase

genes [266]. Interestingly, each of our *tet(W)* findings were associated with iMGEs, namely composite transposons (cTns). However, the interpretation of the mobility potential of these genes is unsure and would require further investigations. Consequently, the public health ramifications of the steady ARG numbers accompanied by gene transfer elements are not clear. In case of *L. lactis*, lincosamide resistance encoding *lmrD* showed the highest prevalence rate within the examined samples. This gene is considered to be of outstanding significance in phenotypically multidrug-resistant (MDR) *L. lactis* strains in interaction with *lmrC* [268]. Just as the majority of our hits, *lmrD* is described to be chromosomally encoded [237]. Nevertheless, twenty samples were associated with plasmid-borne *lmrD*. The plasmidome of *L. lactis* is highly dynamic and of a high economic significance [269]. These findings may have public health importance and may raise awareness of the need for the introduction of advanced surveillance measures. In contrast, no *L. casei* and *L. paracasei* strains contained any ARGs, and *L. delbrueckii*, *L. helveticus* or *L. brevis* were also relatively less ARG-dense. As the presence of AMR markers is an undesired trait for any microbe introduced in humans as a potential probiotic [270], these species could be favored in alimentary products. On the other hand, despite the low ARG numbers and diversity, each ARG detected in the above-mentioned species appeared to be plasmid-associated, and as such, potentially transferable. The middle of the ARG prevalence scale is occupied by *L. plantarum*, *L. mesenteroides* and *S. thermophilus*. These bacteria could have been associated with lower ARG numbers but higher relative ARG diversity. While genomes from *L. mesenteroides* contained no MGE-associated ARGs, the resistome of *S. thermophilus* was predicted to be highly mobile. The uptake of plasmids carrying ARGs, including *ErmB*, encoding the MLSB (macrolide-lincosamide-streptogramin B) phenotype is not without precedent by streptococci [271]. Samples with *L. plantarum* formed the most abundant group, which may have led to the highest ARG diversity. All but two ARGs (*AAC(6')Ii* and *eatAv*) harbored on plasmids or were flanked by iMGEs. Consequently, the public health concern associated with the *L. plantarum* strains is not negligible. The ARGs we identified may undermine several classes of antibiotics, such as rifamycins, tetracyclines, aminoglycosides, phenicols, lincosamides, macrolides, pleuromutilins, streptogramins, cephalosporins, monobactams, penams, and penems. The ARGs we found have resistance mechanisms against many antibiotics in human and animal medicine. The term critically important antimicrobial (CIA) refers to antimicrobials that are the last resort in the treatment of human disease. The WHO produces an updated list of currently used human antimicrobials grouped under three categories according to their importance; CIA, Highly important antimicrobial (HIA) and important antimicrobial (IA). CIAs are further subdivided into high priority CIA (CIA) and highest priority CIA (HPCIA). Most importantly are those listed as HPCIA, which include cephalosporins (3rd, 4th and 5th generation), glycopeptides, macrolides and ketolides, polymyxins and quinolones [272]. Out of the five HPCIA drug groups, we found ARGs that potentially compromise the effectiveness of two (cephalosporins and macrolides). We also found ARGs that have an effect on five CIAs (rifamycins, aminoglycosides, monobactams, penams and penems), six HIAs (tetracyclines, phenicols, lincosamides, streptogramins, cephalosporins) and one IA (pleuromutilins). The EMA also produced a list aimed at restricting the veterinary use of antimicrobials that are important for human medicine [273]. The antimicrobials are listed under the categories; Avoid, Restrict, Caution and Prudence. We found ARGs that threaten three drug groups listed as

avoid (streptogramins, monobactams, streptogramins), one listed as restrict (cephalosporins), seven as caution (rifamycins, aminoglycosides, phenicols, lincosamides, macrolides, pleuromutilins, cephalosporins) and two as prudence (tetracyclines, penams). In addition, the World Organisation for Animal Health (OIE) has a list of critically important antimicrobial agents used in veterinary medicine. The OIE uses three categories; Veterinary Critically Important Antimicrobial Agents (VCIA), Veterinary Highly Important Antimicrobial Agents (VHIA) and Veterinary Important Antimicrobial Agents (VIA) [274]. The ARGs we found have an effect on six VCIAs (tetracyclines, aminogylocosides, phenicols, macrolides, cephalosporins, penams), four VHIAs (rifamycins, lincosamides, pleuromutilins, cephalosporins) and one VIA (streptogramins). Thus, many important human and animal medicine antibiotics could be affected by the ARGs we detected in bacterial strains from probiotic strains from products for human consumption. Nevertheless, it is important to highlight that the presence of ARGs does not necessarily conclude in the phenotypical appearance of AMR.

Further gene expression studies or phenotypical probes (e.g., the assessment of minimal inhibitory concentration values) would be required to evaluate the expressed AMR traits of the examined probiotic bacteria.

Although we consider our results important in the absence of a similar survey study with such a large sample size and a uniform methodology, we must mention its shortcomings and limitations. The foundations of our study are provided by retrospective data collection based on the NCBI SRA system, which is quite permissive regarding the completeness of the metadata of uploaded samples. Hence, uploading detailed information is often neglected, hindering a more thorough presentation and interpretation of the results. We believe that one of the main problems with the extendability of our results is that the exact types of isolation sources were not identified by all samples. Furthermore, it would be very important to know under which conditions (e.g., medium, temperature) each strain was isolated and cultured. It is also unknown whether any antimicrobial agents were used in the cultures to control competing species. If any antimicrobials were used during the culturing process, subpopulations with ARGs could be propagated. The generalizability of our result would also increase if the age of the cultures from which the sequenced strains were isolated was known. Optimally, in a prospective study, these factors could be controlled. Thus the noise in the variation between species and isolation sources could be reduced. Nonetheless, our work could raise awareness of the need for controlled prospective studies.

Our results show that some probiotic bacterial species contain a higher proportion of ARGs, while others represent a lower proportion. We also see that a considerable proportion of the identified ARGs are mobile. In the European Union [88], there are recommendations with methodological suggestions for the WGS analysis of microorganisms in the food chain. However, these recommendations do not provide detailed guidelines for the analysis of the mobilome. Since our results suggest that the prevalence of mobile ARGs is not negligible, considering the development of guidelines for this purpose would be worthy.

