UNIVERSITY OF VETERINARY MEDICINE BUDAPEST

CENTRE FOR BIOINFORMATICS

Study of antimicrobial resistance genes of bacteria in kefir and yoghurt

Joghurt- és kefír-baktériumtörzsek antimikrobiális rezisztencia-gén-tartalmának vizsgálata

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Abstract

Antimicrobial resistance is one of the foremost threats facing the treatment of infectious diseases worldwide. Recent studies have highlighted the potential for antimicrobial resistance genes (ARGs) in fermented foods to contribute to antimicrobial resistance (AMR) via horizontal gene transfer (HGT). The focus of our study was on investigating the ARG content (resistome) and mobility potential of ARGs (mobilome) of bacterial strains commonly used in probiotic products, namely yoghurt and kefir. We performed metagenomic analyses on freely available data sets originating from various kefir and yoghurt strains using next generation sequencing (NGS) in order to gain an insight into the ARG diversity, frequency and mobility. Our study shows that kefir and yoghurt products carry diverse and significant amounts of ARGs and that these genes may often be associated with integrative mobile genetic elements (iMGEs) or plasmids, conferring mobility. Certain bacterial species such as *Bifidobacterium animalis* and *Streptococcus thermophilus* were found to have higher ARG content. Overall, my results support the hypothesis that ARGs are present in fermented foods, namely yoghurt and kefir, and have the potential to contribute to AMR.

Absztrakt

Az antimikrobiális rezisztencia (AMR) világszerte az egyik legjelentősebb kihívás, amely a fertőző betegségek terápiáját nehezíti. A közelmúltban született tanulmányok rávilágítottak arra, hogy a fermentált élelmiszerekben található antimikrobiális rezisztenciagének (ARG) a horizontális géntranszferen (HGT) keresztül hozzájárulhatnak az AMR-hez. Vizsgálatunk középpontjában a probiotikus termékekben, azaz a joghurtban és a kefirben általánosan használt baktériumtörzsek ARG-tartalmának (rezisztom) és mobilitási potenciáljának vizsgálata állt. A különböző kefir-, és joghurt-törzsekből származó, szabadon elérhető, új generációs szekvenálási (NGS) adatállományokon metagenomikai elemzéseket végeztünk, hogy betekintést nyerjünk az azokban előforduló ARG-k diverzitásába, gyakoriságába és mobilitásába. Vizsgálatunk azt mutatja, hogy a kefir- és joghurt-termékek változatos és jelentős mennyiségű ARG-t hordoznak, és ezek a gének gyakran integrált mobilis elemekkel (iMGE) vagy plazmidokkal társulhatnak, ami mobilitást biztosít számukra. Bizonyos baktériumfajok, például a *Bifidobacterium animalis* és a *Streptococcus thermophilus* magasabb ARG-tartalommal bír. Összességében eredményeim alátámasztják azt a hipotézist, hogy az ARG-k jelen vannak a fermentált élelmiszerekben, nevezetesen a joghurtban és a kefirben, és hozzájárulhatnak az AMR-hez.

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1. List of Abbreviations

AMR	Antimicrobial Resistance
ARG	Antimicrobial Resistance Gene
BP	Base Pair
CIA	Critically Important Antimicrobial
CFU	Colony Forming Units
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
dsDNA	Double Stranded Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
EMA	European Medicines Agency
GTA	Gene Transfer Agent
HGT	Horizontal Gene Transfer
HPCIA	Highest Priority Critically Important Antimicrobial
IA	Important Antimicrobial
ICE	Integrative Conjugative Element
i**MGE**	Integrative Mobile Genetic Element
MGE	Mobile Genetic Element
MDR	Multi-drug Resistant
MGE	Mobile Genetic Element
MITES	Miniature Inverted-repeat Transposable Elements
MRSA	Methicillin Resistant Staphylococcus Aureus
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
OIE	World Organisation for Animal Health
ORF	Open Reading Frame
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
SRA	Sequence Read Archive

ssDNA	Single Stranded Deoxyribonucleic Acid
VIA	Veterinary Important Antimicrobial Agents
VCIA	Veterinary Critically Important Antimicrobial Agents
VHIA	Veterinary Highly Important Antimicrobial Agents
WGA	Whole Genome Amplification
WGS	Whole Genome Sequencing
WHO	World Health Organization

2. Introduction

Antimicrobial resistance (AMR) is one of the foremost threats facing the treatment of infectious diseases worldwide, both in human and animal medicine. Dealing with the COVID-19 pandemic of late highlights the significance of this threat. Antimicrobials are used to treat human and animal diseases, and since antimicrobial use has been increasing, so too has AMR causing these antimicrobials to become less effective. It is currently estimated that 700,000 people are dying per annum from AMR related issues, with projections forecasting this number to rise to 10 million by 2050 (IACG, 2019). Identifying potential sources of AMR are thus of utmost importance. AMR can be acquired by bacteria via gene mutations or horizontal gene transfer (HGT) (Partridge *et al.,* 2018). HGT occurs primarily by transformation, conjugation or transduction and involves small packets of DNA being transferred between bacteria. The transfer of antimicrobial resistance genes (ARGs) is enhanced by being linked with mobile genetic elements (MGEs), particularly plasmids (Johansson *et al.,* 2021; Todar, 2020). In this study, as a continuation of our work (Tóth *et al.*, 2020a; Tóth *et al.*, 2021), we explored the possible development of AMR due to HGT during the fermentation of food produce derived from animal sources, namely kefir and yoghurt products. The multiplication of bacteria during the fermentation process is widely known and understood. However, a less studied side to this is that if their genomes harbour ARGs, then their amount is increasing too, which could potentially be aiding the development of AMR.

Probiotics are encouraged to restore natural microbiomes and a healthy gut (Sanders et al. 2018). However, there is some question over their effectiveness (Eloe-Fadrosh, 2015; Suez *et al.,* 2019). Instead of in-situ samples, studies mostly rely on stool samples, which have been shown to often be inaccurate representations of the gut microbiome (Donaldson, Lee & Mazmanian, 2016; Zmora *et al.,* 2018). In addition, there has been some concern over the effects of the bacteria that probiotics harbour (Gopalakrishnan *et al.,* 2018). Recent studies indicate that the genomes of the bacterial composition of fermented foods contain ARGs (Berreta, Baumgardner & Kopper, 2020; Rozman *et al.,* 2020; Selvin *et al.,* 2020; Sharma *et al.,* 2014; Zheng *et al.,* 2017). The gut

microbiome may thus act as a resistome (Montassier *et al.,* 2021). This would give ARGs, even from non-pathogenic bacteria, the opportunity to spread via HGT to pathogenic bacteria that they become physically close to, creating 'superbugs'. In this study, we looked to further explore whether probiotics contribute to this resistome and thus possibly to the development of AMR worldwide. I collated data from other studies to identify the most frequently found bacterial species in kefir and yoghurt products. Using this data, we explored the ARG content of eight bacterial species by performing metagenomic analyses on freely available data sets originating from kefir and yoghurt strains. Using next generation sequencing (NGS) we were able to gain an insight into the ARG diversity, frequency and mobility of those data sets.

3. Literature Review

The concept of ‘one health’ states that the health of humans, animals and the environment are interconnected. Thus a threat to one of these poses a threat to all. This idea was first conceptualized by Rudolph Virchow in the 19th century stating that “between animal and human medicine there are no dividing lines- nor should there be”. A prime example of this is seen today seen with AMR. Antibiotics are known to enter the environment via waste, animal feed and the direct use of antimicrobials. Similar or the same antimicrobials and classes of drugs are often used in both human and veterinary medicine and these are all known to be contributing to AMR. Antibiotics are one of the most commonly used classes of drugs and it is estimated that worldwide antibiotic use in livestock is twice that of human medicine (Aarestrup, 2012). This misuse and overuse of antibiotics in livestock is a contributor to the spread of ARGs and known to be causing AMR in humans (Kunhikannan *et al.*, 2021). Antimicrobials are often used in livestock for mass treatment and also as preventative medicine. In addition, they are still used as growth promoters on farms in many countries today. AMR from livestock is then transmitted to humans via the environment, food and direct contact with the animals (Finley *et al.,* 2013). The U.S.A. and other developed countries where intensive farming has long been established have been the main culprits of antimicrobial use in livestock. However, many developed countries have been developing stricter laws on antimicrobial usage such as banning the use of antimicrobials as growth promoters. Some European countries now produce annual national reports on antimicrobial use in farm sectors and have been reducing usage as encouraged by the European Medicines Agency (EMA). This is most notable in Northern European countries (EMA, 2019b). On the other hand, antimicrobial usage is now increasing in developing countries in Asia and Africa as they turn to intensive farming (Founou, Founou & Essack*,* 2016). Antimicrobial use is forecast to rise by as much as 67% globally between 2010 and 2030 and to double in countries such as South Africa, China, Brazil and India (Van Boeckel e*t al.,* 2015). With these countries exporting animal and animal derived produce, along with tourists and travellers into these countries, the AMR produced is transmitted globally. China, the U.S.A. and Brazil are among the largest contributors to antimicrobial use for livestock (Van Boeckel *et al*., 2017).

