Research Article

TRANSPARENT PROCESS OPEN ACCESS Life Science Alliance

Genomic insights into antibiotic resistance and mobilome of lactic acid bacteria and bifidobacteria

Vita Rozman[1], Petra Mohar Lorbeg[1], Primož Treven[1], Tomaž Accetto[2], Sandra Janežič[3,4], Maja Rupnik[3,4], Bojana Bogovič Matijašić[1]

Lactic acid bacteria (LAB) and *Bifidobacterium* sp. (bifidobacteria) can carry antimicrobial resistance genes (ARGs), yet data on resistance mechanisms in these bacteria are limited. The aim of our study was to identify the underlying genetic mechanisms of phenotypic resistance in 103 LAB and bifidobacteria using whole-genome sequencing. Sequencing data not only confirmed the presence of 36 acquired ARGs in genomes of 18 strains, but also revealed wide dissemination of intrinsic ARGs. The presence of acquired ARGs on known and novel mobile genetic elements raises the possibility of their horizontal spread. In addition, our data suggest that mutations may be a common mechanism of resistance. Several novel candidate resistance mechanisms were uncovered, providing a basis for further in vitro studies. Overall, 1,314 minimum inhibitory concentrations matched with genotypes in 92.4% of the cases; however, prediction of phenotype based on genotypic data was only partially efficient, especially with respect to aminoglycosides and chloramphenicol. Our study sheds light on resistance mechanisms and their transferability potential in LAB and bifidobacteria, which will be useful for risk assessment analysis.

DOI 10.26508/lsa.202201637 | Received 28 July 2022 | Revised 30 January 2023 | Accepted 30 January 2023 | Published online 13 February 2023

Introduction

Antimicrobial (antibiotic) resistance of foodborne pathogenic bacteria is an important food safety problem (1). Commensal bacteria, including lactic acid bacteria (LAB) and *Bifidobacterium* sp. (bifidobacteria), have recently been recognised as a reservoir of resistance genes (ARGs) (2, 3, 4). They are introduced into the agro-food chain as starter and probiotic cultures, protective cultures, and feed additives. Because they come into contact with bacteria residing in gut—a hotspot of microbial horizontal gene transfer (5)—they pose a risk for transmission of ARGs. In scope of the Qualified Presumption of Safety status, such strains must be free of acquired ARGs (6). It was not until 2018 that the guidelines for the characterisation of microorganisms used as feed additives or as production organisms (7) included a requirement for strain characterisation based on whole-genome sequences (WGS). Since then, several studies have analysed resistance genes in LAB and bifidobacteria based on WGS (2, 3, 4, 8, 9), but still only a handful of studies have focused on strains intentionally added to the agro-food chain (10, 11). In addition, these studies often lack data on intrinsic and mutational resistance and transfer capability of ARGs through mobile genetic elements (MGEs). An in-depth understanding of the resistance mechanisms and their potential for transferability is essential to ensure the safety of dietary supplements (probiotics), feed additives, and products manufactured with starter or protective cultures.

Given that most antimicrobials are natural compounds, innate resistance mechanisms have evolved over time. Such natural (intrinsic) antimicrobial resistance is inherent to the species and presents a minimal potential for horizontal spread (12). On the contrary, resistance can be acquired either by a novel genetic mutation of chromosomal genes or by added resistance genes by means of horizontal gene transfer. Resistance acquired through added gene(s) is considered to have a high potential for horizontal dissemination (12). Acquired resistance in *Enterococcus* sp. (enterococci) is widespread and considerably well described, as some strains are important nosocomial pathogens (3, 13). On the contrary, data on resistance, particularly on intrinsic ARGs and mutations, are not as comprehensive in other genera of LAB and bifidobacteria.

In this context, the main goals of our study were not only to determine phenotypic susceptibility of LAB and bifidobacteria from different sources to antimicrobials but also to identify the potential underlying mechanisms of acquired and intrinsic resistance. In addition, we aimed to discover known and novel MGEs using whole-genome sequencing and comparative genomics. To achieve these goals, we analysed 103 strains, mainly commercial cultures but also isolates from human milk and from fermented products of which the genomes of 75 strains were sequenced in-house.