6.2 Other animal source interfaces

6.2.1 Canine saliva

During the bacteriome, resistome and mobilome analysis of the canine saliva samples, a large set of results was obtained that can be examined from a One Health point of view, merging the small animal veterinary sector with the perspective of the human healthcare system.
ARGs were identified in all but one sample (No. 20). No reads of bacterial origin were found in this sample. We speculate that this may be since only those reads generated from sequencing were uploaded to the SRA mapped to the dog genome.
Some of the detected aerobic and anaerobic bacterial genera are saprophytes while others often become isolated from infected dog bite wounds. Dog bite infections are normally polymicrobial, and the bite wound bacteriota consist of bacteria from the animals' oral cavity, the recipients' skin and the environment. The most common pathogens in dog bites are *Pasteurella* spp. (*P. canis* and *P. multocida*), *Staphylococcus* spp., *Streptococcus* spp. and *Capnocytophaga* spp., *Porphyromonas* spp., *Bacteroides* spp., *Fusobacterium* spp. and *Corynebacterium* spp. [275], which all appeared in the analyzed saliva samples. Some other bacterial groups of a relatively higher clinical significance that were detected in the saliva samples, including *Enterococcus* spp., *Moraxella* spp., *Neisseria* spp., *Prevotella* spp. and *Pseudomonas* spp., are also often isolated from dog bite wound infections. The vast majority of other genera isolated in the samples have been mentioned to appear in dog saliva in previous publications with variable abundance rates [110, 276]. Even though members of *Clostridium spp.* were detected in the samples, genome fragments of *C. tetani*, the bacterium responsible for tetanus, were not identified. The number of detected ARGs was relatively high in the salivary bacteriome. Examining eight genera (*Pasteurella* spp., *Staphylococcus* spp., *Streptococcus* spp., *Capnocytophaga* spp., *Porphyromonas* spp., *Bacteroides* spp., *Fusobacterium* spp. and *Corynebacterium* spp.) that were indicated to be the most relevant ones in dog bite infections by other authors [275, 276], we could identify genes that confer resistance against aminoglycosides, carbapenems, cephalosporins, glycylcyclines, lincosamides, macrolides, oxazolidinone, penams, phenicols, pleuromutilins, streptogramins, sulfonamides and tetracyclines, while other antimicrobial groups including fluoroquinolones appeared in *E. coli*, one of the six leading pathogens responsible for the deaths associated with resistance in 2019 worldwide [22].
Such a great number and broad spectrum of ARGs and potentially affected antimicrobial groups associated with the canine saliva samples may be related to the use of antibiotics at small-animal veterinary practices. Antibiotic consumption rates in the companion animal sector are rather difficult to evaluate. However, some systems exist for the surveillance of magnitude of companion animal antibiotic consumption, such as the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) [277], VetCompass [278] or the Small Animal Veterinary Surveillance Network (SAVSNET) [279], these rates are still less well-documented. Moreover, in many countries, antimicrobial use is often just estimated by rough sales data [118]. Nevertheless, according to the two UK-based surveillance systems (VetCompass from Royal Veterinary College, and SAVSNET from Liverpool University) and one EU report (ESVAC), antibiotics are rather frequently prescribed at small-animal clinics. A study states 1 in 4 UK dogs (25.2%, 95%

CI: 25.1–25.3%) were treated with antibiotics in a two-year period [280]. Even though the vast majority of veterinarians are aware of the fact that improper AMU contributes to selection for AMR, and that it is a significant problem according to nationwide surveys [281, 282], there are many factors that influence the antibiotic prescription preferences of veterinarians in addition to the perspectives of antimicrobial stewardship [283–288].

Broad-spectrum amoxicillin-clavulanate is the flagship of antimicrobial agents applied in dogs in many countries, while first-generation cephalosporins are also routinely used [118, 289, 290]. Lincosamides (clindamycin), macrolides, tetracyclines (doxycycline), nitroimidazoles and trimethoprim/sulphonamides have also been reported to be frequently used in small-animal practices [118]. Third- and fourth-generation cephalosoprines, fluoroquinolones and polymixins that belong to cathegory B, 'last resort', or highest-priority Critically Important Antibiotics (HPCIAs) according to the European Medicines Agency [277] should be avoided unless sensitivity testing is conducted and no other antibiotics would be effective. Nevertheless, HPCIAs have been estimated to be prescribed in around 5-6% of total antimicrobial agent usage events. Of the HPCIA category, fluoroquinolones are the most common in dogs, constituting $\sim$4 to 5% of total antibiotic prescriptions [291].

Neverheless, AMR determinants against the above-mentioned antimicrobial compound groups have been detected in and associated with many bacterial species of the examined canine saliva samples. Some of these ARG-associated bacteria can also exert pathogenic effects and are often isolated by dog bite infections. ARGs against cephalosporins were identified in many, often clinically significant bacteria, including but not limited to *Bacteroides* spp., *Capnocytophaga* spp., *E. coli*, *F. ulcerans*, *Porphyromonas* spp. and *P. aeruginosa*. Likewise, ARGs against lincosamides appeared in *Bacteroides* spp., *C. stomatis*, *Enterococcus* spp., *P. cangingivalis*, *P. intermedia* and *Streptococcus* spp.; ARGs against macrolides in *Bacteroides* spp., *C. stomatis*, *Enterococcus* spp., *Porphyromonas* spp., *P. intermedia* and *Streptococcus* spp.; ARGs against tetracyclines in *Bacteroides* spp., *C. stomatis*, *Enterococcus* spp., *P. crevioricanis*, *Prevotella* spp., *P. putida* and *Streptococcus* spp.; ARGs against sulfonamides in *Corynebacterium* spp., *M. bovis*, *P. multocida* and *P. aeruginosa*; and ARGs against fluoroquinolones in *E. coli* (non-exhaustive lists of detected ARG-bacteria associations in the samples, with an emphasis on clinically relevant bacteria). Bacterial associations and the significance of AMR determinants affecting amoxicillin-clavulanate, the most commonly prescribed antibiotic in veterinary medicine, are discussed later on. Even though nitroimidazoles (e.g., metronidazole) are described to be often used in small-animal veterinary practices, no ARGs could be detected in the canine saliva samples against this antibiotic group.

In the current literature, human infections associated to dog bites are better and more frequently documented than the transmission route of licking. Three to thirty percent of dog bites lead to infection [110]. The management of animal bites rests on two pillars: local wound care and adequately applied systemic treatment. Essentials of local therapy include inspection, debridement of the wound accompanied by the removal of possible foreign bodies, e.g., teeth, and irrigation with saline solution. As for the systematic therapy, tetanus boosters (if none given in the past year) and rabies prophylaxis should always be considered. In our study, genome fragments of *C. tetani*, the causative agent of tetanus, were not detected in any of the examined saliva samples. No consensus has yet been found in the use of antibiotics for animal bite

wound care. Prophylactic antibiotics should be considered unless the wound is very superficial and clean. Explicit indications for antibiotic prophylaxis or therapy include presentation at least 8 h after the bite, clear signs of superinfection, moderate or severe wounds with crush injuries or devitalized tissues requiring surgery, deep puncture wounds (exceeding the layer of epidermis), wounds close to joints, diabetes mellitus, asplenic or immunocompromised state, alcohol abuse, or involvement of the genital area, face or hand [292–296]. In the absence of the above reasons, antibiotic therapy may not be necessary. Interestingly, injuries are normally located on the head, neck and face in children and on the hand or upper extremity in adults due to height ratios with the attacking dog [110, 297]. An adequately chosen antibiotic agent is expected to be effective against anaerobic bacteria (*Bacteroides* spp., *Fusobacterium* spp., *Porphyromonas* spp., *Prevotella* spp. etc.), in addition to the *Staphylococcus*, *Streptococcus* and *Pasterurella* species. Prophylactic treatment is normally 3 to 5 days long, while medication for 10 days or longer is recommended if the wound is infected. The first-line choice for oral therapy is amoxicilin-clavulanate, accompanied with a first dose of intravenous antibiotic (e.g., ampicillin/sulbactam, ticarcillin-clavulanate, piperacillin-tazobactam, or a carbapenem) in high-risk patients. Amoxicillin-clavunalate is often combined with metronidazole or clindamycin and is also sometimes replaced with cephalosporins, e.g., cefuroxime, cefotaxime, ceftriaxone or amoxicillin, fluoroquinolones, sulfamethoxazole and trimethoprim, and alternatively, although less effective, azithromycin or doxycycline in this combination [294, 296]. Due to high resistance rates, flucloxacillin, erythromycin and cephalosporins are often ineffective in *Pasteurella* infections, and thus should rather be avoided [293]. In our case, no genes conferring resistance to these agent groups could be identified in *Pasturella* spp.

Data on the outcome of antibiotic prophylaxis in animal bite management by humans is limited and rather controversial and conflicting. While a meta-analysis of eight randomized trials indicated a benefit of antibiotic prophylaxis [298], some studies concluded that antibiotic prophylaxis does not result in a statistically significant difference in the frequency of wound infections among treated and untreated patient groups, except for wounds to the hand [299]. Based on other publications, antibiotic prophylaxis should be recommended for high-risk patient groups only [300, 301].