Nowadays ARGs are ubiquitous. They are not only found in bacteria but also in higher organisms and environmental sources such as soil, water and waste which have been identified as important reservoirs of ARGs (Chen *et al.*, 2016). There is even evidence of antibiotic resistant bacteria from sources dating back 4 million years and they have been identified in Amazonian tribes which have never come into contact with antibiotics (Kunhikannan *et al.*, 2021). However, the threat that we now face from AMR stems from the daily use of antimicrobials in human and animal medicine and, even patients without any prior drug treatment often now have antimicrobial resistant bacteria (Alexander, Hembach & Schwartz, 2020). For example, there are more than 1,000 known β-lactamase variants as a result of β-lactams being some of the most prescribed antibiotic classes (WHO, 2018b). Identifying the sources of ARGs is now essential. As well as medicines, all foods entering the body may be potential sources of ARGs. Studies have shown that ARGs are widespread in food crops such as carrots, lettuce, tomato and peppers as well as in food producing animals (Wang *et al.*, 2020). Bacteria such as *Proteobacteria, Bacteroidetes, Actinobacteria* and *Firmicutes* have been identified as dominant phyla in ready-to-eat foods (Li *et al.,* 2020; Zhou *et al.*, 2020) with multi-drug resistant ARGs being predominant along with chloramphenicol, macrolide-lincosamide-streptogramin, aminoglycoside, bacitracin, tetracycline and β-lactam resistance (Li *et al.,* 2020; Zhou *et al.*, 2020). Several recent studies have also identified ARGs in probiotic foods including yoghurt and kefir (De Alcântara Rodrigues *et al.*, 2020; Machado *et al.,* 2020; Tóth *et al.,* 2020a; Tóth *et al.,* 2021). Commensal bacteria also harbour ARGs and there is evidence that commensal bacteria share ARGs with pathogenic bacteria in the human gut (Blake *et al.*, 2003; Salyers *et al*., 2004). Tóth *et al.* (2020a) found the *poxtA* ARG to be one of the most abundant ARGs in probiotics and it confers resistance to a variety of critical antibiotics. They surmise that the use of certain medications such as phenicols in veterinary medicine may play a role in the presence of *poxtA* in probiotics. However, it is also important to note that not all ARGs are harmful to humans. Several factors are at play when determining ARG risk such as gene mobility and host pathogenicity (Zhang *et al.*, 2021).

Probiotics have become particularly popular in the Western world due to their claimed beneficial health properties (Sanders et al. 2018). However, recently the question of the potential adverse effects of consuming probiotics has been raised (Gopalakrishnan et al. 2018). The number of bacteria multiplies significantly during fermentation, therefore the number of ARGs also has the potential to multiply significantly and provide opportunities for ARG transferral. Thus, probiotics can act not only as a reservoir for ARGs but also as a medium for their exchange. Although kefir is consumed globally, it is not as popular worldwide as yoghurt. However, it is particularly popular in Eastern Europe and Russia whereas yoghurt is particularly popular in countries such as Germany and France. Yoghurt and kefir have minor differences in their fermentation processes. Both are pasteurised and derived from fermented milk but the fermentation of yoghurt only involves bacteria whereas kefir utilises fungi in addition. Yoghurt consists of milk fermented by thermophilic starter lactic acid bacteria, namely *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus* cultures (Behare, Lule & Patil, 2016). Some countries allow other specific bacterial cultures too. Refrigerated yoghurt must contain at least 10^8 bacterial colony forming units (CFU) per gram at the time of manufacturing (IDFA, 2019). Kefir is produced by milk fermented by kefir grains. Kefir grains consist of a wider variety of starter culture microorganisms with lactic acid bacteria at 10^8 CFU per gram, acetic acid bacteria at 10^6-10^7 CFU per gram and yeast at 10^5 CFU per gram (Garrote, Abraham & De Antoni, 2010). The bacteria are of various genera, most commonly *Lactobacillus, Lactococcus* and *Leuconostoc*. The yeasts used are *Kluyveromyces* and *Saccharomyces* (Surono & Hosono, 2011). The microbes from probiotics must remain in the gastrointestinal tract for a certain length of time to exert their effect and there is an antibiotic-dependent effect as well as an individual's metagenome seeming to play an important role (Montassier *et al.,* 2021) as the success of probiotic gut colonisation varies between individuals (Zmora *et al.,* 2018).

As we have been developing antimicrobials, bacteria have been evolving defence mechanisms against antibiotics which lead to AMR. We explored the dominant resistance mechanisms of the ARGs found. These were: (a) antibiotic inactivation, (b) antibiotic

target alteration, (c) antibiotic target replacement, (d) antibiotic target protection, (e) antibiotic efflux and (f) reduced permeability to antibiotics.

(a) Antibiotic inactivation is the most common form. Enzymes are utilized to alter the antibiotic structure and thus inactivate it. It is done mainly by the following enzymes: aminoglycoside modifying enzymes, β-lactamases and chloramphenicol acetyltransferases (Kapoor, Saigal & Elongavan, 2017). These inactivate the antibiotic either by removing the essential reactive center or by impairing target binding of the antibiotic (D'Costa & Wright, 2017). Antibiotic inactivation is the most important method of action for resistance to cephalosporins and penicillins.

(b) Antibiotic target alteration is when the antibiotic target site is changed to make it more resistant to antibiotics. There are many bacterial target sites that can be altered. This is done by point mutations to genes encoding the target site or via enzymes which alter the binding site. This is used against a variety of antibiotics such as rifampin, fluoroquinolones, macrolides and oxazolidinones. It often involves altering the 30S or 50S subunit. Another prime example is the *erm* (erythromycin ribosomal methylation) gene coded enzymatic alteration of the target site conferring macrolide resistance.

(c) Antibiotic target replacement is where the antibiotic target site is substituted by new biochemically similar proteins which are not inhibited by the antibiotic and thus confer antibiotic resistance. This is seen in MRSA (methicillin resistant *staphylococcus aureus*) with *PBP2a* (penicillin binding protein 2a) and with vancomycin resistance in enterococci using *van* genes (Munita & Arias, 2016).

(d) Antibiotic target protection is where an 'antibiotic resistance protein' or 'target protection protein' binds directly to the drug's target site so the antibiotic cannot reach the bacteria. Unlike many of the other forms of antibiotic resistance mechanisms, this does not result in a permanent change to the target site; instead the antibiotic resistance protein must repeatedly bind to the target site to offer protection. This is used against Tetracyclines by the ARGs *Tet(M)* and *Tet(O)* and against fluoroquinolones by *Qnr* (Munita & Arias, 2016).

(e) Antibiotic efflux is also a common method of antibiotic resistance that usually occurs with another antibiotic resistance mechanism such as antibiotic target alteration. It involves genes which encode either generalised multi-drug or antibiotic specific active efflux pumps. These efflux pumps are cytoplasmic membrane proteins which export

antibiotics from the bacterial cell and this mechanism is known to be used against all classes of antibiotics except polymyxin (Kapoor, Saigal & Elongavan, 2017).

(f) Reducing the antibiotic's permeability is particularly used in gram-negative bacteria as they have an outer membrane which they use as an antimicrobial barrier. However, it is also seen in gram-positive bacteria. This mechanism involves altering porin gene expression to reduce permeability of the outer membrane (Peterson & Kaur, 2018). Tetracyclines, β-lactams and fluoroquinolones are particularly affected as they are hydrophilic and often pass through the outer membranes via porins.

The metagenome encompasses all the genetic material in a sample including the bacteriome, virome, resistome and host contaminants. The bacteriome refers to all genetic material within the sample from bacteria and the core bacteriome is the bacteriome that is in all samples. The resistome is the pool of genes that contribute to antibiotic resistance in bacteria, i.e. the antimicrobial genes (ARGs) in a sample. Resistomes can be classified either as extrinsic, acquired and mobile or, as intrinsic and innate (Singh, Verma & Taneja, 2019). The acquired or extrinsic resistome encompasses those genes where genetic mutations are heritable and can be passed on by HGT. Whereas innate or intrinsic resistomes refer to sets of genes which can be acquired via mutations and confer innate resistance but are not developed due to antibiotic exposure nor passed on by HGT (Bello-López *et al.*, 2019). The resistome is a complex pool of genes conferring antibiotic resistance comprising of the resistome from environmental microorganisms (environmental resistome), resistome from pathogens (clinical resistome), intrinsic resistome and ARG precursors (proto-resistance genes) (Bello-López *et al.*, 2019). Resistome analysis is where we collect and describe all the genes in samples that are conferring antimicrobial resistance. This includes ARGs from pathogenic and non-pathogenic bacteria. All genes from the extrinsic resistome can be mobilized by horizontal gene transfer (HGT) for use by pathogenic or non-pathogenic bacteria.