[1]University of Ljubljana, Biotechnical Faculty, Department of Animal Science, Institute of Dairy Science and Probiotics, Domžale, Slovenia [2]University of Ljubljana, Biotechnical Faculty, Department of Microbiology, Chair of Microbial Diversity, Microbiomics and Biotechnology, Ljubljana, Slovenia [3]National Laboratory of Health, Environment and Food, Maribor, Slovenia [4]University of Maribor, Faculty of Medicine, Maribor, Slovenia

Correspondence: vita.rozman@bf.uni-lj.si

Results

Antimicrobial resistance phenotypes

LAB and bifidobacteria can carry mobile ARGs, and when ingested, they can facilitate the transfer of these genes to the resident microbiota in the gut and thus to potential pathogens. Commercial strains are required to be free of acquired (mobile) ARGs (6), but data on the genetic basis for phenotypic resistance in these bacteria are limited. The main objective of our study was to identify the potential underlying mechanisms of acquired and intrinsic resistance in LAB and bifidobacteria using comparative genomics.

The minimum inhibitory concentrations (MICs) of up to 27 antimicrobials were tested using the broth microdilution method for 103 LAB and bifidobacteria (Fig 1). We observed that resistance to kanamycin and resistance to chloramphenicol were the most common clinically relevant phenotypes (Fig 1). In contrast, the lower prevalence of resistance was seen with gentamicin, erythromycin, and ampicillin, whereas atypical vancomycin resistance (7) was not detected (Fig 1). Multidrug resistance frequently occurred in *Enterococcus* sp., *Levilactobacillus brevis*, *Lacticaseibacillus rhamnosus*, and *Pediococcus* sp. Surprisingly, three strains showed resistance to five groups of clinically important antimicrobials (Fig 1).

Whole-genome sequence analysis

Acquired ARGs

Genomic data (n = 103) were mined for the presence of ARGs whose intrinsic or acquired nature was determined by MGEs and pan-genome analyses. Based on the selection criteria, a total of 36 acquired ARGs corresponding to 18 diverse reference ARGs were found in 18 strains (Fig 2 and Table S1). Most of these ARGs (n = 33) were expressed in the resistant phenotype. Collectively, these genes conferred resistance to a broad array of antimicrobial classes (Table S1).

Analysis revealed that the tetracycline resistance gene *tetW* was most frequently detected, particularly in *Bifidobacterium* (*B.*) *animalis* subsp. *lactis* strains and in a probiotic *Limosilactobacillus reuteri* (Fig 2). Phenotypic tetracycline, but not tigecycline, resistance was less frequently conferred by the *tet*(*L*), *tet*(*M*), *tet*(*O*), *tet*(*S*), or *tet*(*U*) genes that were found in isolates from the natural microbiota of fermented products (referred to as non-starter strains) *Enterococcus* (*E.*) *faecalis*, *Lactococcus* (*L.*) *lactis*, and *E. italicus*, and in a probiotic strain of *B. breve* (Fig 2). We found that streptomycin resistance in *E. faecalis* and *L. lactis* (Fig 2) was associated with *ANT*(*6*)-*Ia* that in enterococci appeared to be linked to *SAT-4* and *APH*(*3′*)-*IIIa*, the genes responsible for resistance to streptothricin, and kanamycin and neomycin, respectively. In addition, a bifunctional *AAC*(*6′*)-*Ie*-*APH*(*2″*)-*Ia* that reflected in atypical gentamicin, kanamycin, and neomycin MICs was found in *E. faecalis* (Fig 2).

The MLS_B phenotype in *E. faecalis* and in a probiotic strain *B. longum* (Fig 2) was encoded by *erm*(*B*) and *erm*(*49*), respectively. Markedly, we found a known mutation upstream of *erm*(*B*) in all three enterococcal strains (TAAA duplication between −124 and −127 resulting in a premature stop codon of the leader peptide first reported by Oh et al (14)), which also facilitated resistance to the 16-membered macrolide tylosin, presumably because of the gene overexpression. Chloramphenicol resistance and trimethoprim resistance in *E. faecalis* (Fig 2) were attributed to the *cat* and *dfrG* gene, respectively, whereas the low level of ampicillin and penicillin resistance in *Carnobacterium* (*C.*) *divergens* may be due to the expression of *CAD-1* β-lactamase.