Based on antibiotic prescription data from human and veterinary medical practices described above, amoxicillin-clavulanate and cephalosporins are the most commonly used agents in the treatment of animal patients and dog bite infections, while lincosamides (mostly clindamycin), sulfonamides (mostly potentiated sulfonamides) and fluoroquinolones also appear in both sectors [118, 289–291, 293, 294, 297].

Amoxicillin-clavulanate, the most commonly used antibiotic in small-animal medicine, and the first choice for canine bite wounds, is a member of broad-spectrum penicillins that have been a frequently consumed key antibiotic group in the high-income super-region between 2000 and 2018 according to a global study [302]. All in all, six ARG types were detected in the dog saliva samples that may confer resistance against amoxicillin-clavulanate, which were either the members of the *blaTEM* or *OXA* family [303, 304]. *blaTEM-116* was identified in *E. coli*, while various members of the *OXA* family appeared in in many genera, including *A. baumannii*, *Bacteroides* spp., *Capnocytophaga* spp., *F. ulcerans* and *Pseudomonas* spp., which can have a high clinical relevance in dog bite infections. Moreover, plasmid-borne *blaOXA-2*, *blaOXA-347*

and *blaTEM-116* genes in different bacteria, and *OXA-2* associated with both an iMGE and a plasmid from *P. aeruginosa*, all confer resistance against amoxicillin-clavulanate and have a higher potential to spread from bacteria to bacteria. The accumulation of various mobility factors around the genes may increase the chance of the horizontal transfer of the given ARG. The canine saliva-borne transmission of bacteria harboring mobile ARGs may hamper antimicrobial use in human clinical settings and can also contribute to the spread of AMR among the bacteria derived from pets to the bacteriota appearing in humans.

Cephalosporins, which are also commonly used both in companion animals medicine [118, 290] and human medicine, including in protocols for dog bite infections [294, 297], have been associated with all in all twenty-four bacterial species harboring related ARGs in the canine saliva samples. These bacteria include pathogens such as *A. baumanni*, *Bacteroides* spp., *Capnocytophaga* spp., *F. ulcerans* and *P. aeruginosa* that contain *blaOXA-2*, *blaOXA-347*, *blaOXA-347*, *blaOXA-85* and *blaOXA-2*, respectively. Importantly, *P. aeruginosa*-associated *blaOXA-2*, which was associated with both an iMGE and a plasmid, has a high potential for HGT and can be considered as a major public health concern.

Lincosamides, including clindamycin, are also significant in both veterinary medicine [118, 290] and the treatment of dog bite cases in humans [294, 297]. Genes affecting lincosamides appeared in seventeen bacterial species, many of which, e.g., *B. fragilis* (*ErmF*), *C. stomatis* (*ErmF*), *Porphyromonas* spp. (*ErmF*) and *Streptococcus* spp. (*ErmB*, *lnuB* and *lnuC*), are potentially pathogenic. While the above-mentioned species, which are often associated with dog bite infections, contained no genetic element around these ARGs that could facilitate their transfer, an *Enterococcus* species from the canine saliva samples is linked to carrying *ermB* with prophage and plasmid co-appearance. Thus, the possibility of the transfer of this gene to other bacteria with higher clinical significance is given in the case of lincosamides as well.

Sulfonamide resistance genes appeared in relatively fewer, namely eight, bacterial species, including *P. multocida* and *P. aeruginosa* harboring *sul2* and *sul1*, respectively. In addition to the low ARG counts, none of the sulfonamide resistance genes appeared to be mobile, while, in contrast, HGT has been found to be highly characteristic for these two genes in other publications [305].

In the case of fluoroquinolones, only *E. coli* harbored related ARGs, namely the non-mobile *acrA*, *gadW* and *gadX*. Interestingly, while both *gadW* and *gadX* are *AraC* family regulators that promote *mdtEF* expression to confer multidrug resistance, when they co-occur, *gadW* inhibits *gadX*-dependent activation by repressing *gadX* (CARD).

Furthermore, fosfomycin and tygecyclin, which are often used as last-resort antimicrobial agents [306, 307] and are involved in the list of Critically Important Antibiotics for Human Medicine by WHO [272], also appeared among the affected antibiotic groups, namely due to the presence of *fosA2* and *tetX4*, *tetX5*, respectively. Nonetheless, while the members of the *tet(X)* family are often plasmid-associated [308–311], their plasmid relatedness was not predicted within this study.

While the above-mentioned findings may raise awareness of the potential public health significance associated with canine saliva, this material has been used to promote rapid healing and to reduce bacterial contamination in the past according to the reports of ethnoveterinary and ethnomedicinal practices [312, 313]. Antimicrobial and anti-imflammatory activity of canine

saliva induced by thiocyanate, lysozyme and, indirectly, nitrate, among others [314, 315], can even appear at low concentrations [316]. However, according to our findings, canine saliva can also be associated with public health significance, since salivary bacteria may contaminate the surroundings of people and may also colonize human skin and mucous membranes. Thus, ARG-rich bacteria present in and around humans do not even necessarily need to transfer their ARGs to potentially cause severe harm to various groups of people with weaknesses of the immune system, e.g., extremities in age or diseased state.
As a common trend among many nations, veterinary use of antibiotics is gradually declining [285, 291, 316–318]. In human medicine, antibiotic sales elevated by 65% in low- and middle-income countries and decreased slightly by 4% in high-income countries between 2000 and 2015, which adds up to a rise in global antibiotic consumption rates [302, 319]. As a presumable conclusion, several genes conferring resistance against clinically important antimicrobial groups are present in the salivary bacteriome of dogs that may drift to the genome of bacteria in humans [320]. Encounters with dog saliva and dog bites may serve as an interspecies platform for the migration of bacteria and ARGs. Transmitted bacteria may cause clinical symptoms, and ARGs that they harbor may confer resistance against antimicrobial agents of a clinical relevance.

6.2.2 Pig feces

The comprehensive metagenomic sequencing enabled us to gain profound insights into the composition of the bacteriome and resistome of the fecal samples. Shotgun sequencing, the methodological basis for the analysis of samples containing a large number of bacterial species, and metagenomic analyses of the resulting data are utilized less frequently than culture methods, but rather as a complement to them, and are not part of the current routine diagnostic and monitoring systems. However, this approach also allows the detection of bacterial species that would otherwise remain hidden due to their specific culture requirements. Beyond the comprehensive characterization of bacterial composition, this approach enables the study of relative bacterial abundances. Consequently, fecal analysis of production groups may facilitate a more profound comprehension of the feed-driven changes in the bacterial composition. This knowledge can help reaching the optimal microbiota composition to maximize growth and production efficiency [321]. Furthermore, the method may also facilitate a more profound comprehension of multifactorial diseases. In the present analysis, both saprophytic and facultatively pathogenic bacteria were identified, including the genera of *Escherichia, Prevotella* and *Bacteroides* which were the most prevalent. This is in contrast to the findings of a large-scale study of 287 pig feces samples from France, Denmark and China by Xiao et al. in 2016, which revealed that the gut bacteriota was dominated by *Prevotella, Bacteroides, Clostridium, Ruminococcus* and *Eubacterium spp.* [322]. In a 2019 study of 16 fresh fecal samples in Ireland, the most prevalent genera were *Firmicutes, Bacteroidetes* and *Tenericutes* [323]. The reason for this discrepancy remains to be elucidated, however, the high abundance of *Escherichia coli* can be a sign of dysbacteriosis, a condition that often occurs after antibiotic therapy [324]. There are several approaches to test the antimicrobial susceptibility of microorganisms. However, metagenomic methods based on next-generation sequencing, which are also utilized in this study, may pro-