AMR can be acquired by bacteria via vertical gene transfer (genetic mutations) or HGT (Partridge *et al.,* 2018). Vertical gene transfer involves transposons randomly recombining. Transposons can enter into phages and plasmids and be transferred alongside

them into other cells. HGT refers to the transfer of genetic material between bacteria and without it, the mutation and evolution of bacteria would be very slow and AMR limited. It involves the transferal of small packets of genetic material between related or unrelated bacteria conferring advantages such as ARGs, virulence traits and the ability to utilize certain substrates or survive in certain environments (Bello-López *et al.*, 2019). However, HGT is limited by mechanisms such as surface exclusion which acts as a barrier against conjugation, the CRISPR (clustered, regularly interspaced, short palindromic repeats) system which acts as the prokaryotes immune system and uses Cas (CRISPR-associated) proteins to inhibit plasmids and phages, and also by the restriction modification system where the host uses restriction endonucleases to destroy these packets of DNA (Bello-López *et al.*, 2019). HGT occurs primarily via transformation, transduction and conjugation. Transformation is the direct uptake, incorporation and expression of naked exogenous DNA which occurs between closely related bacteria and is mediated by proteins (Frost *et al.*, 2005). Bacteria must be in a state of competence to uptake the DNA and interestingly, studies have shown that exposure to antibiotics can induce competence (Von Wintersdorff *et al.*, 2016). Transduction can be specialized (involving specific genes) or generalized and involves DNA transfer mediated by independent bacterial viruses known as bacteriophages or phages (Frost *et al.*, 2005). Phages package some of the host's DNA into their capsid which they then inject into the recipient. Gene transfer agents (GTAs) are phage-like structures that can also carry genetic material which can then be released via lysis into the recipient cell. GTAs have several advantages as they can transfer DNA between bacterial phyla while not being constrained by cell to cell contact and they provide good environmental protection to the DNA though they are unable to self-propagate as they predominantly carry host genome fragments (Von Wintersdorff *et al.*, 2016). Conjugation requires conjugative plasmids or integrated conjugative elements (ICEs) and cell to cell contact via cell surface pili or adhesins for the unidirectional transfer of DNA via a bridge or conjugative pore. It is considered to be the main mechanism responsible for HGT and for AMR (Bello-López *et al.*, 2019). Conjugation provides a more efficient method of entering the recipient host than transformation while allowing for a broader host range than transduction (Von Wintersdorff *et al.*, 2016).

The mobilome is the set of mobile genetic elements (MGEs) in a genome, such as insertions sequences, transposons, integrons and plasmids (Bello-López *et al.*, 2019). MGEs are discrete regions of genetic coding encoding enzymes and other proteins that readily move within genomes (vertical gene transfer or intracellular mobility) and between bacterial genomes (horizontal gene transfer or intercellular mobility). MGEs which can integrate into the host's genome are termed integrative mobile genetic elements (iMGEs). The transfer of ARGs is enhanced by being linked with iMGEs (Johansson *et al.,* 2021; Li *et al.,* 2020; Todar, 2020; Zhou *et al.*, 2020). MGEs have been documented to transfer between extremely taxonomically diverse bacteria and enable rapid spread making them very important vectors of ARGs (Von Wintersdorff *et al.*, 2016). Various enzymes such as recombinases, transposases and resolvases may also be encoded by MGEs and aid in their mobility (Bello-López *et al.*, 2019). Transposons and insertion sequences are MGEs that are incorporated into plasmids and chromosomes. Transposons or transposable elements are DNA sequences that can move from one location to another in genomes, are flanked by insertion sequences or repeats, encode transposase enzymes responsible for their mobility and aid ARG transferral. They can be class 1 'copy-and-paste' retrotransposons, class 2 'cut-and-paste' DNA transposons or class 3 MITES (miniature inverted-repeat transposable elements). They can also insert into promoter regions and activate conjugative genes (Sun *et al.*, 2019). Integrons are other genetic elements that capture genetic material by site-specific recombination and carry gene cassettes. They encode integrase enzymes which can assemble genes and provide a promoter for expression (Frost *et al.*, 2005). On the other hand, plasmids are self-replicating bundles of genetic coding organized into 'replicons' and usually don't contain essential genes but contain genes encoding processes distinct from those encoded by the bacterial chromosome (Frost *et al.*, 2005). They are composed of circular dsDNA and less commonly, linear dsDNA and are passed on by undergoing partitioning (Frost *et al.*, 2005). Their function is dependent on the host and may change the host's phenotype (Bello-López *et al.*, 2019). Plasmids with the same replication mechanism cannot coexist in the same cell: this is known as 'Incompatibility' or the 'Inc' trait and provides the basis for plasmid classification (Frost *et al.*, 2005). Phages are the most abundant and rapidly replicating life forms on earth (Frost *et al.*, 2005). They have long been used is bioengineering, molecular biology and genomics and are now drawing

attention for their potential in nanotechnology and antimicrobial therapy. They comprise of 'hijacking' genes which use the host's replicative mechanisms to replicate themselves and genes that encode capsids (Frost *et al.*, 2005). Virulent phages rapidly replicate and destroy the bacterial host. On the other hand, there are also quiescent temperate phages. Some of these replicate autonomously, but most temperate phages usually undergo lysogeny where they integrate into the bacterial chromosome and replicate as a prophage (Frost *et al.*, 2005). Environmental factors can cause phages to switch from quiescent to virulent and relatively large dsDNA phages can enter new hosts by transduction. If they are of the same bacterial species as the host and recombine with the host's DNA they can survive (Frost *et al.*, 2005).

Shotgun metagenomics describes untargeted sequencing to study all genetic material within a sample in order to decipher the entire community of organisms within it. Metagenomics is used when looking to study genetic sequences of microbes which cannot be separated and is often termed 'environmental genomics'. It has allowed genes from environmental samples containing numerous microbes to be studied similarly to if they were a single genome. They are either cloned and inserted into to a vector or sequenced. It enables complex microbiome investigation and pathogen tracking. As the technology has been advancing and speed drastically increasing, the costs have also been decreasing and thus it is gradually moving from research into clinical practice. Shotgun metagenomics comprises of collecting, processing and sequencing samples. Then preprocessing of sequencing reads and finally sequence and post-processing analyses and validation are conducted (Quince *et al.*, 2017). Various experimental and computational techniques are now available to help carry out each step.

A variety of technology is now available to further metagenomic studies. Next generation sequencing (NGS) was founded in 2005 and is a type of whole genome shotgun sequencing that has since revolutionised metagenomic research. Using NGS you can assemble genomes *de novo* or compare your genome to reference genomes, explore molecular evolution and track pathogens etc. Entire genomes are now being sequenced within 24 hours in contrast with the previously used Sanger technology which was

developed in the 1900s and has been used as the gold standard for sequencing. Sanger sequencing uses similar technology but takes a decade to sequence a genome and sequences just one DNA fragment at a time (Behjati & Tarpay, 2013). NGS sequences millions of DNA fragments in parallel and thus hundreds to thousands of genes at a time. NGS also has greater potential than Sanger sequencing to discover novel and rare variants. DNA/RNA is extracted from genomes and broken at random points to be fragmented into shorter sequences of nucleotides. These are then stored in FASTQ files. DNA polymerase adds fluorescent tagged nucleotides one by one. These fragments are pieced together by bioinformatic tools mapping them to reference genomes. Each base is sequenced several times to ensure accuracy. NGS can be used to investigate entire genomes down to specific genes. The clinical use of NGS and metagenomics is yet to be fully taken advantage of due to factors such as cost, speed, personnel experience, quality assurance and uncertainty differentiating contaminants and colonizers from pathogens.

4. Materials and Methods

I first collated data from other studies to determine the most frequently identified bacterial strains found in kefir and yoghurt products (see Appendix). The papers used were Bengoa *et al.,* 2018; Bourrie, Willing & Cotter, 2016; Witthuhn, Schoeman & Britz, 2005; Wyk, 2019; Gueimonde *et al.*, 2004 and Leech *et al*., 2020. Since I found high numbers of species in the literature, we selected the following most common ones for further analyses: *Bifidobacterium animalis, Lacticaseibacillus casei, Lacticaseibacillus paracasei, Lactiplantibacillus argentoratensis, Lactiplantibacillus plantarum, Lactobacillus helveticus, Levilactobacillus brevis* and *Streptococcus thermophilus.* I collected all the available suitable samples for these species from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository. The SRA database is a free international bioinformatics repository and the largest public repository of sequencing data, especially of high throughput short read data for NGS data. It is provided by the International Nucleotide Sequence Database Collaboration in collaboration with the NCBI, the European Bioinformatics Institute and the DNA Data Bank of Japan in order to make an unrestricted platform for bioinformatic analyses. During the search I checked if the reads were suitable using predetermined parameters. I filtered the source by DNA and the platform by Illumina. I selected those with WGS or WGA strategies, genomic sources, random or PCR selections and paired layouts. Only those results with at least a million spots were selected.

4.1 Analysed datasets

We obtained 584 suitable datasets from the NCBI SRA repository. The identifiers of the samples by species are the following:

Bifidobacterium animalis: ERR2221337, ERR2221385, ERR2397402, ERR3931553, ERR3931556, ERR3931557, ERR3931563, ERR3931572, ERR3931573, ERR3931581, ERR3931588, ERR3931756, ERR3931758, ERR4552599, ERR4552600, ERR4552601, ERR4552602, ERR4552603, ERR4552604, SRR11515245, SRR2124893, SRR5310856, SRR5310858, SRR5310869, SRR5310872, SRR5310875, SRR7030678,

SRR7030680, SRR7030681, SRR7030682, SRR7030689, SRR7102022, SRR8060796, SRR8382541, SRR9274925, SRR9275544, SRR9275545.