Intrinsic and candidate ARGs

Most of the ARGs were recognised as intrinsic (140 ARGs in 37 strains, of which 20 were diverse based on gene homology) (Fig 2 and Table S1). Consistent with the intrinsic aminoglycoside resistance phenotype, *E. faecium* strains contained *AAC*(*6′*)-*Ii* and *efmM*, whereas *E. hirae* and *E. durans* possessed *AAC*(*6′*)-*Iid* and *AAC*(*6′*)-*Iih*, respectively. Interestingly, we identified homologs of EfmM with a conserved active site (C185, C235) in the vast majority of the LAB species studied (Fig 2). The observed high aminoglycoside MICs in *B. longum* and *B. breve* appear to be connected to aminoglycoside phosphotransferases, though homologs were also found in *B. animalis*. The *efmA* gene with a surprisingly diverse sequence was present in *E. faecium* strains.

A total of 331 candidate ARGs were discovered (Fig 2 and Table S1), representing 33 diverse genes in 92 strains (37 species). These genes confer resistance to various antibiotics (Table S1). Interestingly, among these genes *arr-4* in *L. lactis* IM145 had conserved amino acid residues His18, Tyr48, and Asp83, which are involved in rifampicin resistance (15). *E. malodoratus* IM1302 encompassed *fosXCC* with conserved amino acid residues in its active site (His7, His64, Glu110, Tyr100, and Arg119), which has been linked to resistance to fosfomycin in *Campylobacter* (16). Although most of these genes were presumably intrinsic, we also found acquired candidate ARGs (e.g., *arr-4*, *catB9*, *lnuA*, *ANT*(*6*), *mefA*, *vga*(*E*)). Their actual involvement in the resistance phenotype remains to be verified in vitro.

Mutations associated with antimicrobial resistance

We provide comprehensive data on mutations in proteins previously reported to be involved in resistance (Table S2). The results suggest that mutations may be an important mechanism of resistance, particularly in bacteria intentionally introduced into the agro-food chain. We discovered known mutations already reported in the studied species, as well as novel mutations in the active (binding) sites of the target (or other) proteins not yet reported in the species or genera considered. Their role in resistance should be further elucidated in vitro.

Multiple sequence alignment of the S12 proteins revealed two substitutions, K43R/N/M and K88Q (*Mycobacterium tuberculosis* numbering), in commercial streptomycin-resistant strains (Fig 3A). Likewise, we discovered *rsmG* point mutations (I55A, G164V, and D67N, *M. tuberculosis* numbering; and G10E and R190H, *Streptomyces coelicolor* numbering, Table S2) involved in low-level streptomycin resistance. Although LAB are generally less susceptible to aminoglycosides, three strains (*Lactobacillus acidophilus* IM116, and *L. lactis* IM1456, IM1341) exhibited a hypersusceptible phenotype. Interestingly, these strains harboured single nucleotide polymorphisms (SNPs) in the F0F1

Figure 1. Phenotypic resistance profiles of 103 lactic acid bacteria and bifidobacteria.
The minimum inhibitory concentrations (MICs) determined by the microdilution tests and the cut-off MICs that define whether a strain is susceptible or resistant to a particular antibiotic are shown as a heatmap. The names of the strains resistant to five different classes of clinically important antimicrobials are highlighted in red. C, cut-off MICs; FD, feed additive; HM, isolate from human milk or colostrum; M, MICs determined by microdilution tests; NS, isolate of natural microbiota from fermented products (non-starter strain); P, probiotic strain; PC, protective culture; R, resistance; Synercid, quinupristin/dalfopristin; S, starter culture.

