Antimicrobial resistance determinants in silage

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ABSTRACT

Animal products may play a role in developing and spreading antimicrobial resistance in several ways. On the one hand, residues of antibiotics not adequately used in animal farming can enter the human body via food. But resistant bacteria may also be present in animal products, which can transfer the antimicrobial resistance genes (ARG) to the bacteria in the consumer's body by horizontal gene transfer. As previous studies have shown that fermented foods have a meaningful ARG content, it is indicated that such genes may also be present in silage used as mass feed in the cattle sector. In our study, we aspired to answer what ARGs occur in silage and what mobility characteristics they have? For this purpose, we have analyzed bioinformatically 40 freely available deep sequenced silage samples from shotgun metagenome next-generation sequencing. A total of 57 ARGs occurred 616 times in the samples. More than half of these ARGs are mobile because they can be linked to integrative mobile genetic elements, prophages or plasmids. We believe that our results point to a substantial source of ARG in the food chain.

Introduction

In intensive cattle farming, silage is an essential component of feed. A dairy cow consumes 25-27 kg per day from this forage, so 12,500 kg per lactation.[1,2] Silage is most commonly produced from maize or legume plants by anaerobic fermentation. In the process, fermenting microorganisms, including bacteria, multiply, causing nutrient-beneficial transformations on the ingredients. If the bacteria involved in the process harbour antimicrobial resistance genes (ARGs), their amount in the silage will also increase. Accordingly, the silage as a mass feed continuously may supply bacteria carrying ARGs into the gastrointestinal tract of animals. Bacteria entering the digestive system may come into contact with the bacteria living there, during which they can exchange genes (e.g. ARGs) by horizontal gene transfer (HGT). HGT may have happened in three different ways: conjugation, transduction and transformation. Excepting transformation, by which the bacterium can take up any gene from its environment, the routes of HGT require particular active delivery processes. By conjugation, cell-to-cell contact provides the opportunity for a copy of a plasmid to translocate to a recipient bacterium.[3] Transduction negates the condition for cell-to-cell contact, as in this case, bacteriophages act as a conduit for shuttling genes among bacteria.[4] The genetic environment of the genes affected by the transfer significantly influences the efficacy of the latter two HGT processes, i.e., on the genes' mobility. If ARGs from silage are transmitted to pathogenic bacteria within an animal's body, they may reduce antibiotic (AB) treatment efficacy on the bacterial diseases they cause. In addition, if ARG-carrier bacteria from silage can colonize the gastrointestinal tract, they may be defecated by faeces, resulting in the appearance and enrichment of ARG-harbouring bacteria in the animal's environment. Decreased efficacy of AB treatments may result in economic loss, and the increased environmental ARG level may have additional veterinary and human health consequences. It is known from previous works that ARGs in fermented dairy products can also be multiplied due to the growth of fermenting bacteria.[5] But, no such data were found for silage in the literature. Our study aimed to learn what ARGs may occur in silage, in what bacteria, and with what mobility characteristics. For this purpose, freely available shotgun metagenome datasets generated by next-generation sequencing (NGS) were analyzed by a unified bioinformatics pipeline.

Materials and Methods

Data

We searched appropriate dataset in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) repository. In August 2021, we found only one BioProject (PRJNA495415) sequenced by shotgun metagenomic that had adequate depth for the assembly on which our study was based. Samples used in our study were sequenced by Inner Mongolia Agricultural University (Table 1). Data providers sampled silage during the fermentation process on days 0, 7, 14 and 28. The paper of the original work is under publication.

Table 1. Analyzed samples. The run column contains the Sequence Read Archive run identifiers. The second and the third columns store the day of sampling and the replication serial number of the samples.

Run	Samplig day	Replication
SRR7990616	0	1
SRR7990612	0	5
SRR7990610	0	9
SRR7990604	0	13
SRR7990608	0	17
SRR7990587	0	21
SRR7990583	0	25
SRR7990591	0	29
SRR7990598	0	33
SRR7990592	0	37
SRR7990617	7	2
SRR7990613	7	6
SRR7990611	7	10
SRR7990605	7	14
SRR7990609	7	18
SRR7990586	7	22
SRR7990582	7	26
SRR7990590	7	30
SRR7990599	7	34
SRR7990593	7	38
SRR7990618	14	3
SRR7990614	14	7
SRR7990602	14	11
SRR7990606	14	15
SRR7990594	14	19
SRR7990589	14	23
SRR7990585	14	27
SRR7990600	14	31
SRR7990596	14	35
SRR7990580	14	39
SRR7990619	28	4
SRR7990615	28	8
SRR7990603	28	12
SRR7990607	28	16
SRR7990595	28	20
SRR7990588	28	24
SRR7990584	28	28
SRR7990601	28	32
SRR7990597	28	36
SRR7990581	28	40

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 bp were retained and taxonomically classified using Kraken2 (v2.1.1)[6] and a database created (24/03/2021) 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 was managed in R[7] using functions of the packages phyloseq[8] and microbiome.[9] The preprocessed reads were assembled to contigs by MEGAHIT (v1.2.9)[10] using default settings. The contigs were also classified taxonomically by Kraken2 with the same database as above. From the contigs all possible open reading frames (ORFs) were gathered by Prodigal (v2.6.3)[11]. The protein translated ORFs were aligned to the ARG sequences of the Comprehensive Antibiotic Resistance Database (CARD, v.3.1.3)[12,13] by Resistance Gene Identifier (RGI, v5.2.0) with Diamond[14] The ORFs classified as perfect or strict were further filtered with 90% identity and 90% coverage. All nudged hits were excluded. The correlation of the bacteria and ARG relative abundance was quantified by Spearman's rho. In keeping with Hendriksen at al.[15] the ARG abundance was expressed as fragments per kilobase per million fragments (FPKM)[16] 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[17] with the parameter of `-very-sensitive-local`. The integrative mobile genetic element (iMGE) content of the ARG harbouring contigs was analyzed by MobileElementFinder (v1.0.3) and its database (v1.0.2).[18] Following the distance concept of Johansson et al.[18] 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)[19] The phage content of the assembled contigs was prediced by VirSorter2 (v2.2.3)[20]. The findings were filtered for dsDNAphages and ssDNAs. All data management procedures, analyses and plottings were performed in R environment (v4.1.0).[7]

Results

Based on the taxon classification performed on a database containing plant complete reference genomes, the dominant plants in the silage belong to the *Medicago* genus. Further analysis results of the shotgun metagenomic sequenced 40 samples (Table 1) is summarised in the following sections. After presenting the bacteriome and the identified AGRs (resistome), predictions regarding the mobility potential of ARGs were also resumed based on genetic characteristics that may play a significant role in HGT.

Bacteriome

By taxon classification, the number of reads aligning to bacterial genomes varied by samples (median: 20.0×10^6, IQR: 2.3×10^6). The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the samples is shown in Figure 1. These dominant genera (with mean abundance) in descending order were *Weissella* (55.8%), *Pantoea* (24.1%), *Pediococcus* (7%), *Lactiplantibacillus* (5.1%), *Companilactobacillus* (2.1%), *Kosakonia* (1%), *Levilactobacillus* (1%), *Enterococcus* (0.8%), *Staphylococcus* (0.7%), *Latilactobacillus* (0.6%), *Bacillus* (0.5%), *Enterobacter* (0.5%), *Pseudomonas* (0.4%), *Leclercia* (0.3%), *Mammaliicoccus* (0.2%).