vide additional information in this area. The extent of the use of a given class of antibiotics and the amount of the class-specific ARGs change proportionally. Thus, the metagenomic analysis of faecal samples can help tracking the population-level changes in AMR resulting from antibiotic use [325]. Accordingly, in the study of Munk and colleagues (2018), countries with high or diverse antibiotic use in livestock medicine (Italy, Spain) exhibited analogous patterns of antibiotic resistance. In contrast, the AMR profiles of nations with lower antibiotic use (Denmark, the Netherlands), which also appeared to be similar to eachother, differed significantly from the above mentioned countries with higher antibiotic use [121]. The hypothesis that the fecal resistome can serve as a reliable indicator of antibiotic usage is also substantiated by the following observation. In the study of Ghanbari and colleagues (2019), the administration of oxytetracycline in therapeutic doses resulted in an increased abundance and diversity of ARGs in swine faecal samples within eight days. Notably, this difference remained statistically significant for two weeks following the administration of antibiotics [326]. In 2012, in the United States of America, the antibiotics used in the largest quantities at pig and poultry farms were tetracyclines (67%), penicillins (11%), macrolides (7%), sulfonamides (6%), aminoglycosides (8%), lincosamides (2%) and cephalosporins (less than 1%) [327]. Meanwhile, an analysis of sales of antibiotics for veterinary use between 2011 and 2014 showed that in 29 European countries, including Hungary, the volume of tetracyclines, penicillins and sulfonamides purchased accounted for 69.9% of the total antibiotic sales. Sales of macrolides, polymixins, aminoglycosides and lincosamides were also significant [328]. A more limited study, specifically covering pig farms in Germany, showed that the most commonly used agents were, as above, tetracyclines, β-lactams and trimethoprim-sulphonamides [329]. At the same time, further studies of the consequences of antibiotic use at pig farms indicated that over the past few decades a significant accumulation of antibiotic resistance genes in the gut microbiome can be observed. The results of a large study by Munk and colleagues (2018) showed that the gut microbiome of the pigs was characterised by the presence of ARGs against tetracyclines, β-lactams and aminoglycosides [121]. Furthermore, Joyce and colleagues (2019) identified 56 types of ARGs in 16 pig fecal samples, the majority of which could affect tetracyclines, the members of the MLS-B group (macrolides, lincosamides, streptogramin B) and aminoglycosides [323]. Xiao and colleagues (2016) studied pig gut samples from three countries of two continents and found that most ARGs were identified against bacitracin, cephalosporins, macrolides, streptogramin B and tetracyclines [322]. Genes affecting these drug classes were also highly present in our samples. ARGs against fluoroquinolones were identified exclusively in Chinese pig fecal samples [322] and in poultry samples [121]. The first detection of ARGs against fluoroquinolones in European pig faecal metagenomes was reported by Joyce and colleagues (2019) [323]. In this study, three genes were identified that primarily encode resistance to fluoroquinolones (*mdtH, emrB, emrK*). The *gyr* and *par* gene families, which are also responsible for phenotypic resistance to fluoroquinolones, could not be detected. The seven ARGs of greatest public health importance with a high probability of bacterial transmission are *AAC(6')-Im, APH(2")-IIa, baeR, CfxA6*, *Escherichia coli marR* encoding AMR, and *marA*. Based on the CARD (Comprehensive Antibiotic Resistance Database) database, *AAC(6')-Im* and *APH(2")-IIa* can potentially affect aminoglycosides through enzymatic inactivation. *BaeR* may also induce aminoglycoside efflux. The *CfxA6* gene can induce the production of a β-lactamase enzyme, which may be specifically respon-

sible for the inactivation of cefamycins. The *Escherichia coli marR* gene variant can reduce the efficacy of fluoroquinolones, tetracyclines, cephalosporins, phenicols, glycylcyclines, penicillins and rifamycins through aspecific mechanisms. Most of these groups (fluoroquinolones, rifamycins, aminoglycosides, cephalosporins, glycylcyclines) are also of major importance in human medicine. *MarA* can also develop resistance to tetracyclines, fluoroquinolones, phenicols, penicillins, carbapenems, cephalosporins, cefamycins, rifamycins, monobactams, carbapenems and glycylcyclines in a similarly aspecific manner by reducing influx and increasing efflux. Bioinformatic tools can be used to determine not only genes, but also their transfer between bacteria, which is crucial for public health risk assessment [30]. Even ARGs with high mobility potential do not necessarily pose a real public health threat. The expression of genes is influenced by many factors. Accordingly, the identification of ARGs does not necessarily indicate phenotypic antibiotic resistance. The risk posed by identified ARGs is influenced not only by their location within the genome (e.g. position within the operon region) that influences gene expression. It is also determined by the location of the gene, since the proximity of MGEs generally increases the likelihood of gene transfer. However, certain mechanisms reduce the natural rate of transmission [24]. On the one hand, genes can only be passed on if members of the carrier and receiver bacterial groups can reproduce in sufficient numbers close to each other. Under natural conditions, there is little chance of gene transfer between two physically distant populations. However, in the case of ARGs, the use of a particular drug can act as a positive selection factor on the cells carrying the gene, so that a gene can be transferred over large physical distances. The overuse of antibiotics in intensive livestock farming can create the right conditions for this to happen. A second factor slowing down the gene spread is the lack of positive selective pressure in the following case. A bacterial population with an ARG against one drug class is not likely acquire a second type of ARG against the same antibiotic compound even if they become physically available. A third aspect to consider when assessing the likelihood of spread of an ARG is that the replication, transcription and translation steps required for gene transfer are energy intensive, which can be detrimental to bacteria in certain circumstances. However, the energy required varies from gene to gene, making some genes more profitable to transfer than others [24]. Nevertheless, the analysis of sequencing results is a very promising method for predicting the spread of a gene, as the phenomenon is difficult to study using culture-based methods.

In conclusion, there is a high degree of convergence in the research findings on the causes and consequences of increasing antibiotic resistance in pig farms. The reasons for the increasing emergence of AMR is the excess use of antibiotics at farms. However, the appearance of ARGs cannot be explained by high antibiotic use alone. Even fecal samples from pigs that had not been treated with antibiotics can have a high abundance of ARGs. Accordingly, ARGs will be present in the gut microbiome even in the absence of antimicrobial agents, and the possible transmission of ARGs to human pathogens cannot be excluded [323]. Although antibiotic susceptibility tests based on the analysis of metagenomes cannot yet compete in all respects with phenotypically expressed AMR-based assays and are not yet widely used in daily practice, the results are promising. Metagenomic analysis can also be used to infer the extent and likelihood of spread of AMR. In addition, the digital nature of the test results allows the data to be re-used or compared with samples taken at a later date or from more distant locations, which could

form the basis of monitoring and surveillance programs for both pathogens and AMR, even on a global scale. Momentarily, pig feces is only considered as a potential food hygiene hazard, as it is often responsible for meat contamination at slaughterhouses. However, it may also act as an indicator of the public health significance associated with the accumulation of ARGs in animal farms and a knowledge source for the optimization of animal feeding [321].

7. New scientific findings

7.1 Food and dietary supplements

7.1.1 Raw milk

Several ARGs were identified in publicly available raw milk samples including the phage integrase-associated *blaZ* that was predicted to derive from a plasmid. Some of these ARGs were detected in contigs from *Acinetobacter spp.*. In conclusion, the consumption of raw milk may have significant implications for public health.