Lacticaseibacillus casei: SRR3944187, SRR3944188, SRR5518762, SRR5518764, SRR5518765, SRR5518846, SRR5518847, SRR5518848, SRR5518849, SRR5518850, SRR5518851, SRR5518852, SRR5518853, SRR5518854, SRR5518855, SRR5518856, SRR5518857, SRR5518858, SRR6790308, SRR6790309, SRR6790310, SRR6790312, SRR6790313, SRR6790314, SRR6790315, SRR6790316, SRR6790317, SRR6790318, SRR6790319, SRR6790320, SRR6790321, SRR6790322, SRR6790323, SRR6790324, SRR6790325, SRR6790326, SRR6790327, SRR6790328, SRR6790329, SRR6790330, SRR6790331, SRR6790332, SRR6790333, SRR6790334, SRR6790335, SRR6790336, SRR6790337, SRR6790338, SRR6790339, SRR6790340, SRR6790341, SRR6790342, SRR6790343, SRR6790344, SRR6790345, SRR6790346, SRR6790347, SRR6790348, SRR6790349.

Lacticaseibacillus paracasei: SRR3944189, SRR3944190.

Lactiplantibacillus argentoratensis: ERR387522, SRR1151228.

Lactiplantibacillus plantarum: ERR1158396, ERR1158397, ERR1554589, ERR1554590, ERR1554591, ERR2221349, ERR2286790, ERR2286794, ERR2286891, ERR2286898, ERR2286899, ERR298627, ERR298635, ERR298703, ERR298714, ERR3151426, ERR3159182, ERR3162803, ERR3162986, ERR3283901, ERR3330792, ERR3330865, ERR3330866, ERR3330867, ERR3330868, ERR3330869, ERR3330870, ERR3330937, ERR386058, ERR386059, ERR3899072, ERR433488, ERR4593526, ERR4593527, ERR4593528, ERR4593530, ERR4593549, ERR4593550, ERR4833518, ERR485022, ERR485030, ERR485098, ERR485109, ERR570145, ERR570151, ERR570177, ERR570181, ERR570183, ERR570281, ERR570284, ERR570285, SRR10291920, SRR10357779, SRR10442245, SRR10605764, SRR10605765, SRR10605766, SRR10605767, SRR10605768, SRR10605769, SRR10605770, SRR10605771, SRR10605772, SRR10605773, SRR10605774, SRR10605775, SRR10605776, SRR10605777, SRR10605778, SRR10605779, SRR10605780, SRR10605781, SRR10605782, SRR10605783, SRR10605784, SRR10605785, SRR10605786, SRR10605787, SRR10605788, SRR10605789, SRR10605790, SRR10605791, SRR10605792, SRR10605793, SRR10605794, SRR10605795,

SRR10605796, SRR10605797, SRR10605798, SRR10605799, SRR10605800, SRR10605801, SRR10605802, SRR10605803, SRR10605804, SRR10605805, SRR10605806, SRR10605807, SRR10605808, SRR10605809, SRR10605810, SRR10605811, SRR10605812, SRR10605813, SRR10605814, SRR10605815, SRR10605816, SRR10605817, SRR10605818, SRR10605819, SRR10605820, SRR10605821, SRR10605822, SRR10605823, SRR10605824, SRR10605825, SRR10605826, SRR10605827, SRR10605828, SRR10605829, SRR10605830, SRR10605831, SRR10605832, SRR10605833, SRR10605834, SRR10605835, SRR10605836, SRR10605837, SRR10605838, SRR10605839, SRR10605840, SRR10605841, SRR10605842, SRR10605843, SRR10605844, SRR10605845, SRR10605846, SRR10605847, SRR10605848, SRR10605849, SRR10605850, SRR10605851, SRR10605852, SRR10605853, SRR10605854, SRR10605855, SRR10605856, SRR10605857, SRR10605858, SRR10605859, SRR10605860, SRR10605861, SRR10605862, SRR10605863, SRR10605864, SRR10605865, SRR10605866, SRR10605867, SRR10605868, SRR10605869, SRR10605870, SRR10605871, SRR10605872, SRR10671639, SRR10671640, SRR10671641, SRR10671642, SRR10902843, SRR1151193, SRR11910127, SRR11910128, SRR11910136, SRR11910143, SRR11910158, SRR11910162, SRR11910168, SRR11910178, SRR11910187, SRR11910196, SRR11910200, SRR11910204, SRR11910205, SRR11910217, SRR11910220, SRR11910248, SRR11910255, SRR11910257, SRR11910269, SRR11910275, SRR11910286, SRR11910296, SRR11910298, SRR11910303, SRR11910305, SRR11910311, SRR11910337, SRR11910344, SRR11910354, SRR11910358, SRR11910365, SRR11910395, SRR11910414, SRR11910423, SRR11910429, SRR11910437, SRR12145645, SRR12559458, SRR12559459, SRR12559460, SRR12559461, SRR12559462, SRR12559464, SRR12559465, SRR12559467, SRR12559468, SRR12559469, SRR12559470, SRR12559471, SRR12559472, SRR12559473, SRR12559474, SRR12559476, SRR12559477, SRR12559478, SRR12559479, SRR12559480, SRR12559481, SRR12559482, SRR12559483, SRR12559484, SRR12559485, SRR12559487, SRR12559488, SRR12559489, SRR12559490, SRR12559491, SRR12559707, SRR12559708, SRR12559710, SRR12559711, SRR12559712,

SRR12559713, SRR12559714, SRR12559715, SRR12559716, SRR12559717, SRR12559718, SRR12559719, SRR12559721, SRR12559722, SRR12559723, SRR12559724, SRR12559725, SRR12559726, SRR12559727, SRR12559728, SRR12559729, SRR12559730, SRR12559732, SRR12559733, SRR12559734, SRR12559735, SRR12559736, SRR12559737, SRR12559738, SRR12559739, SRR12559740, SRR12559741, SRR12559743, SRR12559744, SRR12559745, SRR12559746, SRR12559747, SRR12559748, SRR12559749, SRR12559750, SRR12559751, SRR12559752, SRR12559755, SRR12559756, SRR12559757, SRR12559758, SRR12559759, SRR12559858, SRR12559859, SRR12559860, SRR12559861, SRR12559862, SRR12559864, SRR12559865, SRR12559867, SRR12559868, SRR12559869, SRR12559870, SRR12559871, SRR12559872, SRR12559873, SRR12559875, SRR12559876, SRR12559877, SRR12559878, SRR12559879, SRR12559880, SRR12559881, SRR12559882, SRR12559883, SRR12559884, SRR12559886, SRR12559887, SRR12559888, SRR12559889, SRR12559890, SRR12560026, SRR12560027, SRR12560028, SRR12560029, SRR12560030, SRR12560032, SRR12560033, SRR12560034, SRR12875629, SRR13060588, SRR13213118, SRR13442537, SRR13442538, SRR13442539, SRR1552084, SRR1552590, SRR1552611, SRR1552612, SRR1552613, SRR1552614, SRR1552615, SRR1552616, SRR2142235, SRR4124949, SRR4124950, SRR4124954, SRR4124955, SRR5518758, SRR5518859, SRR5518863, SRR5518864, SRR5518865, SRR5518866, SRR5518867, SRR5518868, SRR5518869, SRR5518870, SRR5518871, SRR5518872, SRR5518873, SRR5518874, SRR5518875, SRR5724505, SRR5914586, SRR7550999, SRR7551002, SRR7551003, SRR7551004, SRR7551010, SRR8182735, SRR8252890, SRR8252891, SRR8382543, SRR8693953, SRR9107669, SRR9861755.

Lactobacillus helveticus: ERR204044, ERR298639, ERR298656, ERR298711, ERR3283916, ERR387534, ERR485034, ERR485051, ERR485106, SRR10332348, SRR1151128, SRR11910141, SRR11910150, SRR11910390, SRR12560068, SRR12560069, SRR4450492, SRR5724508, SRR9866100, SRR9866101, SRR9866102, SRR9866103, SRR9866104, SRR9866105, SRR9866106, SRR9866107, SRR9866108, SRR9866109, SRR9866110, SRR9866111, SRR9866112, SRR9866113, SRR9866114, SRR9866115, SRR9866116, SRR9866117, SRR9866118, SRR9866119, SRR9866120,

SRR9866121, SRR9866122, SRR9866123, SRR9866124, SRR9866125, SRR9866126, SRR9866127, SRR9866128, SRR9866129, SRR9866130, SRR9866131, SRR9866132, SRR9866133, SRR9866134, SRR9866135, SRR9866136, SRR9866137, SRR9866138, SRR9866139, SRR9866140, SRR9866141.

Levilactobacillus brevis: ERR2305654, ERR3283759, ERR386054, SRR1151178, SRR11910179, SRR11910198, SRR11910213, SRR11910306, SRR11910310, SRR11910336, SRR11910381, SRR11910417, SRR11910436, SRR11910439, SRR12559992, SRR12559993, SRR12560016, SRR4450488, SRR5724506, SRR5724507, SRR5724509, SRR6918185, SRR6918186, SRR6918187, SRR6918188, SRR6918189, SRR6918190, SRR7551000, SRR7551001.

Streptococcus thermophilus: ERR3330766, SRR11276977, SRR11277033, SRR11277066, SRR11910208, SRR11910216, SRR11910219, SRR11910241, SRR11910242, SRR11910258, SRR11910321, SRR11910326, SRR11910350, SRR11910360, SRR11910376, SRR11910392, SRR11910418, SRR12037890, SRR5310871, SRR5310876, SRR5310877, SRR6319282, SRR6319283, SRR6319284, SRR6319285, SRR6319286, SRR7850709.