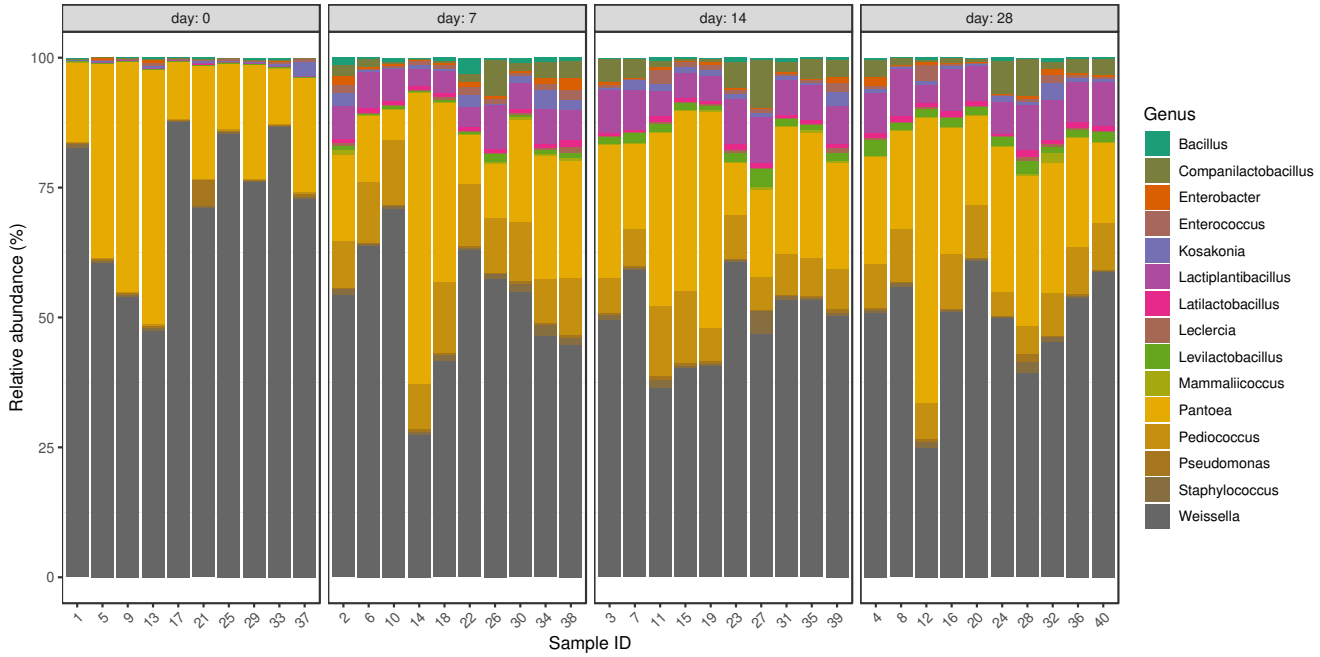

Figure 1. Silage core bacteriome. The relative abundances of genera that achieved more than 1% of the bacterial hits in any of the samples. These dominant genera (with mean prevalence) in descending order were *Weissella* (55.8%), *Pantoea* (24.1%), *Pediococcus* (7%), *Lactiplantibacillus* (5.1%), *Companilactobacillus* (2.1%), *Kosakonia* (1%), *Levilactobacillus* (1%), *Enterococcus* (0.8%), *Staphylococcus* (0.7%), *Latilactobacillus* (0.6%), *Bacillus* (0.5%), *Enterobacter* (0.5%), *Pseudomonas* (0.4%), *Leclercia* (0.3%), *Mammaliicoccus* (0.2%).

Resistome

The median length of the filtered contigs harbouring ARGs constructed by de novo assembly was 2,472 bp (IQR: 3,303). The number of ARGs found on the contigs ranged from 1 to 3. Besides 16 perfect ARG matches, a further 600 hits were classified

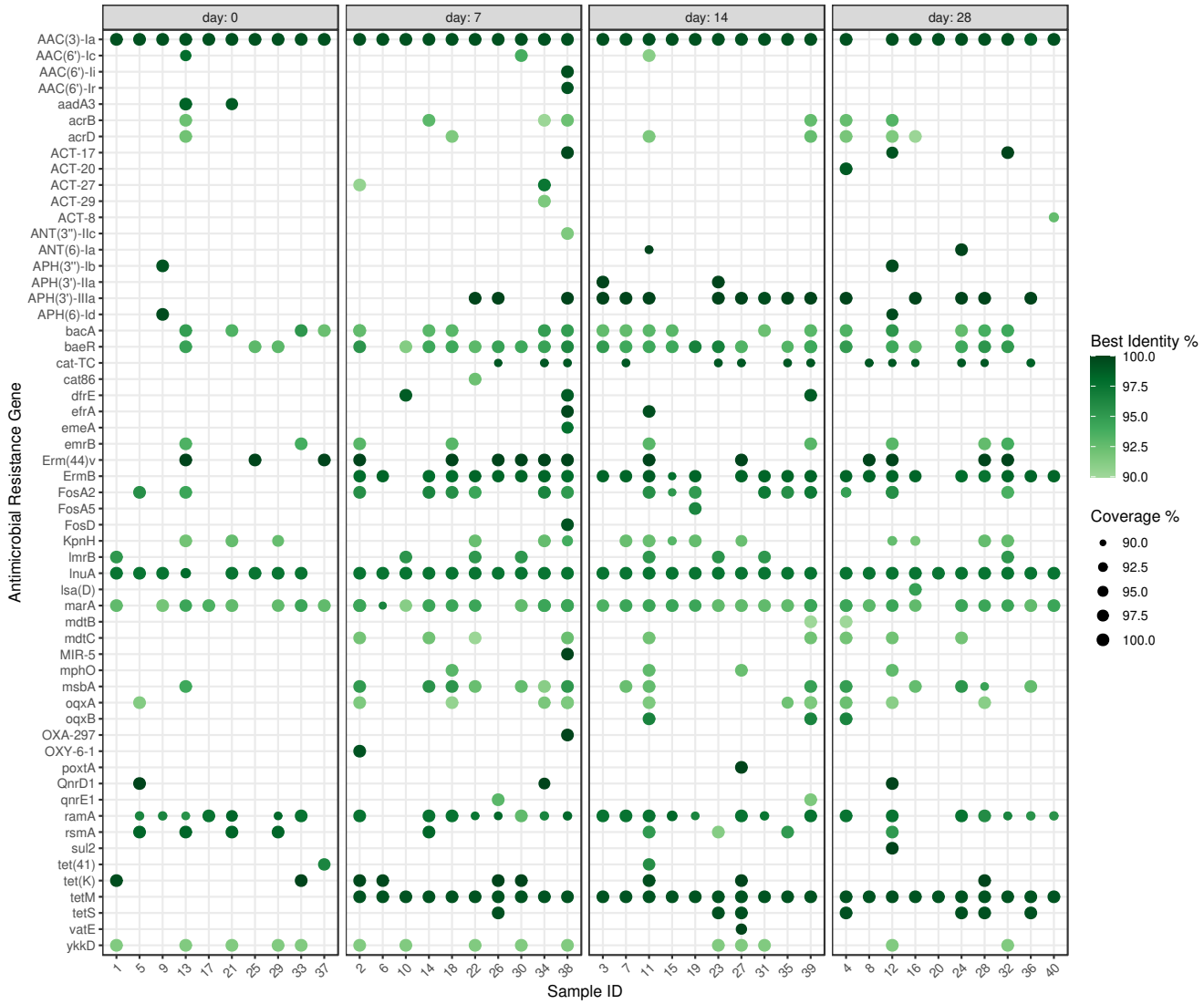

Figure 2. Identifed antimicrobial resistance genes (ARGs) by samples. For each sample-ARG combination, only the best finding is plotted. The size and the colour of the dots correspond to the coverage and the sequence identity of hits on reference genes, respectively. The gene names that are too long have been abbreviated (cat-TC: *Lactobacillus reuteri cat-TC*; cat86: *Bacillus pumilus cat86*; KpnH: *Klebsiella pneumoniae KpnH*).