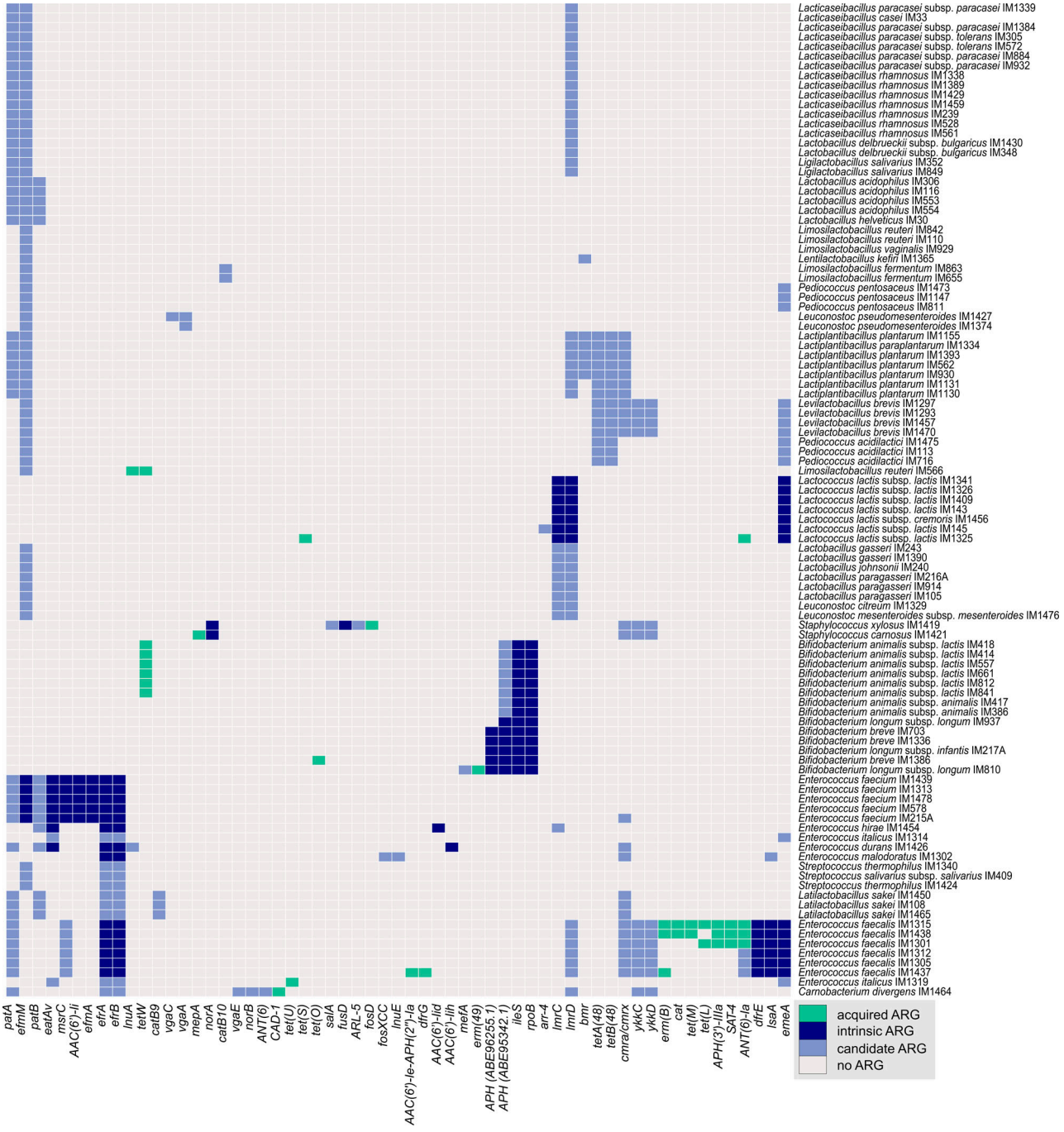

Figure 2. Acquired, intrinsic, and candidate antimicrobial resistance genes (ARGs) found in 103 bacterial strains.
A gene was annotated as an ARG based on the best BLAST hit with a sequence similarity threshold greater than 70%. The intrinsic and acquired nature of ARGs was determined with the aid of mobile genetic element prediction and pan-genome analyses. Candidate (homologous) ARGs were identified based on additional analyses of the hits with lower BLAST similarities (sequence similarity threshold between 40% and 70%).