7.1.2 Probiotic dairy products

In bacteria associated with the fermentation process of yoghurt and kefir, 23 ARG types were identified, including ones that were mobile such as (*lmrD*) or act against antibiotics that are critically important for human medicine. Considering that dairy products often derive from environments where antibiotics are applied and the ARG content of fermented foods appeared to be able to grow due to bacterial multiplication, the starting culture strains of fermented foods should be monitored and selected carefully in order to decrease the intake of ARGs via foods.

7.1.3 Other probiotic products

Based on the large-scale study *Bifidobacterium animalis* and *Lactococcus lactis* appeared to be highly rich in ARGs. In contrast, no *Lactobacillus casei* or *Lactobacillus paracasei* strains contained any ARGs, and in *Lactobacillus delbrueckii, Lactobacillus helveticus* and *Lactobacillus brevis*, ARGs were relatively less frequent. A high proportion of the identified ARGs appeared to be mobile. While acquiring mobile ARGs does not always confer AMR, extending current recommendations to detect potential functional traits of concern, including the selection of less ARG-rich bacterial species and strains used for food fermentation could be considered, with screening for mobile ARGs in probiotic bacteria.

7.2 Other animal source interfaces

7.2.1 Canine saliva

In the genome of potentially pathogenic bacterial species, which are some of the most relevant bacteria in dog bite infections, 69 ARGs were detected. Several ARGs, including ones against amoxicillin–clavulanate, the most commonly applied antimicrobial agent for dog bites, were predicted to be potentially transferable. According to our findings, canine saliva may be a source of transfer for ARG-rich bacteria that can either colonize the human body or transport ARGs to the host bacteriota, and thus can be considered significant in the interspecies spread of AMR.

7.2.2 Pig feces

Throughout the metagenomic analysis of swine fecal samples, 54 ARG types, including potentially mobile ones were detected. Similar surveillance studies at large-scale farms may lead to valuable discoveries in connection with the appearance and spread of AMR.

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9. Publications related to the PhD

1. Tóth A. G., Csabai I., Krikó E., Tőzsér D., Maróti G., Patai Á. V., Makrai L., Szita G., Solymosi N. (2020a): Antimicrobial resistance genes in raw milk for human consumption. In: *Scientific Reports*, 10. 7464.
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5. Tóth A. G., Tóth I., Rózsa B., Dubecz A., Patai Á. V., Németh T., Kaplan S., Kovács E. G., Makrai L., Solymosi N. (2022a): Canine saliva as a possible source of antimicrobial resistance genes. In: *Antibiotics*, 11. 1490.
6. Tóth A. G., Judge M. F., Nagy S. Á., Papp M., Solymosi N. (2023a): A survey on antimicrobial resistance genes of frequently used probiotic bacteria, 1901 to 2022. In: *Eurosurveillance*, 28. 2200272.
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9. Tóth A. G., Tóth I., Rózsa B., Dubecz A., Patai Á. V., Németh T., Makrai L., Solymosi N. (2022b): Canine saliva: a possible interspecies medium for mobile antimicrobial resistance genes. Presented at the 47th World Small Animal Veterinary Association Congress and XVIII FIAVAC Congress. Conference Poster. Lima, Peru.
10. Tóth A. G., Tóth D. L., Remport L., Tóth I., Németh T., Dubecz A., Patai Á. V., Makrai L., Solymosi N. (2024b): A One Health approach metagenomic study on the antimicrobial resistance traits of canine saliva. Presented at the Antimicrobial Resistance – Genomes, Big Data and Emerging Technologies Conference. Conference Poster. Wellcome Genome Campus, Hinxton, UK.

10. Further publications during the PhD programme

1. Tóth A. G. (2021): Az antimikrobiális rezisztencia kommunális szennyvízre alapozott, globális megfigyelése. In: *Magyar Állatorvosok Lapja*, 141. 351–352.
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3. Orbán É., Tóth A. G., Farkas R., Solymosi N. (2021): Bizonyítékokon alapuló állatorvoslás In: *Kamarai Állatorvos* 16. 32-38.
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11. Educational activities during the PhD programme

Throughout my PhD years, I taught an elective course called 'Practicls in Genomics I.' for undergraduate students for two years. I also gave plenary lectures on genomics in the Department of Animal Breeding and Genetics in each year of my PhD.

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13. Supplementary materials

13.1 Additional information

Addition to Table 1

The list of analyzed samples obtained from NCBI SRA. In the unified names of the samples the first character corresponds to the type of the sample (k and y, kefir and yoghurt, respectively), the second character comes from the first letter of the source (g, p and s for grain, product and strain, respectively), while the last tag is a sequence number. Column 'Sample' shows the available attribute data of the biosamples. The columns show the year and country of submission, sequencing device name and sequencing layout for each sample.

Sample ID	BioProject	Run	Type	Source	Sample	Year	Country	Sequencing device	Layout
k_g_01	PRJEB15432	ERR1653138	kefir	grain	Fr1 grain	2016	Ireland	Illumina MiSeq	Single
k_g_02	PRJEB15432	ERR1653139	kefir	grain	Ick grain	2016	Ireland	Illumina MiSeq	Single
k_g_03	PRJEB15432	ERR1653140	kefir	grain	UK3 grain	2016	Ireland	Illumina MiSeq	Single
k_g_04	PRJNA644779	SRR12171332	kefir	grain	kefir seed culture	2020	Hungary	Illumina NextSeq 500	Paired
k_p_01	PRJEB15432	ERR1653129	kefir	product	UK3, 8 hours	2016	Ireland	Illumina MiSeq	Single
k_p_02	PRJEB15432	ERR1653130	kefir	product	Fr1, 24 hours (replicate 2)	2016	Ireland	Illumina MiSeq	Single
k_p_03	PRJEB15432	ERR1653131	kefir	product	Ick, 24 hours (replicate 2)	2016	Ireland	Illumina MiSeq	Single
k_p_04	PRJEB15432	ERR1653132	kefir	product	UK3, 24 hours (replicate 2)	2016	Ireland	Illumina MiSeq	Single
k_p_05	PRJEB15432	ERR1653135	kefir	product	Fr1, 24 hours (replicate 3)	2016	Ireland	Illumina MiSeq	Single
k_p_06	PRJEB15432	ERR1653136	kefir	product	Ick, 24 hours (replicate 3)	2016	Ireland	Illumina MiSeq	Single
k_p_07	PRJEB15432	ERR1653137	kefir	product	UK3, 24 hours (replicate 3)	2016	Ireland	Illumina MiSeq	Single
k_p_08	PRJEB15432	ERR1653141	kefir	product	Fr1, 24 hours (replicate 1)	2016	Ireland	Illumina MiSeq	Single
k_p_09	PRJEB15432	ERR1653142	kefir	product	Ick, 24 hours (replicate 1)	2016	Ireland	Illumina MiSeq	Single
k_p_10	PRJEB15432	ERR1653143	kefir	product	UK3, 24 hours (replicate 1)	2016	Ireland	Illumina MiSeq	Single
k_p_11	PRJEB15432	ERR1653145	kefir	product	Fr1, 8 hours	2016	Ireland	Illumina MiSeq	Single
k_p_12	PRJEB15432	ERR1653146	kefir	product	Ick, 8 hours	2016	Ireland	Illumina MiSeq	Single
k_p_13	PRJNA288044	SRR2082409	kefir	product	KEFIR.shotgun	2013	Canada	Illumina MiSeq	Paired
k_p_14	PRJNA388572	SRR7287342	kefir	product	Metagenome from probiotic beverage K03	2019	Chile	Illumina HiSeq 2500	Paired
k_p_15	PRJNA388572	SRR8282406	kefir	product	Metagenome from probiotic beverage K02	2019	Chile	Illumina HiSeq 2500	Paired
k_s_01	PRJDB4955	DRR064132	kefir	strain	*Lactobacillus parakefiri JCM 8573*	2017	Japan	Illumina MiSeq	Paired
k_s_02	PRJNA222257	SRR1151211	kefir	strain	*Lactobacillus kefiranofaciens subsp. kefiranofaciens DSM 5016*	2014	China	Illumina HiSeq 2000	Paired
k_s_03	PRJNA222257	SRR1151212	kefir	strain	*Lactobacillus kefiranofaciens subsp. kefirgranum DSM 10550*	2014	China	Illumina HiSeq 2000	Paired
k_s_04	PRJNA222257	SRR1151213	kefir	strain	*Lactobacillus kefiri DSM 20587*	2014	China	Illumina HiSeq 2000	Paired
k_s_05	PRJNA222257	SRR1151226	kefir	strain	*Lactobacillus parakefiri DSM 10551*	2014	China	Illumina HiSeq 2000	Paired
k_s_06	PRJNA635855	SRR11965732	kefir	strain	*Acetobacter syzygii str. K03D05*	2020	Chile	Illumina MiSeq	Paired
k_s_07	PRJNA635872	SRR11966381	kefir	strain	*Lactobacillus plantarum K03D08*	2020	Chile	Illumina MiSeq	Paired
m_01	PRJEB15432	ERR1653133	milk	milk	0 hours (replicate 1)	2016	Ireland	Illumina MiSeq	Single
m_02	PRJEB15432	ERR1653134	milk	milk	0 hours (replicate 2)	2016	Ireland	Illumina MiSeq	Single
m_03	PRJEB15432	ERR1653144	milk	milk	0 hours (replicate 3)	2016	Ireland	Illumina MiSeq	Single
y_g_01	PRJNA644779	SRR12171305	yoghurt	grain	yoghurt seed culture	2020	Hungary	Illumina NextSeq 500	Paired
y_p_01	PRJEB30083	ERR2982980	yoghurt	product	Yoghurt-A	2018	Ireland	Illumina MiSeq	Paired
y_p_02	PRJEB30083	ERR2982981	yoghurt	product	Yoghurt-B	2018	Ireland	Illumina MiSeq	Paired
y_p_03	PRJEB30083	ERR2982982	yoghurt	product	Yoghurt-C	2018	Ireland	Illumina MiSeq	Paired
y_p_04	PRJEB30083	ERR2982983	yoghurt	product	Yoghurt-D	2018	Ireland	Illumina MiSeq	Paired
y_p_05	PRJEB30083	ERR2982984	yoghurt	product	Yoghurt-E	2018	Ireland	Illumina MiSeq	Paired