strict (RGI) and met the criteria of having 90% coverage and 90% sequential identity. All samples contained ARGs, ranging between 4 and 44 (Figure 2). The resistance mechanism of identified ARGs was in descending order of frequency the antibiotic inactivation (32.7%), antibiotic efflux (31.2%), antibiotic efflux with reduced permeability to antibiotic (13.6%), antibiotic target alteration (12.7%), antibiotic target protection (9.0%), antibiotic target replacement (0.8%).

The identified ARGs associated with bacteria by species are as follows. *Acinetobacter baumannii*: *AAC(3)-Ia*, *aadA3*; *A. dispersus*: *AAC(6')-Ir*; *A. gyllenbergii*: *ANT(3")-IIc*; *A. lwoffii*: *OXA-297*; *Amylolactobacillus amylophilus*: *ErmB*, *lnuA*; *Bacillus mojavensis*: *lmrB*, *ykkD*; *B. safensis*: *cat86*; *B. subtilis*: *APH(3')-IIa*; *Brachybacterium saurashtrense*: *mphO*; *Citrobacter freundii*: *AAC(3)-Ia*; *Companilactobacillus futsaii*: *lnuA*; *Enterobacter asburiae*: *acrD*; *E. cancerogenus*: *acrB*, *acrD*, *ACT-17*, *bacA*, *baeR*, *emrB*, *FosA2*, *KpnH*, *marA*, *mdtC*, *msbA*, *ramA*; *E. cloacae*: *acrB*, *acrD*, *ACT-27*, *ACT-29*, *ACT-8*, *bacA*, *baeR*, *emrB*, *FosA2*, *KpnH*, *marA*, *mdtB*, *mdtC*, *msbA*, *oqxA*, *oqxB*, *qnrE1*, *ramA*; *E. hormaechei*: *acrB*, *acrD*, *ACT-17*, *ACT-20*, *ACT-27*, *bacA*, *baeR*, *emrB*, *FosA2*, *KpnH*, *marA*, *mdtB*, *mdtC*, *msbA*, *oqxA*, *oqxB*, *ramA*; *E. kobei*: *bacA*; *E. ludwigii*: *ramA*; *E. roggenkampii*: *MIR-5*; *Enterococcus casseliflavus*: *ErmB*; *E. faecalis*: *APH(3')-IIIa*, *dfrE*, *efrA*, *emeA*; *E. faecium*: *AAC(6')-Ii*, *ErmB*, *tetS*; *Escherichia coli*: *APH(3")-Ib*, *APH(6)-Id*, *sul2*; *Klebsiella aerogenes*: *FosA5*; *K. grimontii*: *marA*; *K. michiganensis*: *APH(3")-Ib*, *APH(6)-Id*, *bacA*; *K. oxytoca*: *oqxA*; *K. sp. MPUS7*: *OXY-6-1*; *Kluyvera intermedia*: *marA*;

Kosakonia cowanii: *baeR*, *KpnH*, *marA*, *mdtC*, *msbA*; *K. sp. CCTCC M2018092*: *KpnH*, *marA*, *msbA*; *Lacticaseibacillus manihotivorans*: *ANT(6)-Ia*; *Lactiplantibacillus argentoratensis*: *tetM*; *L. plantarum*: *cat-TC*, *ErmB*, *lnuA*, *tetM*; *Lactococcus garvieae*: *lsa(D)*; *Latilactobacillus curvatus*: *lnuA*; *L. sakei*: *tetM*; *Leclercia adecarboxylata*: *bacA*, *emrB*, *KpnH*, *marA*, *msbA*, *rsmA*; *L. sp. 29361*: *acrB*, *acrD*, *bacA*, *baeR*, *emrB*, *marA*, *mdtC*, *msbA*, *oqxA*; *L. sp. 4-9-1-25*: *marA*; *L. sp. W6*: *marA*; *Lelliottia amnigena*: *FosA2*, *KpnH*; *Leuconostoc kimchii*: *lnuA*; *L. mesenteroides*: *tetM*; *Levilactobacillus brevis*: *ErmB*, *tetM*; *L. salivarius*: *lnuA*; *Limosilactobacillus reuteri*: *lnuA*; *Loigolactobacillus backii*: *lnuA*; *Pantoea agglomerans*: *marA*, *rsmA*; *Pediococcus acidilactici*: *tetM*; *P. claussenii*: *cat-TC*; *P. pentosaceus*: *ErmB*; *Providencia rettgeri*: *QnrD1*; *Pseudescherichia vulneris*: *marA*; *Pseudomonas fulva*: *rsmA*; *P. oryzihabitans*: *rsmA*; *P. putida*: *AAC(3)-Ia*, *aadA3*; *Rhodopseudomonas palustris*: *APH(3')-IIa*; *Serratia marcescens*: *AAC(6')-Ic*, *tet(41)*; *S. sp. SCBI*: *tet(41)*; *Staphylococcus aureus*: *lnuA*, *tet(K)*; *S. carnosus*: *Erm(44)v*; *S. epidermidis*: *FosD*; *S. equorum*: *lnuA*; *S. kloosii*: *tet(K)*; *S. nepalensis*: *Erm(44)v*; *S. pseudoxylosus*: *Erm(44)v*; *S. saprophyticus*: *Erm(44)v*; *Tetragenococcus halophilus*: *APH(3')-IIIa*, *ErmB*; *Weissella cibaria*: *lnuA*.