ATPase genes (Table S2). F0F1 ATPase is reportedly involved in aminoglycoside transport into cells (17) that could be hampered by these mutations. The effects of these mutations on resistance have yet to be confirmed in vitro.

Several resistant probiotic strains had SNPs in *16S rRNA* (A1408G, C1054T, and A1197T, *E. coli* numbering) that presumably confer resistance to aminoglycosides or tetracycline (Fig 3B and C). We also identified a SNP (A986T, *E. coli* numbering) near the primary tetracycline binding site in the representatives of LAB (Table S2) displaying high-end tetracycline MICs. Among four SNPs in *23S rRNA* (Fig 3B and C), G2057T, A2058G, and C2610T presumably encode resistance to MLS_B, whereas A2062T encodes resistance to tylosin,

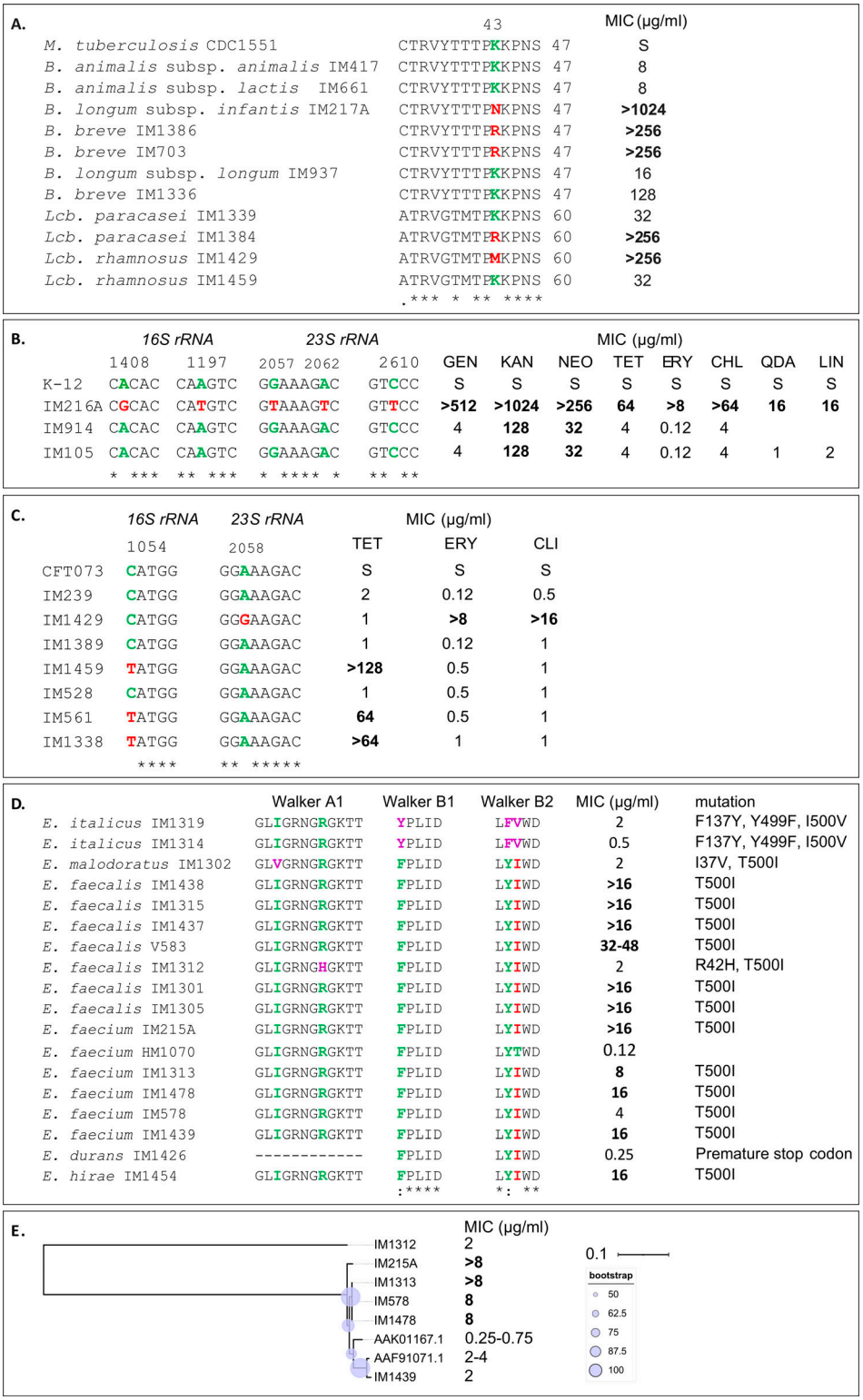

Figure 3. Polymorphisms in S12, Lsa(A), and MsrC in *16S* and *23S rRNA*.
Shown is a section of the sequence alignment in which the mutations presumably associated with resistance are highlighted in red. **(A)** Substitution of amino acid K43 in S12 was associated with streptomycin resistance. Polymorphisms in *16S rRNA* and *23S rRNA* in strains of **(B)** *Lactobacillus paragasseri* and **(C)** *Lacticaseibacillus rhamnosus* confer resistance to different groups of antimicrobials. K-12 and CFT073 represent *Escherichia coli* strains. **(D)** Polymorphisms in key motifs of Lsa(A) and homologs were associated with clindamycin resistance (SNPs highlighted in red) and susceptibility (SNPs highlighted in purple). **(E)** Phylogenetic tree of MsrC protein sequences of *E. faecium* strains. Shown are the minimum inhibitory concentrations (MICs) of erythromycin. The tree was rooted