4.2 Bioinformatic analysis

Quality based filtering and trimming of the raw short reads was performed by TrimGalore (v.0.6.6, https://github. com/FelixKrueger/TrimGalore) setting 20 as a quality threshold. Only reads longer than 50 base pair (bp) were retained. The preprocessed reads were assembled to contigs by MEGAHIT (v1.2.9) (Li *et* al., 2015) using default settings. From the contigs having more than 500 bp, all possible open reading frames (ORFs) were gathered by Prodigal (v2.6.3) (Hyatt *et al.*, 2010). The protein translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v.3.1.1) (McArthur *et al.*, 2013; Jia *et al.*, 2017) by Resistance Gene Identifier (RGI, v5.1.1) with Diamond (Buchfink, Xie & Huson, 2015). The ORFs classified as perfect or strict were further filtered with 90% identity and 60% coverage. All nudged hits were excluded. The iMGE content of the ARG harbouring contigs was analyzed by MobileElementFinder (v1.0.3). Following the distance concept of Johansson *et al.* (2021) for each bacterial species, those with a distance threshold defined within iMGEs and ARGs

were considered associated. The plasmid origin probability of the contigs was estimated by PlasFlow (v.1.1) (Krawczyk, Lipinski & Dziembowski, 2018). The phage content of the assembled contigs was predicted by VirSorter2 (v2.2.1) (Guo, J. *et al*, 2021). The findings were filtered for dsDNA phages and ssDNAs. All data management procedures, plotting and analyses were performed in R environment (v4.1.0) (R Core Team, 2021).

5. Results

The analysis of the short read datasets (n=584) from the metagenomic samples of *Bifidobacterium animalis, Lacticaseibacillus casei, Lacticaseibacillus paracasei, Lactiplantibacillus argentoratensis, Lactiplantibacillus plantarum, Lactobacillus helveticus, Levilactobacillus brevis* and *Streptococcus thermophilus* are summarised in the two following sections: Resistome and Mobilome. Following the presentation of the identified ARGs (resistome), the mobility potential of the ARGs (mobilome) is summarized based on the identification of iMGEs in the sequence context of the ARGs and contigs harbouring the ARGs being identified as plasmid originated. We did not find any phage-associated ARGs.

5.1 Resistome

The resistome results are summarized in *Table 1* and *Figures 1* and *2*. In *Table 1* we listed all ARGs found by species. The ARG names which were too long have been abbreviated. *Bifidobacterium animalis* had the lowest diversity of ARGs with two distinct ARGs identified and *Streptococcus thermophilus* had the highest with twenty-seven.

The ARGs belonging to the genome of *Bifidobacterium animalis* may play a role in the appearance of resistance against rifamycin and tetracycline; *Lacticaseibacillus casei*: aminoglycoside, elfamycin; *Lactiplantibacillus plantarum*: aminoglycoside, fluoroquinolone, lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline; *Lactobacillus helveticus*: fluoroquinolone, lincosamide, macrolide; *Levilactobacillus brevis*: fluoroquinolone, lincosamide, macrolide; *Streptococcus thermophilus*: aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, fosfomycin, glycylcycline, lincosamide, macrolide, monobactam, oxazolidinone, penam, penem, phenicol, pleuromutilin, rifamycin, streptogramin, tetracycline, triclosan.

Table 1. ARGs by species. The gene names that are too long have been abbreviated (*cat-TC*: *Lactobacillus reuteri cat-TC*; *EF-Tu*: *Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin*; *rpoB*: *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin; *soxS*: *Escherichia coli soxS* with mutation conferring antibiotic resistance)

Bifidobacterium animalis	rpoB, tet(W/N/W)
Lacticaseibacillus casei	AAC(6')-Ib10, AAC(6')-Ic, ANT(3")-IIa, EF-Tu
Lactiplantibacillus plantarum	AAC(6')-Ii, ANT(3")-IIa, ANT(6)-Ia, cat-TC, catA8, eatAv, ErmB, lnuA, msrC, patA, patB, pmrA, poxtA, RlmA(II), tetM, tetS
Lactobacillus helveticus	lnuA, patA, patB, pmrA, RlmA(II)
Levilactobacillus brevis	lnuA, patA, patB, pmrA, RlmA(II)
Streptococcus thermophilus	AAC(6')-Ie-APH(2")-Ia, AAC(6')-Ii, aadS, AcrE, APH(2")-IVa, CblA-1, CfxA2, CfxA3, CfxA6, CTX-M-90, dfrF, eatAv, ErmB, ErmF, ErmG, FosA3, lnuC, lsaE, soxS, tet(40), Tet(X1), tet32, tetO, tetQ, tetS, tetW,

The proportions of resistance mechanisms were calculated based on the ARG diversity. The dominant mechanisms of identified ARGs were antibiotic target protection (30.72%), antibiotic inactivation (26.8%), antibiotic target alteration with antibiotic target replacement (23.53%), antibiotic target alteration (9.15%), antibiotic efflux (7.19%), antibiotic target replacement (1.96%) and, antibiotic target alteration with antibiotic efflux and reduced permeability to antibiotics (0.65%).

Figure 1 represents the proportion of positive and negative ARG samples of each of the eight bacterial species. These results show that all species contained ARGs except *Lactiplantibacillus argentoratensis and Lacticaseibacillus paracasei. Levilactobacillus brevis, Lactobacillus helveticus, Lactiplantibacillus plantarum* and *Lacticaseibacillus casei* all had a low proportion of ARG positive samples. This is in contrast with *Bifidobacterium animalis* which had a higher proportion of ARG positive samples.

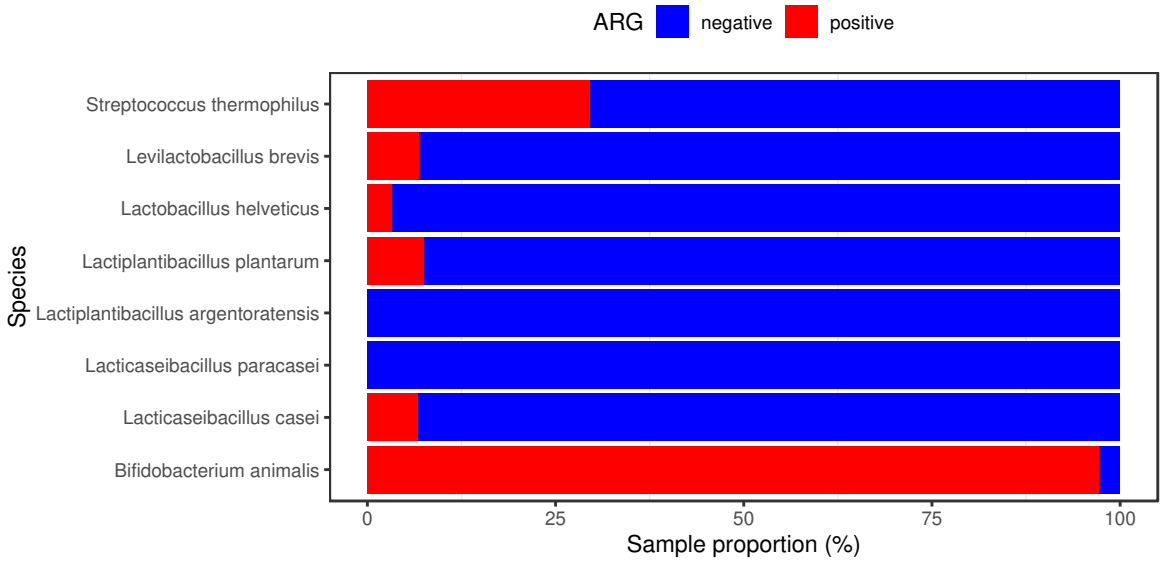

Figure 1 Antimicrobial resistance gene (ARG) positive sample frequencies. The proportion of ARG positive samples by species.

Figure 2 illustrates all identified ARGs by species and sample. The size of dot indicates coverage (the proportion of the reference ARG sequence covered by the ORF). The colour indicates the percentage sequence identity to the reference ARG. The most common ARGs were *rpoB* mutants conferring resistance to rifampicin and *tet(W/N/W)* which were detected in 36 and 20 samples respectively, all in *Bifidobacterium animalis*. The number of distinct ARGs found within a given sample ranged from 1 to 20 with the highest number of ARGs found in sample SRR11277066 obtained from *Streptococcus thermophilus*.