The ARGs belonging to the genome of *Acinetobacter baumannii* may play a role in the appearance of resistance against aminoglycoside; *A. dispersus*: aminoglycoside; *A. gyllenbergii*: aminoglycoside; *A. lwoffii*: carbapenem, cephalosporin, penam; *Amylolactobacillus amylophilus*: lincosamide, macrolide, streptogramin; *Bacillus mojavensis*: aminoglycoside, lincosamide, phenicol, tetracycline; *B. safensis*: phenicol; *B. subtilis*: aminoglycoside; *Brachybacterium saurashtrense*: macrolide; *Citrobacter freundii*: aminoglycoside; *Companilactobacillus futsaii*: lincosamide; *Enterobacter asburiae*: aminoglycoside; *E. cancerogenus*: aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, fluoroquinolone, fosfomycin, glycylcycline, macrolide, monobactam, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline, triclosan; *E. cloacae*: aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, fosfomycin, glycylcycline, macrolide, monobactam, nitrofuran, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline, triclosan; *E. hormaechei*: aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, fosfomycin, glycylcycline, macrolide, monobactam, nitrofuran, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline, triclosan; *E. kobei*: peptide; *E. ludwigii*: carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, monobactam, penam, penem, phenicol, rifamycin, tetracycline, triclosan; *E. roggenkampii*: cephalosporin, monobactam; *Enterococcus casseliflavus*: lincosamide, macrolide, streptogramin; *E. faecalis*: acridine dye, aminoglycoside, diaminopyrimidine, disinfecting agents and intercalating dyes, fluoroquinolone, macrolide, rifamycin; *E. faecium*: aminoglycoside, lincosamide, macrolide, streptogramin, tetracycline; *Escherichia coli*: aminoglycoside, sulfonamide; Klebsiella aerogenes: fosfomycin; *Klebsiella grimontii*: carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, monobactam, penam, penem, phenicol, rifamycin, tetracycline, triclosan; *K. michiganensis*: aminoglycoside, peptide; *K. oxytoca*: diaminopyrimidine, fluoroquinolone, glycylcycline, nitrofuran, tetracycline; *K. sp. MPUS7*: cephalosporin, monobactam, penam; *Kluyvera intermedia*: carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, monobactam, penam, penem, phenicol, rifamycin, tetracycline, triclosan; *Kosakonia cowanii*: aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, macrolide, monobactam, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline, triclosan; *K. sp. CCTCC M2018092*: aminoglycoside, carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, macrolide, monobactam, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline, triclosan; *Lacticaseibacillus manihotivorans*: aminoglycoside; *Lactiplantibacillus argentoratensis*: tetracycline; *L. plantarum*: lincosamide, macrolide, phenicol, streptogramin, tetracycline; *Lactococcus garvieae*: lincosamide, macrolide, oxazolidinone, phenicol, pleuromutilin, streptogramin, tetracycline; *Latilactobacillus curvatus*: lincosamide; *L. sakei*: tetracycline; *Leclercia adecarboxylata*: aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, glycylcycline, macrolide, monobactam, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline, triclosan; *L. sp. 29361*: aminocoumarin, aminoglycoside, carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, glycylcycline, monobactam, nitrofuran, nitroimidazole, penam, penem, peptide, phenicol, rifamycin, tetracycline, triclosan; *L. sp. 4-9-1-25*: carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, monobactam, penam, penem, phenicol, rifamycin, tetracycline, triclosan; *L. sp. W6*: carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, monobactam, penam, penem, phenicol, rifamycin, tetracycline, triclosan; *Lelliottia amnigena*: aminoglycoside, carbapenem, cephalosporin, fluoroquinolone, fosfomycin, macrolide, penam, penem, peptide; *Leuconostoc kimchii*: lincosamide; *L. mesenteroides*: tetracycline; *Levilactobacillus brevis*: lincosamide, macrolide, streptogramin, tetracycline; *Ligilactobacillus salivarius*: lincosamide; *Limosilactobacillus reuteri*: lincosamide; *Loigolactobacillus backii*: lincosamide; *Pantoea agglomerans*: carbapenem, cephalosporin, cephamycin, diaminopyrimidine, fluoroquinolone, glycylcycline, monobactam, penam, penem, phenicol, rifamycin, tetracycline, triclosan; *Pediococcus acidilactici*: tetracycline; *P. claussenii*: phenicol; *P. pentosaceus*: lincosamide, macrolide, streptogramin; *Providencia rettgeri*: fluoroquinolone; *Pseudescherichia vulneris*: carbapenem, cephalosporin, cephamycin, fluoroquinolone, glycylcycline, monobactam, penam, penem, phenicol, rifamycin, tetracycline, triclosan; *Pseudomonas fulva*: diaminopyrimidine, fluoroquinolone, phenicol; *P. oryzihabitans*: diaminopyrimidine, fluoroquinolone, phenicol; *P. putida*: aminoglycoside; *Rhodopseudomonas palustris*: aminoglycoside; *Serratia marcescens*: aminoglycoside, tetracycline; *S. sp. SCBI*: tetracycline; *Staphylococcus aureus*: lincosamide, tetracycline; *S. carnosus*:

lincosamide, macrolide, streptogramin; *S. epidermidis*: fosfomycin; *S. equorum*: lincosamide; *S. kloosii*: tetracycline; *S. nepalensis*: lincosamide, macrolide, streptogramin; *S. pseudoxylosus*: lincosamide, macrolide, streptogramin; *S. saprophyticus*: lincosamide, macrolide, streptogramin; *Tetragenococcus halophilus*: aminoglycoside, lincosamide, macrolide, streptogramin; *Weissella cibaria*: lincosamide.

The proportion of species harbouring ARGs within all bacteria was 49.1% (SD: 15.2). For *marA* and *ykkD*, we found that the abundance of the genes and bacteria carrying them showed a significant positive correlation, with $r_s = 0.44$ ($p<0.001$) and $r_s = 0.84$ ($p=0.0013$), respectively.

Mobilome

We found a total of 330 ARGs that can be assumed to be mobile. Eight of these ARGs are linked to integrative mobile genetic elements (iMGE). Further, 25 ARGs were detected in prophages and 296 on plasmids. The frequencies of ARGs associated with iMGEs, phages and plasmids by bacteria species of origin are summarized in Figure 3.

Discussion

The carrier bacteria predicted for the identified ARGs can be classified according to their presence in silage. In the literature, one can find the following bacteria as a characteristic feature of silage: *Acinetobacter lwoffii*[21], *Amylolactobacillus amylophilus*[22], *Escherichia coli*[23], *Lactiplantibacillus plantarum*[24], *Levilactobacillus brevis*[25–27], *Ligilactobacillus salivarius*[28], *Loigolactobacillus backii*[29], *Pediococcus acidilactici*[30], *P. claussenii*[31], *P. pentosaceus*[32], *Weissella cibaria*[33]. The genera of these species dominate the bacteriome of the samples. Other identified bacteria may be of plant origin: *Bacillus mojavensis*[34], *B. safensis*[35], *Brachybacterium saurashtrense*[36], *Enterococcus casseliflavus*[37], *Klebsiella michiganensis*[38], *K. sp. MPUS7*[39], *Kosakonia cowanii*[40], *K. sp. CCTCC M2018092*[41], *Pantoea agglomerans*[42], *Pseudomonas fulva*[43], *P. putida*[44], *Serratia marcescens*[45]. Additional species may join the silage from animals, soil, or water: *Acinetobacter gyllenbergii*[46], *Citrobacter freundii*[47], *Enterobacter asburiae*[48], *E. cloacae*[49], *E. roggenkampii*[50], *E. sichuanensis*[51], *Enterococcus faecalis*[52], *Klebsiella aerogenes*[53], *K. oxytoca*[54], *Kluyvera intermedia*[55], *Lactococcus garvieae*[56], *Latilactobacillus curvatus*[57], *Leclercia adecarboxylata*[58], *Lelliottia amnigena*[59], *Limosilactobacillus reuteri*[28], *Providencia rettgeri*[60], *Pseudescherichia vulneris*[61], *Pseudomonas oryzihabitans*[62], *Rhodopseudomonas palustris*[63], *Serratia sp. SCBI*[64], *Staphylococcus aureus*[46], *S. kloosii*[65], *S. pseudoxylosus*[66], *S. saprophyticus*[46], *Enterococcus faecium*[52]. However, it can be assumed that other species may be present as contaminants: *Acinetobacter baumannii*, *A. dispersus*, *Bacillus subtilis*, *Enterobacter hormaechei*, *E. kobei*, *E. ludwigii*, *E. cancerogenus*, *Klebsiella grimontii*, *Lacticaseibacillus manihotivorans*, *Latilactobacillus sakei*, *Lactiplantibacillus argentoratensis*, *Leclercia sp. 29361*, *L. sp. 4-9-1-25*, *L. sp. W6*, *Leuconostoc kimchii*, *Leuconostoc mesenteroides*, *Staphylococcus carnosus*, *S. epidermidis*, *S. equorum*, *S. nepalensis*, *Tetragenococcus halophilus*. Nevertheless, some of these bacteria are members of the *Lactobacillaceae* family, the *Leuconostoc* or *Enterobacter* genera. Which groups' numerous species are typical of fermented food and feed components.