with an outgroup (*E. faecalis* IM1312). The exceeded cut-off MICs are shown in bold. MIC, minimum inhibitory concentration; S, susceptible; GEN, gentamicin; KAN, kanamycin; NEO, neomycin; TET, tetracycline; ERY, erythromycin; CHL, chloramphenicol; AMP, ampicillin; QDA, quinupristin/dalfopristin; LIN, linezolid.

chloramphenicol, quinupristin/dalfopristin, and linezolid. The coverage and variances of *16S* and *23S rRNA* SNPs have been validated by mapping the sequenced reads to the assembled sequences (Table S3).

As reported before, enterococci exhibit clindamycin resistance because of the T500I substitution in the Walker B2 motif of the intrinsic protein Lsa(A) or its homologs (18). The susceptibility of our strains was likely related to novel mutations in key motifs of these

Table 1. Amino acids of PBP5 proteins associated with ampicillin susceptibility of *E. faecium*.

	Amino acid																					S	R	MIC (μg/ml)
Strains	24	27	34	66	68	85	100	144	172	177	204	216	324	466	485	496	499	525	586	629	667			
Com15[a]	V	S	R	G	A	E	E	K	T	L	D	A	T	/	M	N	A	E	V	E	P	21	0	0.5-1
IM1313	V	S	R	G	A	E	E	K	T	L	D	A	T	/	M	N	A	E	V	E	P	21	0	1
IM1478	V	S	R	G	A	E	E	K	T	L	D	A	T	/	M	N	A	E	V	E	P	21	0	2
IM578	V	S	R	G	A	E	E	K	T	L	D	A	T	/	M	N	A	E	V	E	P	21	0	1
IM215A[b]	A	G	R	G	A	E	E	Q	A	L	D	A	A	/	M	N	A	E	V	E	P	16	5	>16
IM1439	A	G	Q	E	A	E	Q	Q	A	I	D	S	A	/	M	K	I	D	V	E	P	8	13	2
TX2043	A	G	Q	E	A	E	Q	Q	A	I	D	S	A	/	M	K	T	D	V	E	P	8	13	4
C68[c]	A	G	Q	E	T	D	Q	Q	A	I	G	S	A	S	A	K	T	D	V	V	S	1	20	256

The WT amino acids are shaded green, and amino acid changes, red. MIC, minimum inhibitory concentration; S, susceptible; R, resistant.
[a]PBP-S.
[b]Insertion upstream of *PBP5* (transposase).
[c]PBP-R.