Addition to Table 2

The list of analysed samples obtained from NCBI SRA. In the unified names of the samples the first character corresponds to the type of the sample (s and m, isolate and metagenome, respectively), the second tag is a sequence number. Except the signed (*) all samples were paired end sequenced. Column 'Description' shows available information of the biosamples. The columns show the year and country of submission, sequencing device name and sequencing layout for each sample.

Sample ID	BioProject	Run	Description	Year	Country	Sequencing device	Layout
Isolates							
s01	PRJEB14693	ERR1554589	*Lactiplantibacillus plantarum*	2017	Italy	Illumina MiSeq	Paired
s02	PRJEB14693	ERR1554590	*Lactiplantibacillus plantarum*	2017	Italy	Illumina MiSeq	Paired
s03	PRJEB14693	ERR1554591	*Lactiplantibacillus plantarum*	2017	Italy	Illumina MiSeq	Paired
s04	PRJEB38007	ERR4421718	*Pseudomonas* sp. RGM2144	2020	Chile	Illumina NovaSeq 6000	Paired
s05	PRJNA312743	SRR3205957	*Limosilactobacillus fermentum*	2016	Malaysia	Illumina HiSeq 2000	Paired
s06	PRJNA347617	SRR4417252	*Limosilactobacillus fermentum*	2016	Thailand	Illumina HiSeq 2000	Paired
s07	PRJNA635872	SRR11966381	*Lactiplantibacillus plantarum*	2020	Chile	Illumina MiSeq	Paired
s08	PRJNA639653	SRR12037315	*Lactobacillus delbrueckii* subsp. *bulgaricus*	2020	Canada	Illumina MiSeq	Paired
s09	PRJNA639653	SRR12037316	*Lactobacillus delbrueckii* subsp. *bulgaricus*	2020	Canada	Illumina MiSeq	Paired
s10	PRJNA639653	SRR12037890	*Streptococcus thermophilus*	2020	Canada	Illumina MiSeq	Paired
s11	PRJNA649814	SRR12375795	*Enterococcus faecalis*	2020	USA	Illumina HiSeq X Ten	Paired
s12	PRJNA649814	SRR12375796	*Enterococcus faecalis*	2020	USA	Illumina HiSeq X Ten	Paired
s13	PRJNA649814	SRR12375797	*Enterococcus faecalis*	2020	USA	Illumina HiSeq X Ten	Paired
s14	PRJNA650131	SRR12376423	*Escherichia coli*	2020	USA	Illumina HiSeq X Ten	Paired
s15	PRJNA650131	SRR12376425	*Escherichia coli*	2020	USA	Illumina HiSeq X Ten	Paired
s16	PRJNA650131	SRR12376427	*Escherichia coli*	2020	USA	Illumina HiSeq X Ten	Paired
s17	PRJNA650131	SRR12376429	*Escherichia coli*	2020	USA	Illumina HiSeq X Ten	Paired
s18	PRJNA650131	SRR12376431	*Escherichia coli*	2020	USA	Illumina HiSeq X Ten	Paired
s19	PRJNA650131	SRR12376433	*Escherichia coli*	2020	USA	Illumina HiSeq X Ten	Paired
s20	PRJNA639653	SRR12412204	*Lacticaseibacillus rhamnosus*	2020	Canada	Illumina MiSeq	Paired
Microbiota							
m01	PRJNA474998	SRR8132838	probiotic powder (FC13678)	2019	China	Illumina HiSeq X Ten	Paired
m02	PRJNA475000	SRR8138827	probiotic powder (FC13669)	2019	China	Illumina HiSeq X Ten	Paired
m03	PRJNA474989	SRR8140233	probiotic powder (FC13655)	2019	China	Illumina HiSeq X Ten	Paired
m04	PRJNA474995	SRR8140386	probiotic powder (FC13628)	2019	China	Illumina HiSeq X Ten	Paired
* m05	PRJNA508569	SRR8289759	probiotic product (2)	2019	South Korea	Ion GeneStudio S5	Single
m06	PRJNA508569	SRR8289760	probiotic product (1)	2019	South Korea	Ion GeneStudio S5	Paired
* m07	PRJNA508569	SRR8289761	probiotic product (4)	2019	South Korea	Ion GeneStudio S5	Single
* m08	PRJNA508569	SRR8289762	probiotic product (3)	2019	South Korea	Ion GeneStudio S5	Single
* m09	PRJNA508569	SRR8289763	probiotic product (6)	2019	South Korea	Ion GeneStudio S5	Single
* m10	PRJNA508569	SRR8289764	probiotic product (5)	2019	South Korea	Ion GeneStudio S5	Single
m11	PRJNA542229	SRR9040978	dietary supplement (PB4)	2019	China	Illumina HiSeq 2000	Paired
m12	PRJNA542229	SRR9040979	dietary supplement (PB10)	2019	China	Illumina HiSeq 2000	Paired
m13	PRJNA542229	SRR9040980	dietary supplement (PB11)	2019	China	Illumina HiSeq 2000	Paired
m14	PRJNA542229	SRR9040981	dietary supplement (PB2)	2019	China	Illumina HiSeq 2000	Paired
m15	PRJNA542229	SRR9040982	dietary supplement (PB14)	2019	China	Illumina HiSeq 2000	Paired
m16	PRJNA542229	SRR9040983	dietary supplement (PB13)	2019	China	Illumina HiSeq 2000	Paired
m17	PRJNA542229	SRR9040984	dietary supplement (PB16)	2019	China	Illumina HiSeq 2000	Paired
m18	PRJNA542229	SRR9040986	dietary supplement (PB18)	2019	China	Illumina HiSeq 2000	Paired
m19	PRJNA542229	SRR9040987	dietary supplement (PB17)	2019	China	Illumina HiSeq 2000	Paired
m20	PRJNA542229	SRR9040988	dietary supplement (PB8)	2019	China	Illumina HiSeq 2000	Paired
m21	PRJNA542229	SRR9040989	dietary supplement (PB19)	2019	China	Illumina HiSeq 2000	Paired
m22	PRJNA542229	SRR9040990	dietary supplement (PB12)	2019	China	Illumina HiSeq 2000	Paired
m23	PRJNA542229	SRR9040991	dietary supplement (PB9)	2019	China	Illumina HiSeq 2000	Paired
m24	PRJNA542229	SRR9040992	dietary supplement (PB6)	2019	China	Illumina HiSeq 2000	Paired
m25	PRJNA542229	SRR9040993	dietary supplement (PB5)	2019	China	Illumina HiSeq 2000	Paired
m26	PRJNA542229	SRR9040994	dietary supplement (PB7)	2019	China	Illumina HiSeq 2000	Paired
m27	PRJNA644361	SRR12153424	probiotic capsule	2021	Hungary	Illumina NextSeq 500	Paired