The description and previous appearance of the identified ARGs grouped by bacterial families can be found below.

YkkD, encoding a small multidrug resistance (SMR) protein subunit of the *ykkCD* multidrug efflux pump is associated with *Bacillus* spp. Species level identification in *Bacillus mojavensis* is a pioneer finding. *lmrB*, a chromosomally-encoded gene encoding an efflux pump confers resistance to lincosamides[13]. This ARG was originally identified in *Bacillus subtilis*[13], that is the same genus as our finding, *Bacillus mojavensis*. The description of *cat86*, a variant of the chloramphenicol acetyltransferase cat gene first took place in *Bacillus pumilus*[67]. According to our result, the gene appeared in *Bacillus safensis* in the silage samples. *APH(3')-IIa*, an aminoglycoside phosphotransferase, to our knowledge, has not detected in *Bacillus subtilis* up till now.

Brachybacterium saurashtrense deriving *mphO*, a chromosomal macrolide phosphostransferase gene was first identified in an other species of the genus, namely *Brachybacterium paraconglomeratum*.

AAC(3)-Ia, an aminoglycoside acetyltransferase, has not yet been written down in *Citrobacter freundii*, although other members of the *AAC(3)* AMR gene family appear in the literature related to this species[68]. Within *Enterobacter* spp., a large set of ARGs was found. By *Enterobacter* spp. (*E. cancerogenus*, *E. cloacae*, *E. hormaechei*, *E. ludwigii*) *BacA*, conferring resistance to bacitracin, *emrB*, a translocase in the *emrB-TolC* multidrug efflux protein, *fosA2*, enzyme that confers resistance to fosfomycin, *KpnH*, a translocase in the *KpnGH-TolC* multridrug efflux protein, *marA* conferring AMR by modulating multridrug efflux pump and porin expression, *mdtB* and *mdtC*, transporters that form a complex with eachother to become a multidrug transporter, *qnrE1*, a (fluoro)quinolone-resistance gene and ramA a positive regulator of *AcrAB-TolC* efflux pump leading to high level multidrug resistance[69,70] high level activator of multidrug resistance all tend to appear in the *Entrobacteriaceae* family[13]. Further ARGs identified in *Enterobacter* spp., such as *ACT-8*, *ACT-17*, *ACT-20*, *ACT-27*, and *ACT-29* all encode beta-lactameses and are very common in this genus[13]. A further findings was *MIR-5* in *E. roggenkampii*. *KpnF*, a subunit of *KpnEF*, is an SMR-type efflux pump involved in broad-spectrum antimicrobial resistance originally deriving from *Klebsiella pneumoniae*[13]. *MIR-5* encodes a beta-lactamase and has already been identified in *Enterobacter* spp.[71]. *AcrD*,

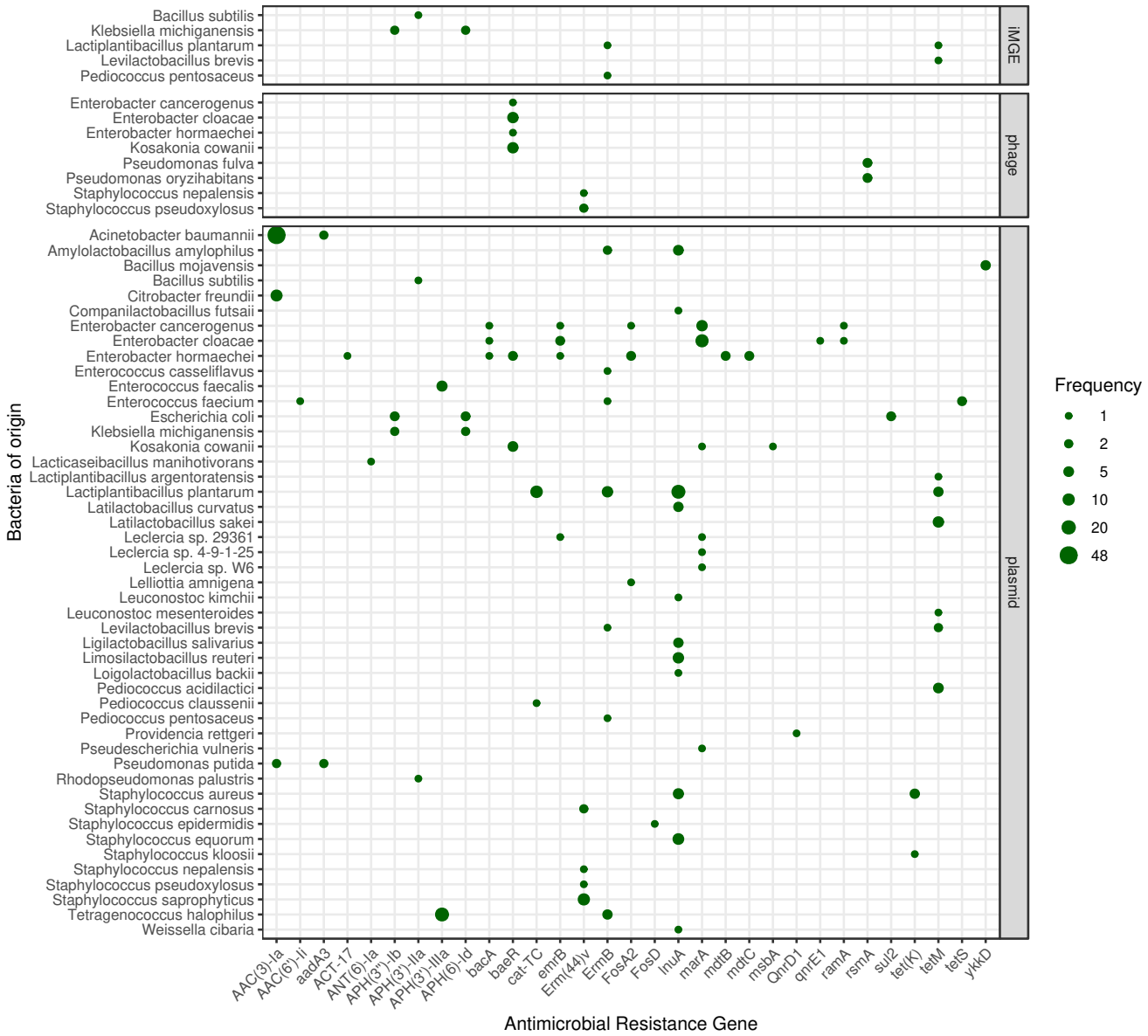

Figure 3. Mobile antimicrobial resistance gene frequency by bacteria of origin. The size of the dots indicates the occurrence frequency of the given gene flanked by iMGE, positioned in plasmid or phage.