Table 2. Known or novel mutations associated with resistance to antimicrobials not included in the EFSA list (7).

Protein	Amino acid change	Phenotype	Species of origin
DfrG	F98Y/L	trimethoprim	*Staphylococcus aureus* (22)
DfrG	P21A	trimethoprim	*E. coli* (23)
DfrG	N/H23Y, D27E, A7S (*E. coli* numbering)	trimethoprim	novel mutations in the active site
GyrA	S83T	ciprofloxacin	*E. coli* (24)
FusA	V90I, G451A/S, H457Q, L461I/M	fusidic acid	*Staphylococcus aureus* (25)
FolP	V48I	sulphamethoxazole	*Mycobacterium leprae* (26)
LiaF	S48Y	daptomycin	novel mutation
LiaS	G226E, V351I	daptomycin	novel mutations
LiaR	E45V	daptomycin	novel mutation
GdpD	A249T, P307Q, F478L, D552N	daptomycin	novel mutations

proteins (see Fig 3D). Furthermore, mutations in *lsa(A)* also affect the MIC of streptogramin, as observed in a non-starter isolate *E. faecalis* IM1301 that carried a previously reported substitution in the −10 promoter region (A-131T) (19).

The phylogenetic tree of intrinsic MsrC proteins (Fig 3E and Table S2) indicates that variations in sequence (including a novel substitution in a Walker A1 motif, T45S) may have an impact on erythromycin MIC and resistance in *E. faecium*. A WT strain (TX1330, AAK01167.1) had a MIC of 0.25–0.75 μg/ml, whereas our strains coding for mutated MsrC exhibited MICs of at least 2 μg/ml. Resistant strains (MIC ≥ 8 μg/ml) contained additional mutations that were reflected in two clades of the phylogenetic tree, the erythromycin-susceptible and erythromycin-resistant strains.

E. faecium strains exhibited ampicillin or penicillin resistance as a result of variations in 20 (or 21) amino acids of PBP5 or its promoter region described before (20, 21). Despite a hybrid PBP5 sequence (see Table 1), the probiotic strain *E. faecium* IM215A exhibited higher ampicillin and penicillin MICs compared with other *E. faecium* strains. We hypothesise that an insertion, which bears partial similarity to a transposase gene, between regions −10 and −35 of the *PBP5* promoter affects overexpression of the gene and leads to resistance in this strain.

Resistance to antimicrobials not included in the EFSA list (7) was commonly associated with known or novel mutations (Table 2). Interestingly, most species of LAB do not carry a FolP homolog, which we believe to be a reason for the extreme MICs of sulphamethoxazole.

Phenotype–genotype agreement

In total, 1,496 phenotypic tests were performed for 103 strains, yet resistance and susceptibility could be determined for 1,314 MICs. The resulting catalogue is shown schematically in Fig 4 and described in detail in Table S2. We observed an overall high agreement (92.4%) between the presence and absence of (candidate) ARGs and mutations and the corresponding phenotypic resistance or susceptibility, respectively (Table 3). Phenotypic resistance was validated in 65.0% of the cases by genetic analyses. In fact, all exceeded cut-off values for six antibiotics could be elucidated (Table 3). All but three acquired resistance genes (*tetW*) are expressed in phenotypic resistance.

All in all, our method for predicting phenotype from genotypic data was only partially efficient. Even though positive (97.8%) and