Addition to Table 3

PRJNA648123: 2020, USA, Illumina HiSeq 3000, Paired

PRJNA683923: 2021, USA, Ilumina HiSeq X Ten, Paired

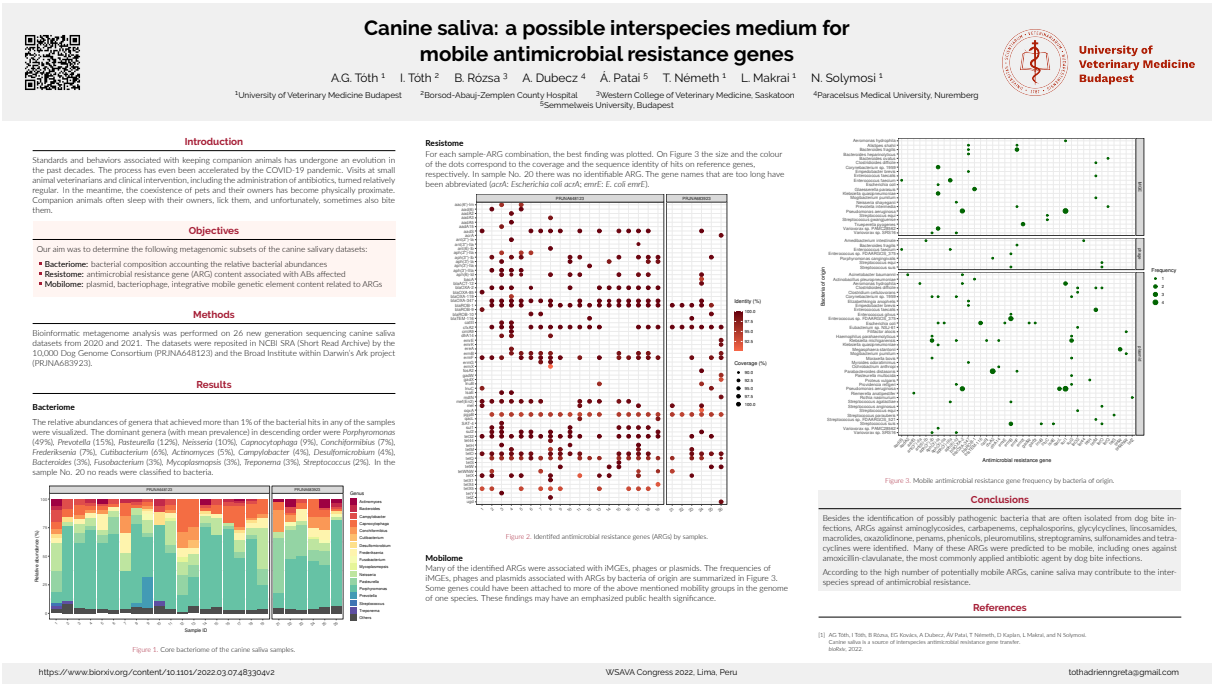
13.2 Conference presentations

One Health Antimicrobial Stewardship Conference, Alberta, Canada, 2021

A One Health approach study on alimentary products, sources in the process of animal-to-human antimicrobial resistance gene transfer

13.3 Conference posters

47th World Small Animal Veterinary Association Congress XVIII FIAVAC Congress, Lima, Peru, 2022



Canine saliva: a possible interspecies medium for mobile antimicrobial resistance genes

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Introduction

Standards and behaviors associated with keeping companion animals has undergone an evolution in the past decades. The process has even been accelerated by the COVID-19 pandemic. Visits at small animal veterinarians and clinical intervention, including the administration of antibiotics, turned relatively regular. In the meantime, the coexistence of pets and their owners has become physically proximate. Companion animals often sleep with their owners, lick them, and unfortunately, sometimes also bite them.

Objectives

Our aim was to determine the following metagenomic subsets of the canine salivary datasets:

- **Bacteriome:** bacterial composition accounting the relative bacterial abundances
- **Resistome:** antimicrobial resistance gene (ARG) content associated with ABs affected
- **Mobilome:** plasmid, bacteriophage, integrative mobile genetic element content related to ARGs

Methods

Bioinformatic metagenome analysis was performed on 26 new generation sequencing canine saliva datasets from 2020 and 2021. The datasets were reposited in NCBI SRA (Short Read Archive) by the 10,000 Dog Genome Consortium (PRJNA448123) and the Broad Institute within Darwin's Ark project (PRJNA683923).

Results

Bacteriome

The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the samples were visualized. The dominant genera (with mean prevalence) in descending order were *Porphyromonas* (49%), *Prevotella* (15%), *Pasteurella* (12%), *Neisseria* (10%), *Capnocytophaga* (9%), *Conchiformibius* (7%), *Frederiksenia* (7%), *Cutibacterium* (6%), *Actinomyces* (5%), *Campylobacter* (4%), *Desulfomicrobium* (4%), *Bacteroides* (3%), *Fusobacterium* (3%), *Mycoplasmopsis* (3%), *Treponema* (3%), *Streptococcus* (2%). In the sample No. 20 no reads were classified to bacteria.

Figure 1. Core bacteriome of the canine saliva samples.

Resistome

For each sample-ARG combination, the best finding was plotted. On Figure 3 the size and the colour of the dots correspond to the coverage and the sequence identity of hits on reference genes, respectively. In sample No. 20 there was no identifiable ARG. The gene names that are too long have been abbreviated (*acrA: Escherichia coli acrA; emrE: E. coli emrE*).

Figure 2. Identified antimicrobial resistance genes (ARGs) by samples.

Mobilome

Many of the identified ARGs were associated with IMGEs, phages or plasmids. The frequencies of IMGEs, phages and plasmids associated with ARGs by bacteria of origin are summarized in Figure 3. Some genes could have been attached to more of the above mentioned mobility groups in the genome of one species. These findings may have an emphasized public health significance.