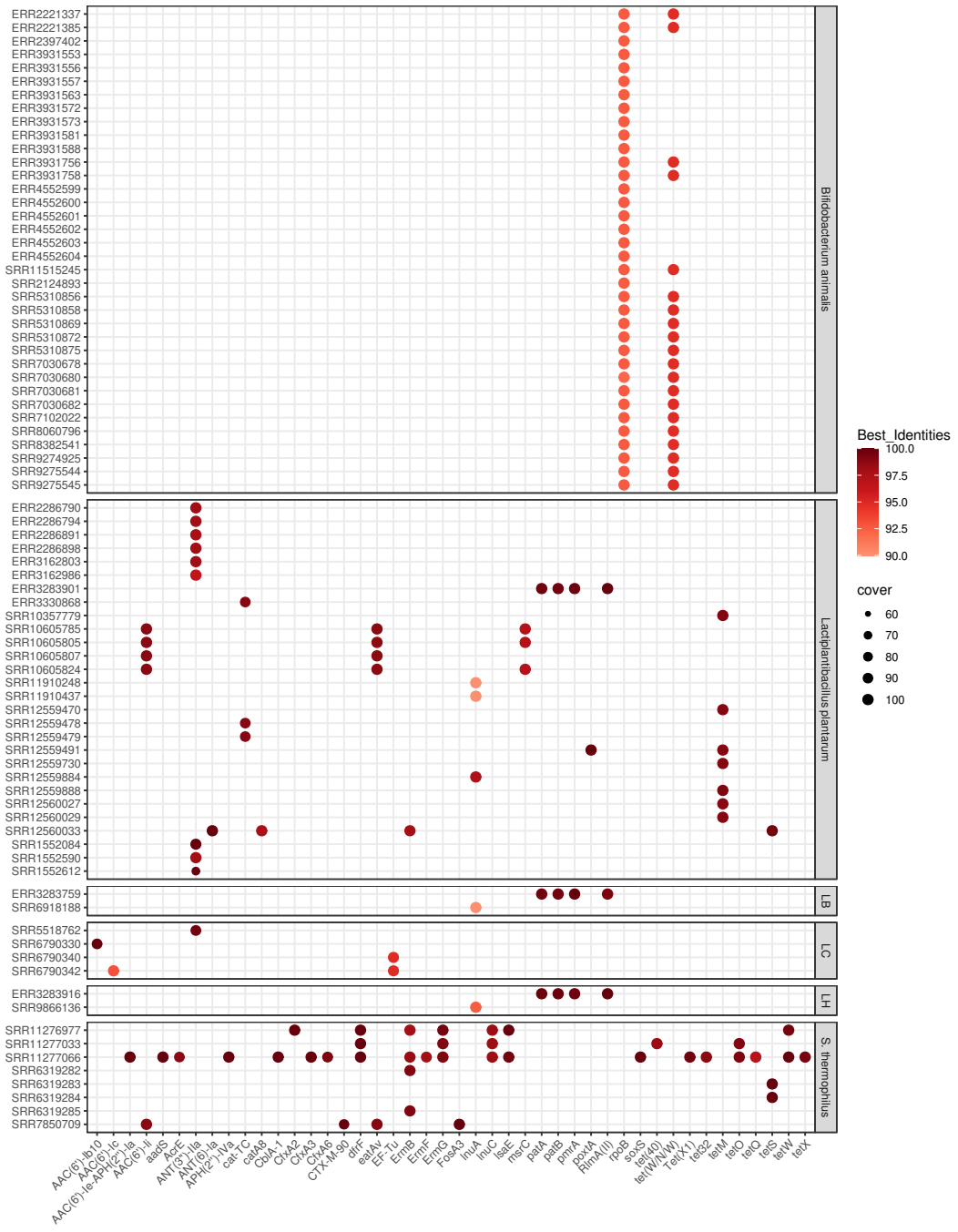

Figure 2 Identified ARGs by sample. For each sample-ARG combination, only the best finding is plotted. The size of the dot corresponds to the coverage and the colour to the sequence identity of hits on reference genes. Abbreviated species names: LB: *Levilactobacillus brevis*; LC: *Lacticaseibacillus casei*; LH: *Lactobacillus helveticus*. Gene names which are too long have been abbreviated (*cat-TC*: *Lactobacillus reuteri cat-TC; EF-Tu*: *Escherichia coli EF-Tu mutants conferring resistance to Pulvomycin*; *rpoB*: *Bifidobacterium adolescentis rpoB* mutants conferring resistance to rifampicin; *soxS*: *Escherichia coli soxS* with mutation conferring antibiotic resistance*)*

5.2 Mobilome

The frequency of iMGEs and plasmids associated with the ARGs is summarized by bacteria of origin in *Figure 3.* This figure represents the mobility of the ARGs identified. The size of the dot indicates the number of occurrences of the given mobile ARG.

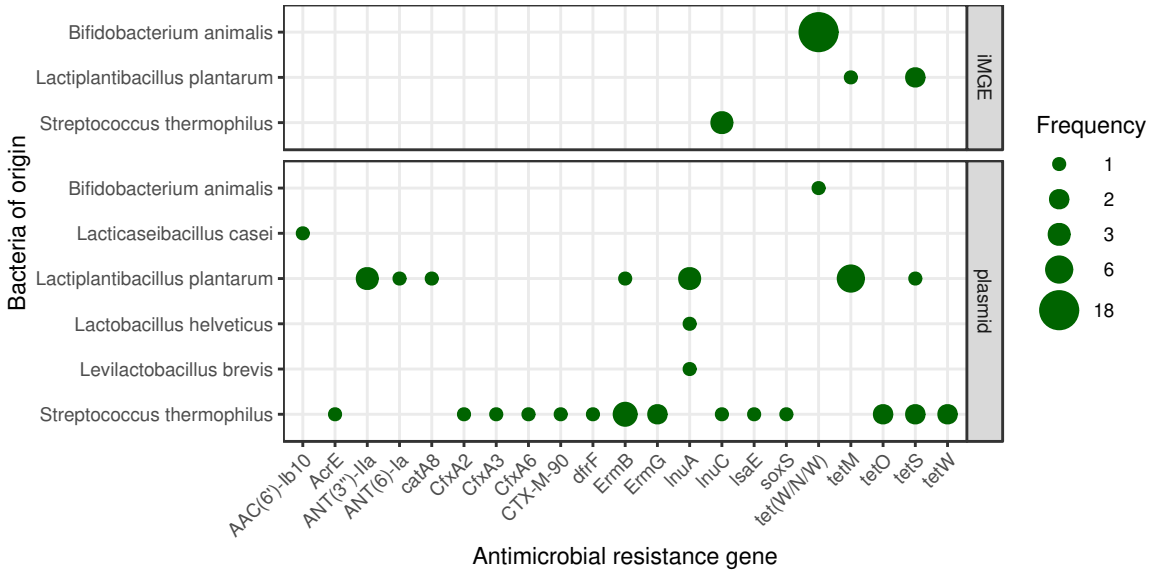

Figure 3. Mobile ARG frequency by bacteria of origin. The size of the dot indicates the occurrence frequency of the given gene flanked by iMGE or positioned in a plasmid. No ARGs were detected in phages.

5.2.1 iMGEs

Our results show that iMGE associated ARGs were detected in three species (*Bifidobacterium animalis, Lactiplantibacillus plantarum and Streptococcus thermophilus).* These iMGEs were physically close to the ARG in the chromosome, thus providing mobility potential. In 18 metagenomic samples we found *tet(W/N/W)* associated with iMGEs on contigs classified as *Bifidobacterium animalis* originated. In three further samples *inuC* is linked to an iMGE on *Streptococcus thermophilus* originated contigs. The ARGs *tetM* and *tetS* were also linked with iMGEs on *Lactiplantibacillus plantarum* originated contigs.

5.2.2 Plasmids

Using another technique, we predicted if contigs with ARGs were coming from plasmids. This prediction shows the number of times each gene occurred on plasmid sequences in the bacterial species. In *Bifidobacterium animalis,* we identified a plasmid associated contig with *tet(W/N/W)*. We also identified a plasmid associated contig with *AAC(6')-lb10* classified as *Lacticaseibacillus casei*. In both the *Lactobacillus helveticus and Levilactobacillus brevis* samples, one contig of plasmid origin had the *InuA* gene. In the *Lactiplantibacillus plantarum* samples, the genes *ANT(3'')-IIa, ANT(6)-Ia, catA8, ErmB, lnuA, tetM* and *tetS* were detected in 3, 1, 1, 1, 3, 6 and 1 contigs of plasmids respectively. In the *Streptococcus thermophilus* samples a plasmid associated contig harboured each of the genes *AcrE, CfxA2, CfxA3, CfxA6, CTX-M-90, dfrF, lnuC, lsaE* and *soxS*. Furthermore, in the *Streptococcus thermophilus* samples, the genes *ErmB, ErmG, tetO tetS* and *tetW* were detected in 3, 2, 2, 2 and 2 contigs of plasmids respectively.

6. Discussion & Conclusions

Our study confirms that numerous ARGs are present in kefir and yoghurt products and that many of them are mobile. Thus, yoghurt and kefir have the potential to contribute to AMR. *Figure 1* shows the proportion of ARG positive samples by bacterial species. We found that *Levilactobacillus brevis, Lactobacillus helveticus, Lactiplantibacillus plantarum* and *Lacticaseibacillus casei* all had low proportions of ARG positive samples (<10%). *Lactiplantibacillus plantarum* is one of the most commonly used strains in probiotics so it is important to note that it had a low proportion of ARG positive samples indicating that it is a good choice for continued use in probiotics. This is in contrast with *Bifidobacterium animalis* which was found in fewer samples but had proportionally higher ARG positive samples with nearly all samples containing ARGs and thus our study suggests that it is not an optimum choice for probiotic use. In addition, no ARGs were found in *Lactiplantibacillus argentoratensis and Lacticaseibacillus paracasei* indicating that these are also good choices for probiotic cultures though there was only two samples of each so further studies would be needed to determine their suitability. In *Table 1* we can see the ARGs found listed by species. It shows that although *Bifidobacterium animalis* had the highest proportion of ARGs (*Figure 1*), it had the lowest diversity of ARGs with only two distinct ARGs identified. Sixteen ARGs were identified in *Lactiplantibacillus plantarum* but it is important to bear in mind that it had by far the largest sample size which may skew results. Interestingly, the same five ARGs were identified in both *Levilactobacillus brevis* and *Lactobacillus helveticus* and these were the only ARGs found in both species. We identified a further four ARGs in *Lacticaseibacillus casei*. Furthermore, twenty-six distinct ARGs were identified in *Streptococcus thermophilus* making its ARG content the most diverse. In addition, in *Figure 2* we can see that the number of distinct ARGs found within a given sample ranged from 1 to 20 with the highest number of ARGs being found in sample SRR11277066 from *Streptococcus thermophilus*.