an aminoglycoside efflux pump gene regulated by *baeRS*[13] was found in *Enterobacter asburiae*, *E. cancerogenus*, *E. cloacae* and *E. hormaechei*. This gene has been described in *Enerobacteriaceae* before. *Enterobacter cancerogenus*, *E. cloaceae* and *E. hormaechei* had further characteristics in common, explained as follows. *AcrD* was always detected coexsisting with *baeR* (in case of *E. hormaechei*, a plasmid-mediated *baeR*), indicating their direct binding in line with former publications[72]. Besides this, all three species harbored, a gene encoding a subunit of *AcrAB-TolC* multidrug efflux complex, *AcrB*. *AcrB* has already been identified in *Enterobacter* spp. by other authors as well[69]. Moreover, in these *Enterobacter* species *ramA* that is a positive regulator of *AcrAB-TolC* was also detected besides these genes. All three species shared the common feature of having *MDR-ABC* multidrug resistance transporter homolog *msbA* as well, a common finding by *Enterobacteriaceae*[13] which is the only bacterial *ABC* (ATP-binding cassette) transporter essential for cell viability. Moreover, within these species *baeR*, a response regulator promoting the expression of *MdtABC* efflux protein[13] co-occurred with *MdtB* and *MdtC*. *MdtB* is a transporter that forms a multidrug transporter complex with *MdtC*. *MdtBC* is part of the *MdtABC-TolC* efflux complex that is a a multidrug efflux system in Gram-negative bacteria[13]. *Enterobacter cloaceae* and *E. hormaechei* harbored *oqxA* and *oqxB* that are common ARGs among *Enterobacteriaceae* encoding efflux pumps and conferring resistance to fluoroquinolones[73]. Interestingly, to our knowledge species-specific associations of *KpnH* in *Enterobacter* genus pioneered in our study. *Escherichia coli* deriving ARGs, namely *APH(3")-Ib*, an aminoglycoside phosphotransferase in *Enterobacteriaceae* and *Pseudomonas* spp., *APH(6)-Id*, an aminoglycoside phosphotransferase appearing in many species, including *E. coli* and *Sul2*, a sulfonamide resistant dihydropteroate synthase of Gram-negative bacteria are commonly described in this species[13]. *Pseudoescherichia vulneris*, or *Escherichia vulneris*, another member of *Enterobacteriaceae* also contained *marA* that often appears in this family[13]. *FosA5*, a fosfomycin resistance gene detected in *Klebsiella aerogenes* has already been associated to *Klebsiella* species, including *K. aerogenes*[74]. Our ARG findings in *Klebsiella michiganensis* were in line with a recent study[75], describing the appearance of aminoglycoside phosphotransferase *APH(3")-Ib* and *APH(6)-Id* in this species. *BacA* conferring bacitracin resistance and *marA* have already been identified in *Klebsiella* spp. by other authors as well[76,77]. *OqxA* was detected in the genom of *Klebsiella oxytoca*. This gene had associations with *Klebsiella* spp. in other publications as well[78]. *OXY-6-1*, a beta-lactamase gene found in *Klebsiella sp. MPUS7*, originates from *Klebsiella oxytoca*[79]. *Kluyvera intermedia*, a member of *Enterobacteriaceae* was detected in *marA*, that is a a new association. Sequences from *Kosakonia cowanii*, formerly known as *Enterobacter cowanii*, contained *marA* and *msbA* which are both a common finding by *Enterobacteriaceae*[13]. While *KpnH* is also typical for the *Enterobacteriaceae* family, its appearance in *Kosakonia* spp. is a new finding. *MdtC-baeR* associations were observable by *Kosakonia cowanii* too. Sequence variants of *marA* have been described in *Leclercia* spp.[13], while *acrB* and *ermB* have been mentioned related to this species[80,81]. In contrast, *BacA*, *KpnH*, *msbA* and *oqxA* has not been mentioned to appear within the genom of *Leclercia* spp. to our knowledge. In *Leclercia sp. 29361 baeR* was also detected, that regulates two of the detected genes, *acrD* and *mdtC*. These two genes were also present of the genom of *Leclercia sp. 29361* and also described to appear in this genus by other authors[81]. *Lelliottia amnigena*, previously classified as *Enterobacter amnigena*, itself has not yet been described to carry either *FosA2* or *KpnH*, but both genes appear in *Enterobacter* spp.[13,82].

Based on previous publications[83], the appearance of *ermB* in *Enterecoccus* spp. is not surprising. Other genes found in this genus, such as *AAC(6')-Ii*, that is an aminoglycoside acetyltransferase in *Enterococcus* spp., *tetS*, a common tetracycline resistance gene and *APH(3')-IIIa*, an aminoglycoside phosphotransferase in *Staphylococcus aureus* and *Enterococcus* spp. are also in line with previous publications[84]. *Enterococcus faecalis* harbored further ARGs, namely *dfrE*, a chromosome-encoded dihydrofolate reductase[85], *efrA*, a part of the *EfrAB* efflux pump that is inactive without *EfrB*[86] and *emeA* encoding a multidrug efflux pump[87] all originting from *Enterococcus faecalis*[13]. Besides the bacterial species mentioned above, *APH(3')-IIIa* was also detected in *Tetragenococcus halophilus*. This ARG is often appears in *Enterococcaceae*[13] but has not yet been written down in this species. Even though *ErmB* is an ARG that is written down in a wide range of bacterial species, no currently available publications mention its detection in *Tetragenococcus halophilus*.

To our knowledge *Pantoea agglomerans* has not yet been associated with *marA* and *rsmA* in the currently available literature. *Rsma* a gene regulating virulence of *Pseudomonas aeruginosa* has negative effect on the overexpression of *MexEF-OprN* efflux pumps involved in the development of antibiotic resistance[13,88,89]. *Rsma* has a homolog in *Escherichia coli* called *csrA* that is rather common by some members of *Enterobacteriaceae*[13].

Neither macrolide-lincosamide-streptogramin B (MLSB) resistance phenotype encoding *ErmB* nor nucleotidyltransferase *lnuA* was previously detected in *Amylolactobacillus amylophilus*. *LnuA* is a nucleotidyltransferase up till now mostly related to *Staphylococcus chromogenes*[13], was detected in *Companilactobacillus futsaii* within our study. *ANT(6)-Ia*, an aminoglycoside nucleotidyltransferase gene appears in many species, including *Lactobacillus* spp.[90]. Its species-specific association with *Lacticaseibacillus manihotivorans* has not been described in any publications. Neither *ErmB*, nor *tetM* have ever been described to derive from the genetic material of *Lactiplantibacillus* species. Genus-level identification of *tetM* in *Lactobacillaceae* has recently happened[91], but species-level identification and association with *Levilactobacillus brevis* or with *Lactiplantibacillus argentoratensis* has been executed for first. *Latilactobacillus sakei* and *Lactiplantibacillus plantarum* have already been found to harbour *tetM* by other publications[92,93]. In *Lactiplantibacillus plantarum*, *Loigolactobacillus backii*, *Limosilactobacillus*