Figure 3. Mobile antimicrobial resistance gene frequency by bacteria of origin.

Conclusions

Besides the identification of possibly pathogenic bacteria that are often isolated from dog bite infections, ARGs against aminoglycosides, carbapenems, cephalosporins, glycylcyclines, lincosamides, macrolides, oxazolidinone, penams, phenicols, pleuromutilins, streptogramins, sulfonamides and tetracyclines were identified. Many of these ARGs were predicted to be mobile, including ones against amoxicillin-clavulanate, the most commonly applied antibiotic agent by dog bite infections.

According to the high number of potentially mobile ARGs, canine saliva may contribute to the interspecies spread of antimicrobial resistance.

References

[1] AG Tóth, I Tóth, B Rózsa, EG Kovács, A Dubecz, ÁV Patai, T Németh, D Kaplan, L Makrai, and N Solymosi. Canine saliva is a source of interspecies antimicrobial resistance gene transfer. bioRxiv, 2022.

https://www.biorxiv.org/content/10.1101/2022.03.07.483304v2 WSAVA Congress 2022, Lima, Peru tothadriennegreta@gmail.com

Antimicrobial Resistance – Genomes, Big Data and Emerging Technologies, Wellcome Genome Campus, Hinxton, UK, 2024

A One Health approach metagenomic study on the antimicrobial resistance traits of canine saliva

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Introduction

Canine saliva can be considered as an interspecies transfer medium for several microorganisms, including bacteria with antimicrobial resistance genes (ARGs). Public health risk associated with ARGs depends on several factors (e.g., affected antibiotic class, mobility potential, host traits).

Objectives

Our aim was to assess the resistome (set of ARGs) of canine saliva samples considering the risk associated with the individual ARGs (mobility potential, affected antibiotic drug class, pathogen relatedness) and the canine physical and behavioral traits affecting the presence of ARGs.

Methods

The shotgun metagenomic sequencing dataset of 1830 canine saliva samples was bioinformatically analyzed. Bacteriome and resistome results, including the ARG subsets of higher public health risk ARGs and ESKAPE pathogen-related (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) higher public health risk ARGs were studied in light of survey data of the physical and behavioral traits of the dogs.

Results

Bacteriome properties

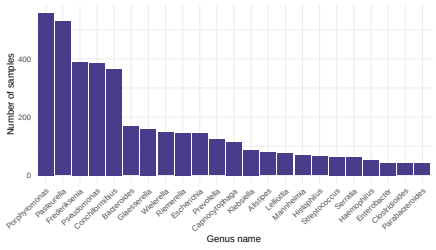

Figure 1. Bacterial genera associated with AMR determinants that appeared in at least 2% of all 1682 samples with the number of samples in which they were detected (axis Y).

Resistome properties

In the 1682 samples associated with sufficient ARG detection rates, 318 ARG types were identified.

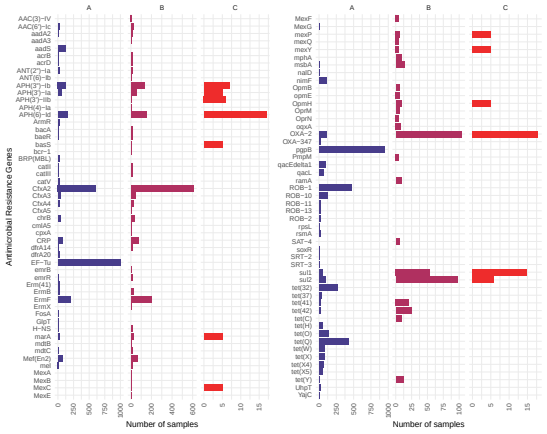

Figure 2. ARGs detected in more than 10 samples (A), higher public health risk ARGs detected in at least 5 samples (B) and higher public health risk ARGs deriving from ESKAPE pathogens detected in at least 5 samples (C). *EF-Tu* is the abbreviation for *Escherichia coli EF-Tu* mutants conferring resistance to Pulvomycin, *UhpT* for *E. coli UhpT* with mutation conferring resistance to fosfomycin, *GlpT* for *E. coli GlpT* with mutation conferring resistance to fosfomycin, *soxR* for *Pseudomonas aeruginosa soxR*, and *rpsL* for *Mycobacterium tuberculosis rpsL* mutations conferring resistance to streptomycin.

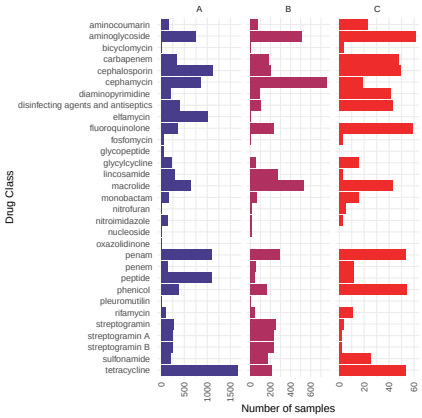

Figure 3. Antibiotic groups against which ARGs were detected in any metagenomic samples (A), antibiotic groups against which higher public health risk ARGs were detected in any samples (B) and antibiotic groups against which ESKAPE pathogen related higher public health risk ARGs appeared in any samples (C). The number of samples in which ARGs against the presented antibiotic groups were detected is shown on the horizontal axis. Antibiotic compounds affected by multidrug resistance are displayed separately.

Figure 4. Antimicrobial resistance mechanism abundances of ARGs detected in any metagenomic samples (A), antimicrobial resistance mechanism abundances of higher public health risk ARGs detected in any samples (B) and antimicrobial resistance mechanism abundances of ESKAPE pathogen related higher public health risk ARGs in any samples (C). The number of occasions when ARGs with the given antimicrobial resistance mechanisms were detected is presented on the horizontal axis.

The detected ARGs can affect 31 antibiotic drug classes by various resistance mechanisms. ARGs against tetracyclines and cephalosporins appeared in the highest number of samples, followed by peptides, that are Critically Important Antimicrobials for Human Medicine (WHO).

ARG associations of canine physical and behavioral traits

Certain characteristics and physical traits, such as being sterilized, purebred, under knee-high, having white or diluted (merle, gray) fur color, heterochromia or long fur was associated with higher ARG presence by one or more ARG detection approaches (A, B or C). Regarding behavioral traits, dogs characterized with decreased activity and decreased aggression more often harbored ARGs. Reduced playfulness (indicated as 'motor pattern' on *Figure* 5.) could be specifically associated with higher public health risk ARG presence.

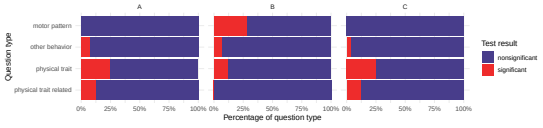

Figure 5. Proportions of significant ($p \leq 0.05$) and nonsignificant associations by question groups and approaches (ARGs detected in all metagenomic samples (A), higher public health risk ARGs detected in any samples (B) ESKAPE pathogen-related higher public health risk ARGs in any samples (C)).

Conclusions

Even though the oral microbiome of the owners is unknown, One Health and public health implications of the close human-pet bonds and factors potentially underlying the rise in salivary ARG numbers should be considered, mostly in the light of the presence of ARGs affecting Critically Important Antimicrobials for Human Medicine (e.g., peptides, aminoglycosides, fluoroquinolones, macrolides).

Tóth, A.G., et al. A One Health approach metagenomic study on the antimicrobial resistance traits of canine saliva. bioRxiv (2024): 2024-01. tothadriennngreta@gmail.com