In *Figure 3* we identified the mobile ARG frequencies by bacterial species. The mobility potentials of the ARGs were predicted based on identifying iMGEs and plasmids as these may play a significant role in HGT. Where iMGEs are identified in the sequence

context of an ARG, greater mobility can be assumed. The case is the same if the contig harbouring an ARG is plasmid originated. There were 18 occurrences of tet(W/N/W) where iMGEs were close to the ARG in the chromosome and one incidence where it was positioned in a plasmid. This was by far the most frequently identified mobile ARG but the only bacterial species it was found in was *Bifidobacterium animalis*. It is also important to note that despite its abundance, it was the only mobile ARG we could find in *Bifidobacterium animalis*. In contrast, in *Streptococcus thermophilus* twenty occurrences of plasmid originated ARGs were identified and three of ARGs being flanked by iMGEs, totalling twenty-three occurrences of mobile ARGs from fourteen distinct ARGs. *Lactiplantibacillus plantarum* had three incidences of ARGs being flanked by iMGEs and sixteen of plasmid originated ARGs, totalling nineteen incidences of mobile ARGs from nine distinct ARGs. Six of these mobile ARG occurrences were the *TetM* gene thus making it the ARG with the second highest mobility potential. *Lacticaseibacillus casei, Levilactobacillus brevis* and *Lactobacillus helveticus* all had one incidence of plasmid originated ARG with the same ARG identified in *Levilactobacillus brevis* and *Lactobacillus helveticus*, *InuC.*

In *Table 1* we listed the ARGs found by species. The ARGs found in *Bifidobacterium animalis* (*Bifidobacterium adolescentis rpoB mutants* conferring resistance to rifampicin and *tet(W/N/W)*) have been previously identified as occurring in this species. To my knowledge, none of the four ARGs found in *Lacticaseibacillus casei* (*AAC(6')-Ib10, AAC(6')-Ic, ANT(3")-IIa* and *Escherichia coli EF-Tu mutants* conferring resistance to Pulvomycin) have been previously identified as occurring in *Lacticaseibacillus casei* in the literature associated with this topic. Also, of the ARGs found in *Lactiplantibacillus plantarum* (*AAC(6')-Ii, ANT(3")-IIa, ANT(6)-Ia, Lactobacillus reuteri cat-TC, catA8, eatAv, ErmB, lnuA, msrC, patA, patB, pmrA, poxtA, RlmA(II), tetM* and *tetS*), none are identified in the literature as occurring in this species according to my knowledge. Furthermore, none of the ARGs found in *Lactobacillus helveticus* (*lnuA, patA, patB, pmrA* and *RlmA(II)*) nor in *Levilactobacillus brevis* (*lnuA, patA, patB, pmrA* and *RlmA(II)*) have been previously identified as occurring in these species as far as I have been able to ascertain. In addition, according to my knowledge this is the first time the

ARGs found in *Streptococcus thermophilus* (*AAC(6')-Ie-APH(2'')-Ia, AAC(6')-Ii, aadS, AcrE, APH(2'')-IVa, CblA-1, CfxA2, CfxA3, CfxA6, CTX-M-90, dfrF, eatAv, ErmB, ErmF, ErmG, FosA3, lnuC, lsaE, soxS, tet(40), Tet(X1), tet32, tetO, tetQ, tetS, tetW* and *tetX*) have been identified in this species.

In *Figure 2* we can see all samples where ARGs were detected. This shows that the most common ARGs were *rpoB* mutants conferring resistance to rifampicin and *tet(W/N/W)* detected in 36 and 20 samples respectively, all in *Bifidobacterium animalis*. *Bifidobacterium adolescentis rpoB mutants* conferring resistance to rifampicin is a rifamycin-resistant beta-subunit of RNA polymerase (*rpoB*). It has the resistance mechanisms of antibiotic target alteration and antibiotic target replacement. It is capable of conferring resistance to the Rifamycin drug class. This gene is published in the literature as being found in the following bacteria: *Bifidobacterium animalis, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium thermophilum, Gardnerella vaginalis* and *Streptococcus pneumoniae*. Similarly to what we found in the literature, we also identified it as existing in *Bifidobacterium animalis***.** We identified *tet(W/N/W)* as having the highest mobility potential of all ARGs found. *tet(W/N/W)* is a mosaic tetracycline resistance gene and ribosomal protection protein. It has the resistance mechanism of antibiotic target protection and has an effect on tetracylines. In the literature this gene has been identified in a large variety of bacterial species. Our finding is similar to the previous findings published by other authors as we also found it in *Bifidobacterium animalis***.**

The ARGs we identified may undermine several classes of antibiotics such as rifamycin, tetracycline, aminoglycoside, elfamycin, phenicol, fluoroquinolone, lincosamide, macrolide, oxazolidinone, pleuromutilin, streptogramin, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fosfomycin, glycylcycline, monobactam, penam, penem and triclosan. The ARGs we found have resistance mechanisms against some of the most important antibiotics, both in human and animal medicine. The term critically important antimicrobial (CIA) refers to antimicrobials which are last resorts in the treatment of human disease (DAFM, 2018). 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 includes cephalosporins (3rd, 4th and 5th generation), glycopeptides, macrolides and ketolides, polymyxins and quinolones (WHO, 2018a). Out of the five HPCIA drug groups, we found ARGs which compromise the effectiveness of three (fluoroquinolones, cephalosporins and macrolides). We also found ARGs which have an effect on eight CIAs, five HIAs and one IA. The EMA also produced a list aimed at restricting the veterinary use of antimicrobials which are important for human medicine (EMA, 2019a). The antimicrobials are listed under four categories: Avoid, Restrict, Caution and Prudence. We found ARGs that threaten eight drug groups listed as avoid, one listed as restrict, six as caution and one as prudence. 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). The ARGs we found have an effect on five VCIAs, five VHIAs and one VIA. Thus, many of the most important antibiotics in human and animal medicine could be affected by the ARGs we detected in bacterial strains from kefir and yoghurt products.

The results of our study indicate that the use of bacteria such as *Bifidobacterium animalis* and *Streptococcus thermophilus* in probiotics should be reconsidered due to their high ARG content. *Streptococcus thermophilus* had the highest diversity of ARGs as well as the highest abundance and diversity of mobile ARGs. *Bifidobacterium animalis* had the lowest diversity of ARGs but the highest proportion of ARG positive samples with nearly all samples containing ARGs. It was also the only bacterial species to contain the ARG which we found to have the highest mobility potential, *tet(W/N/W)*. Thus, our study suggests that these are not optimum choices for continued use in probiotic cultures. In contrast, our findings suggest that the use of other species such as *Lactiplantibacillus plantarum* may be better choices for continued use. *Lactiplantibacillus plantarum* is one of the most commonly used strains in probiotics so it is important to note that it had a low proportion of ARG positive samples. However, we did identify sixteen distinct ARGs in

addition to nineteen occurrences of mobile ARGs in *Lactiplantibacillus plantarum*, though the large sample size may somewhat skew results. No ARGs were found in *Lactiplantibacillus argentoratensis and Lacticaseibacillus paracasei* indicating that these may be good choices for probiotic cultures, though there were only two samples of each so further studies with larger sample sizes would be needed to determine their suitability. The same ARGs and the same mobile ARG were identified in both *Levilactobacillus brevis* and *Lactobacillus helveticus* indicating they have similar effects on the resistome and mobilome. *Levilactobacillus brevis, Lactobacillus helveticus* and *Lacticaseibacillus casei* all had low proportions of ARG positive samples indicating they are potentially good options for use in probiotics. Further studies assessing the ARG diversity, frequency and mobility of bacterial strains used in fermented foods are needed to better assess the danger of the currently used strains and to explore potential alternative bacterial strains suitable for use. In this study, I curated the data and selected and downloaded the appropriate datasets from the SRA database; this could also be extended by further bioinformatic steps.

In conclusion, our study highlights the need for starting cultures of probiotics, such as yoghurt and kefir products, to be strictly monitored and bacteria of low ARG content selected for use. We found numerous and diverse ARGs in commonly used bacterial strains of kefir and yoghurt products. Thus, the results of our study support the findings of several other recent studies which have also identified ARGs in probiotics (De Alcântara Rodrigues *et al.*, 2020; Machado *et al.,* 2020; Tóth *et al.,* 2020b; Tóth *et al.*, 2021). We also found that many of them have the potential to be mobile. Thus, fermented foods can act not only as a reservoir for ARGs but also as a medium for their exchange. During the fermentation process, the ARG content of yoghurt and kefir increases and, with the aid of plasmids and iMGEs such as we found, a potential hotspot for AMR development is created. Given the popularity of probiotics worldwide and the urgent threat of AMR, it is of utmost importance to fully investigate the risks associated with the consumption of probiotics. Considering the direct interactions humans have with animals, the interactions with the environment and the consumption of these animals and their produce, the implementation of a one health approach is needed. The prudent use of antimicrobials in human medicine and the strict monitoring of antimicrobial use in livestock with the aim of

reduction is vital. In addition to medications, investigating potential environmental and animal sources of ARGs is essential to help tackle this threat for the sake of both veterinary and human medicine. Our study helps bring to light the fact that foods entering the body should be regarded as potential sources of ARGs, especially in light of the important classes of drugs these ARGs found are known to affect. Thus, going forward, there is a need for further studies with larger sample sizes of more commonly and lesser used probiotic bacterial strains.