reuteri, *Ligilactobacillus salivarius* or *Levilactobacillus brevis cat-TC*, *ErmB* or *lnuA* were also detected. Unlike other genes of the *cat* family[90], chloramphenicol acetyltransferase *cat-TC* was up till now only found in *Lactobacillus reuteri*[13], while the diverse species-level *Lactobacillus* association of *ermB* has been described in previous publications[94]. *LnuA* had previously been described in *Lactobacillus* spp.[90], but not specifically in *Lactiplantibacillus plantarum*, in *Latilactobacillus curvatus*, in *Loigolactobacillus backii*, in *Limosilactobacillus reuteri*, in *Ligilactobacillus salivarius*. The presence of *lnuA* in *Leuconostoc kimchii*, to our best knowledge, is not mentioned in any previous publications, while *tetM* has already been associated with *Leuconostoc mesenteroides*[95]. *LnuA* has not yet been identified in *Weisella cibaria* up till now. *Pediococcus acidilactici* associated tetracyclin resistance encoding *tetM* is already described in the literature[96]. Even though, *cat-TC*, a variant of the chloramphenicol acetyltransferase encoding cat gene is interconnected with *Lactobacillus reuteri* according to CARD[13], within our study it was identified in *Pediococcus claussenii*. *Pediococcus pentosaceus* associated *ErmB* has also been mentioned in the publication of Thumu et al.[97].

QnrD1, a gene encoding a quinolone resistance protein that is normally detected in *Salmonella enterica*[13], has already been found in *Providencia* spp.[98] and was attached to *Providencia rettgeri* in our study as well.

While *AAC(3)-Ia* is a common finding in *Acinetobacter baumanni*, *aadA3* is rather typical in *E. coli*[13], nevertheless, it has already been reported in *Acinetobacter baumanni* as well[99]. *AAC(6')-Ir* is a chromosomal-encoded aminoglycoside acetyltransferase was originally isolated from *Acinetobacter colistiniresistens*[13], and now was found in *Acinetobacter dispersus*. The identification of *ANT(3")-IIc* in *Acinetobacter gyllenbergii* and *OXA-297* in *Acinetobacter lwoffii* is in line with the current literature, as this ARG is supposed to normally occur in *Acinetobacter* spp.[100,101].

Pseudomonas putida associated *AAC(3)-Ia* and *aaDA3* had been identified in *Pseudomonas* spp. before[102,103], although not in this very species. *RsmA* that was first identified in *Pseudomonas aeruginosa*[13] was identified in *P. fulva* and in *P. oryzihabitans* in our samples. *APH(3')-IIa* is an aminoglycoside phosphotransferase having possible relatedness to *Rhodopseudomonas palustris* mentioned in other publications as well[104].

Staphylococcus aureus could have been associated with two ARGs, *lnuA* and *tetK* encoding a tetracycline efflux protein, that are both common findings in *Staphylococcus* spp.[105,106]. *lnuA* was also detected in *S. equorum* and *tetK* in *S. kloosii*. Although, *Erm(44)v* was first detected in the *S. saprophyticus*[107], no literature could be found about the appearance of this gene neither in *S. carnosus*, in *S. nepalensis* nor in *S. pseudoxylosus* species. *FosD* encoding fosfomycin resistance was found in *S. epidermidis*, that is in line with previous publications mentioning the gene in *Staphylococcus* spp.[108].

Lsa(D) identified in *Lactococcus garvieae* confers resistance to streptogramins A, pleuromutilins and lincosamides and also derives from this species[13].

Serratia spp. carried *AAC(6')-Ic* and *tet(41)* are both commonly found in this species[109,110].

Throughout our study, several ARGs were predicted to be co-occurring with genetic elements facilitating mobility. The bioinformatic analysis of the mobility characteristics relied upon the identification of three major mobility determination groups, namely iMGEs, phages and plasmids.

iMGE associated ARGs were detected in seven species (*Bacillus subtilis*, *Enterobacter cloacae*, *Klebsiella michiganensis*, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *Pantoea agglomerans*, *Pediococcus pentosaceus*). While the identification of amynoglycoside resistance encoding *APH(3')-IIa* in *Bacillus subtilis* is a unique finding, the gene is described to be located on transposons in other bacterial species[13] and mentioned to spread with MGEs by other publications as well[111,112]. Moreover, according to our investigations, iMGE associated *APH(3')-IIa* harbored on a plasmid. Such a co-occurence of an iMGE and a plasmid can provide *APH(3')-IIa* with a high mobility potential and a high likelyhood of the contribuition to the spread of AMR.

KpnH detected in *Enterobacter cloaceae* was flanked by an iMGE. To our best knowledge, this is a new finding of gene *KpnH*. Our findings on *APH(3")-Ib* and *APH(6)-Id* are in line with the current literature, as these aminoglycoside phosphotransferases are described to be normally encoded by plasmids, transposons, integrative conjugative elements and chromosomal genomic islands in many bacterial species[13], including *Klebsiella michiganensis*[75]. *ErmB*, that confers the MLSB resistance phenotype, and *tetM* has long been known to be carried on transposable DNA elements in *Bacteroides*[113] and in *Escherichia coli*[114], respectively, but the the coexistence of transposons and these genes has now been described first in Lactiplantibacillus species. In line with the findings of Thumu et al.[97] *Pediococcus pentosaceus* associated *ErmB* was found likely to be spread by horizontal gene transfer. Plasmid-encoded *tetM* has been identified in *Lactobacillaceae*[91], and also linked to transposable elements[115] but up till now not exactly in the species *Levilactobacillus brevis*, to our knowledge.

Erm(44)v, originally described in *Staphylococcus saprophyticus*, has not been detected with any relics of mobile genetic elements in the sequences flanking but fragments of phage DNA were found in the vicinity by other research groups as well[116,117]. To our knowledge, no previous publications include *S. nepalensis* and *S. pseudoxylosus* specifically harboring this gene accompanied with phages. *RsmA* detected in the genom of *Pseudomonas fulva* and *P. oryzihabitans*. To our knowledge this gene has not yet reported to be detected flanked by phages up till now. *BaeR*, a regulator gene detected in *Enterobacter cancerogenus*, *E. cloaceae*, *E. hormaechei* and *Kosakonia cowanii* co-exsisted with phages. As this gene itself does not encode

antibiotic resistance the interpretation of this finding is rather questionable.