7. Summary

The focus of this study was to investigate the ARG content of bacterial strains used in probiotic products, namely yoghurt and kefir. Recent studies have shown that ARGs are found in fermented foods and may contribute to AMR via HGT (De Alcântara Rodrigues *et al.*, 2020; Machado *et al.,* 2020; Tóth *et al.,* 2020b). We investigated the ARG content (resistome) and mobility potential of the ARGs (mobilome) of eight bacterial strains commonly used in yoghurt and kefir cultures. The species were as follows: *Bifidobacterium animalis, Lacticaseibacillus casei, Lacticaseibacillus paracasei, Lactiplantibacillus argentoratensis, Lactiplantibacillus plantarum, Lactobacillus helveticus, Levilactobacillus brevis* and *Streptococcus thermophilus*. In order to select these species, I collated data from previous studies to identify the most commonly used bacterial species in kefir and yoghurt stater cultures (see Appendix). Using next generation sequencing, we performed metagenomic analyses based on freely available data sets from kefir and yoghurt strains which I curated from the NCBI SRA database. Our results identify numerous and diverse ARGs as present in kefir and yoghurt products. We also discovered that many of the ARGs identified have the potential to be mobile as they were associated with plasmids or iMGEs, although no phage-associated ARGs were found. The ARGs we identified are known to have resistance mechanisms against many CIAs such as the HPCIAs fluoroquinolones, cephalosporins and macrolides. Thus, our results show that ARGs in probiotics have the potential to contribute to AMR and undermine the effectiveness of vitally important classes of drugs. Going forward, bacterial strains used in probiotics need to be strictly monitored and selected for and the use of certain bacteria such as *Bifidobacterium animalis* and *Streptococcus thermophilus* reassessed due to their high ARG content. Further studies are needed assessing the ARG diversity, frequency and mobility of bacterial species used in fermented foods to better assess the impacts of the current commonly used species.

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10. Statements

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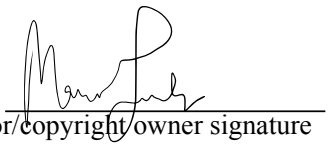

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- *facilitate professional relations and collaboration;*
- *support open access.*

10.2 Supervisor Counter-Signature Form

I hereby confirm that I am familiar with the content of the thesis entitled

Study of antimicrobial resistance genes of bacteria in kefir and yoghurt

written by Maura Judge (student name) which I deem suitable for submission and defence.

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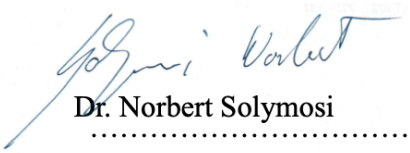

Dr. Norbert Solymosi

…. Supervisor name and signature

Centre for Bioinformatics

…. Department

11. Appendix

Table indicating the number of studies that identified each bacterial species as occurring in kefir or yoghurt products. Duplicate sources were omitted.

Microbial species	Bengoa *et al.*, 2018	Bourrie *et al.*, 2016	Witthuhn *et al.*, 2005	Wyk *et al.*, 2019	Gueimonde *et al.*, 2004	Leech *et al.*, 2020	Total number of times identified
Acetobacter spp.		3					**3**
A. aceti				2			2
A. fabarum				3			3
A. orientalis	1			4			5
A. lovaniensis	1			4			5
A. pasteurianus				2			2
A. rasens				1			1
A. sicerae		1					1
A. syzygii				5			5
Acinetobacter spp.							
A. calcoaceticus				1			1
A. rhizosphaerae				1			1
Bacillus spp.				2			**2**
B. amyloliquefaciens				1			1
B. megaterium				1			1
B. methylotrophicus				1			1
B. siamensis				1			1
B. tequilensis				1			1
Bifidobacterium spp.		1					**1**
B. animalis					1		1
B. bifidum				3			3
B. breve		1					1
B. choerinum		1					1
B. lactis					1		
B. longum		1					1
B. pseudolongum		1					1
B. psychraerophilum				1			1
Enterobacter spp.							
E. amnigenus				1			1
E. hormaechei				1			1
E. soli				1			1
Enterococcus spp.	1						**1**
E. durans				4			4
E. faecalis				3			3
E. lactis				1			1
Escherichia coli				3		1	4
Gluconoacetobacter diazotrophicus				1			1

Microbial species	Bengoa *et al.*, 2018	Bourrie *et al.*, 2016	Witthuhn *et al.*, 2005	Wyk *et al.*, 2019	Gueimonde *et al.*, 2004	Leech *et al.*, 2020	Total number of times identified
Gluconobacter spp.							
G. frateurii	1			1			2
G. japonicus				3			3
G. oxydans				1			1
Lactobacillus spp.							
L. acidophilus		4			1		5
L. amylovorus	2			1			3
L. apis				1			1
L. brevis	1	4		4			9
L. brevis 3			1	5			6
L. buchneri	2			3			5
L. bulgaricus				4			4
L. casei	2	4		5	1		12
L. crispatus	2			5			7
L. curvatis		1					1
L. curvatus	3			1			4
L. delbrueckii		4					4
L. delbrueckii ssp. bulgaricus				4			4
L. delbrueckii ssp. delbrueckii			1	2			3
L. delbrueckii ssp. lactis	1			1			2
L. diolivorans		1		3			4
L. fermentum	1	1	1	5			8
L. fructivorans				3			3
L. gallinarum		1		3			4
L. garvieae		1					1
L. gasseri		2		2			4
L. helveticus	2	4		7			13
L. hilgardi				5			5
L. hordei				3			3
L. intestinalis		1					1
L. jensenii				1			1
L. johnsonii		1		1	1		3
L. kalixensis		1					1
L. kefir		6					6
L. kefiranofacien	2						2
L. kefiranofaciens	1	8		11			20
L. kefiranofaciens ssp kefiranofaciens				2			2
L. kefiranofaciens ssp kefirgranum	2			3			5
L. kefirgranum		2		4			6
L. kefiri	7	6		16			29
L. mali				3			3
L. mesenteroides				1			1
L. nagelii				2			2

Microbial species	Bengoa *et al.*, 2018	Bourrie *et al.*, 2016	Witthuhn *et al.*, 2005	Wyk *et al.*, 2019	Gueimonde *et al.*, 2004	Leech *et al.*, 2020	Total number of times identified
L. otakiensis		1		3			4
L. parabuchneri		2		1			3
L. paracasei	4	3		4	1		11
L. parafarraginis		1					1
L. parafarraginis				3			3
L. parakefir		2		6			8
L. parakefiri	4	5					9
L. pentosus		1					1
L. perolens				2			2
L. plantarum	1	3	1	6			11
L. rapi		1					1
L. reuteri		1		2			3
L. rhamnosus		1		3			4
L. rossiae		1					1
L. sakei		1		1			2
L. salivarius		1					1
L. sanfranciscensis				1			1
L. satsumensis		1		4			5
L. sunkii		1		3			4
L. ultunensis				1			1
L. uvarum				3			3
L. viridescens		1		2			3
L. xiangfangensis						1	1
L. zeae					1		1
Lactococcus spp.							
L. filant				1			1
L. garvieae		1					1
L. lactis	6			8		1	14
L. lactis ssp cremoris		2		3			5
L. lactis ssp cremoris/lactis	1						1
L. lactis ssp lactis	1	8		5			14
L. lactis ssp lactis 1			1				1
L. lactis ssp lactis 2			1				1
L. lactis ssp lactis biovar diacetylactis		1		1			2
L. raffinolactis				2		1	3
Leucoconstoc spp.	1	1					**2**
L. carnosum				1			1
L. citreum				2			2
L. gelidum				1			1
L. kimchii				1			1
L. lactis				3			3
L. mesenteroides	2	5		10		1	18
L. mesenteroides subsp. cremoris		1	1	1			3

Microbial species	Bengoa *et al.*, 2018	Bourrie *et al.*, 2016	Witthuhn *et al.*, 2005	Wyk *et al.*, 2019	Gueimonde *et al.*, 2004	Leech *et al.*, 2020	Total number of times identified
L. mesenteroides ssp. mesenteroides/dextranicum	1						1
L. mesenteroides ssp. mesenteroides		2					2
L. pseudomesenteroides	1	1		1			3
Lysinibacillus sphaericus				1			1
Oenococcus oeni		1		1			2
Pediococcus spp.	1						**1**
P. acidilactici				2			2
P. claussenii				1			1
P. damnosus				1			1
P. dextrinicus				2			2
P. halophilus				1			1
P. lolii				1			1
P. pentosaceus				3			3
Propionibacterium acnes				1			1
Pseudomonas spp.				1			**1**
P. aeruginosa				1			1
P. azotoformans				1			1
P. otitidis				1			1
Streptococcus spp.							
S. cremoris				2			2
S. durans				2			2
S. faecalis				1			1
S. infantarius						1	1
S, salivarius subsp. thermophilus		1					1
S. thermophilus	1	2		10		1	14
Tetragenococcus halophilus	1						1