The majority of mobile ARGs were predicted to have plasmid origins. A species-based literature review was performed to evaluate our results. Aminoglycoside acetyltransferase *AAC(3)-Ia* and aminoglycoside nucleotidyltransferase *aadA3*, that had both been described in *Acinetobacter baumanni* before[13,99], are normally encoded by plasmids, transposons or integrons. Since *Acinetobacter baumanni* often appears in the hospital environment, and hence has a great public health significance[118], the detection of mobile ARGs in its genome is of outstanding importance. The fact, that the mobility characeristics of MLSB encoding *ErmB* can derive from various mobile genetic elements, including plasmids, have already been indicated in various publications[113,119]. Nevertheless, there have been no written sources of the appearance and mobility of this gene in *Amylolactobacillus amylophilus*, and neither of that of *lnuA*, that is a plasmid-mediated nucleotidyltransferase[13]. In contrast to the previous publications[120], *ykkD* harbored on a plasmid in *Bacillus mojavensis*. As mentioned earlier, *Bacillus subtilis* deriving *APH(3')-IIa* could have been associated with an iMGE and a plasmid, what emphasizes its spreading potential and public health significance. *AAC(3)-Ia*, that is encoded by plasmids, transposons, integrons according to CARD[13] as well, was found in *Citrobacter freundii*. Our findings regarding the mobilome of *Companilactobacillus futsaii* are in line with CARD[13], as it states *lnuA* is plasmid mediated. In *Enterococcus* spp., a great range of plasmid borne ARGs were found. The plasmid association of *BacA*, *FosA2*, *qnrE1* is beyond question[121–123]. In contrast, the natural plasmid occurrence of *EmrB*, *mdtB* and *mdtC* was not revealed up till now, while *ramA* and *marA* are normally rather chromosomally encoded[124]. Interestingly, there are no sources mentioning the mobility characteristcs of *KpnH*, that according to our examinations was associated to plasmids by *E. hormaechei* and *E. cancerogenus* and flanked an iMGE by *E. cloaceae*. *ACT-17* from *E. hormaechei* was also predicted to have plasmid origins. Members of the *ACT* beta-lactamases, also known as *AmpC* beta-lactamases, are normally encoded by genes located on the chromosomes[13], but recently have also been found on plasmids[125,126]. Furthermore, *E. hormaechei* had regulator gene *baeR* associated to a plasmid. Taking into consideration that *baeR* itself is just a regulatory gene, the interpretation of this result is questionable. Examining the mobilome of *Enterococcus* spp., *ermB* was predicted to be of plasmid origins by *E. casseliflavus* and *E. faecium*, that is in line with other publications in the topic[83]. *E. faecium* also carried *AAC(6')-Ii* and *tetS* on plasmids. This finding is unexpected in case of *AAC(6')-Ii*, that is rather chromosomally encoded[13], while *tetS* has already been observed to appear on plasmids[119]. *E. faecalis* associated *APH(3')-IIIa* was predicted to be plasmid-encoded, that is in line with the CARDciteJia2017 definition of this gene. By *Escherichia coli* all the detected resistance genes (*APH(3")-Ib*, *APH(6)-Id*, *sul2*) appeared to harbor on plasmids. *E. coli* associated *APH(3")-Ib*, *APH(6)-Id* are encoded by plasmids, transposons, integrative conjugative elements and chromosomal genomic islands, while *Sul2* is usually found on small plasmids[13]. By the species that have been associated with *dfrG*, the gene has always been encoded on plasmids, that is in line with our findings in *Gracilibacillus sp. SCU50*[13]. *Klebsiella aerogenes* deriving *fosA5* was associated to a plasmid, that is in line with other findings regarding this ARG[74]. *APH(3")-Ib* and *APH(6)-Id* detected in *Klebsiella michiganensis* are of an emphasized public health significance, as they both were associated to iMGEs and harboured by plasmids. Of the genes associated with *Kosakonia cowanii*, *msbA* has already been identified on plasmids[127], while *marA* is referred to as being chromosomally encoded[124]. *BaeR*, a regulator gene found in *Kosakonia cowanii* was also predicted to have plasmid origins, but the interpretation of this result is difficult. Similarly to the cae of *marA*, the plasmid association of *KpnH* from *Kosakonia sp. CCTCC M2018092* is an interesting finding. The possible plasmid origin of *Lacticaseibacillus manihotivorans* deriving *ANT(6)-Ia* is supported by the CARD description[13]. The identification of plasmid-encoded *tetM* has happened in *Lactobacillaceae*[91], but species level association to *Levilactobacillus brevis*, *Lactiplantibacillus argentoratensis*, *Latilactobacillus sakei* or *Lactiplantibacillus plantarum* has been executed for first. Moreover, in *Levilactobacillus brevis*, plasmid-mediated *tetM* appeared to be flanked by an iMGE. Other *Lactiplantibacillus plantarum*, *Latilactobacillus curvatus*, *Loigolactobacillus backii*, *Limosilactobacillus reuteri*, *Ligilactobacillus salivarius* or *Weisella cibaria* related ARGs such as *cat-TC*, *ermB*, *lnuA* that were predicted to be mobile are in line with previous studies describing the plasmid-related genomic position of these genes[13]. By *Leclercia* spp. *ermB* was again predicted to be positioned on plasmids. Nonetheless, exceeding the expectations, *marA* was described plasmid-mediated in this species too. The plasmid association of *Lelliottia amnigena* originated *FosA2* is in line with other publications[122]. *LnuA* and *tetM* that were found in *Leuconostoc kimchii* and *L. mesenteroides*, respectively, both normally appear on plasmids[13,91]. The plasmid association of *Pediococcus acidilactici* deriving *tetM* is in line with the current literature[13,96]. *Cat-TC* that was interestingly identified in *Pediococcus clausenii* is a plasmid-borne cat variant according to CARD as well[13]. Plasmid-borne *ErmB* has already been described to appear in *Pediococcus* spp.[128]. In line with CARD[13], *QnrD1* of *Providentia rettgeri* appeared to be plasmid-mediated. Surprisingly, *marA* was predicted to be plasmid-borne in *Pseudescherichia/Escherichia vulneris* as well. In line with CARD[13], we found *AAC(3)-Ia* and *aadA3* of *Pseudomonas putida* plasmid-mediated. *APH(3')-IIa* is normally transposon-encoded[13], but publications of its detection in plasmids also exsist[104]. *Staphylococcus aureus* and *S. equorum* deriving *lnuA* and *S. aureus* and *S. kloosii* originated *tetK* are both plasmid-associated genes that often appear in *Staphylococcus* spp.[105,106]. *Erm(44)v* deriving from *Staphylococcus carnosus*, *S. nepalensis*, *S. pseudoxylosus* or *S. saprophyticus* has not been detected on plasmids up till now[107,117], even though some other members of the *Erm* gene family are often plasmid-borne[116]. *Erm(44)v* of *Staphylococcus nepalensis* and *S.*

pseudoxylosus was also flnaked by phages, what may emphasize their public health significance. *S. epidermidis* deriving FosD has been described flanking mobile, transposon-like elements[108], and was also detected on plasmids in *Staphylococcus* spp.[129]. According to the description of CARD[13], *APH(3')-IIIa* is plasmid-mediated, that is in line with our finding in *Tetragenococcus halophilus*. The second plasmid finding from *Tetragenococcus halophilus* is *ErmB*, that often harbores on plasmids, but has not yet been interconnected with this species up till now[119].

Antimicrobial resistance is an emerging threat to public health worldwide. This concern affects not only the healthcare sector but also agriculture. The usage of antibiotics in livestock exceeds the rate of human applications. Consequently, the antibiotics used in food animal medicine are considered a grave risk to humans through the food chain. The effects of the veterinary administered antimicrobials on the food chain are well documented, whereas the presence of the ARGs in dairy cattle nutrition is underrepresented in the literature. Still, the microbial mass contained in fermented feeds could play an essential role in the ARGs shifting through the food chain.

Nonetheless, our work has limitations. Because of the lack of previous research studies on the topic, we cannot compare the findings, and further research is needed to understand the importance of nutrition. Furthermore, we have had limited access to data about the samples (sampling history, exact location, timing, etc.). This is very much the key component in future attempts to understand the role of the fermented feed on ARG shift.

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Author contributions statement

NS takes responsibility for the integrity of the data and the accuracy of the data analysis. AGT, MP, NS and SÁN conceived the concept of the study. AGT, MP, NS and SÁN participated in the bioinformatic analysis. AGT, KS, MP, NS and SÁN participated in the drafting of the manuscript. AGT, KS, MP, NS and SÁN carried out the critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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