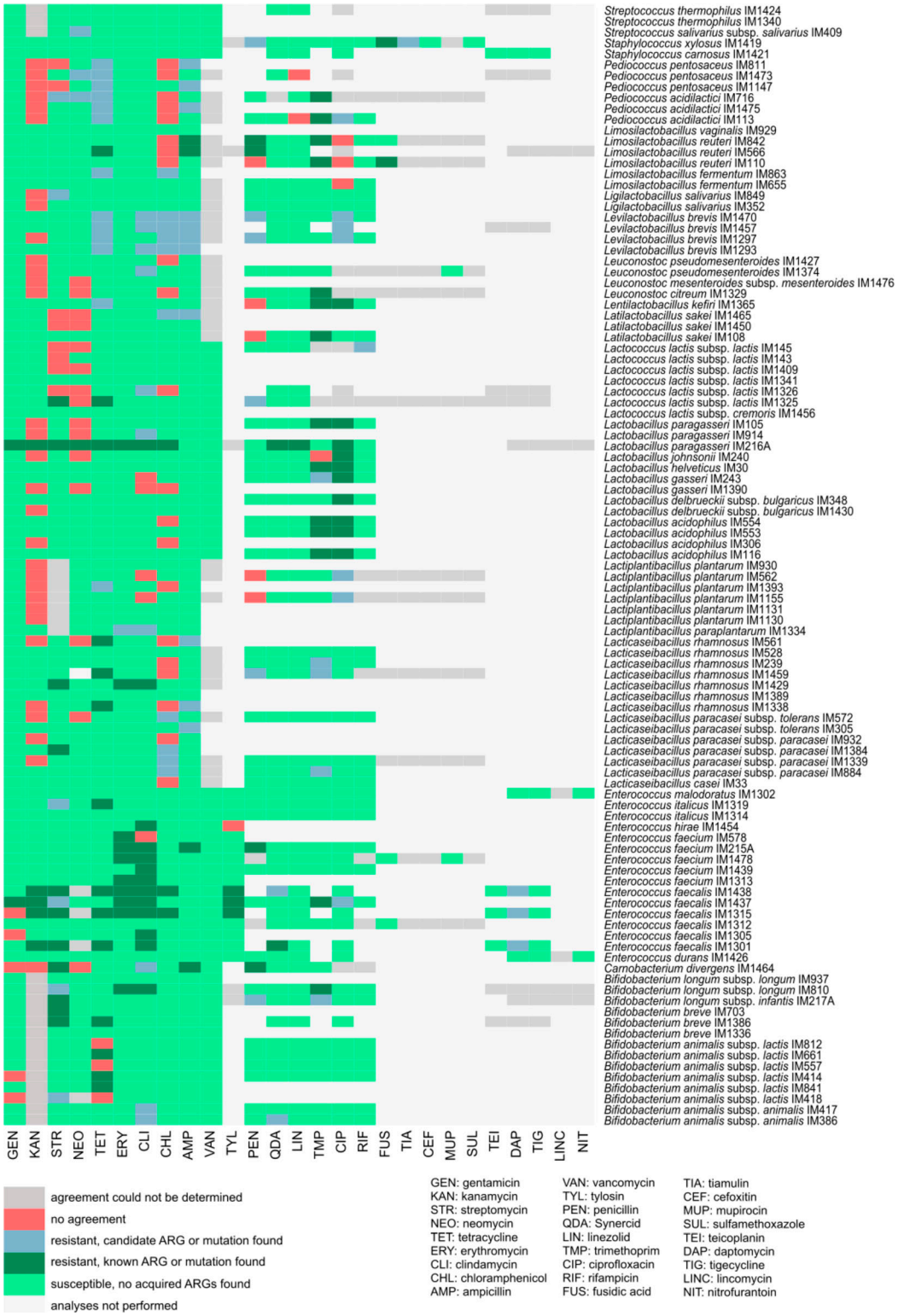

Figure 4. Phenotype–genotype agreement analysis of 103 strains of lactic acid bacteria and bifidobacteria.
In cases where no cut-off minimum inhibitory concentration was defined and in cases where the minimum inhibitory concentration was outside the concentration range of the microdilution test, agreement was not determined (shown in dark grey). ARG, resistance gene; Synercid, quinupristin/dalfopristin.

Table 3. Phenotype–genotype agreement analysis of 103 strains for individual antibiotics.

	Phenotype–genotype agreement (%)[a]	Validated phenotypic resistance (%)[b]
Gentamicin	95.1	28.6
Kanamycin	64.0	13.9
Streptomycin	91.7	68.0
Neomycin	84.7	21.1
Tetracycline	97.1	100
Erythromycin	100	100
Clindamycin	95.1	85.2
Chloramphenicol	79.6	38.2
Ampicillin	100	100
Vancomycin	100	/
Tylosin	93.8	75.0
Penicillin	90.2	66.7
Quinupristin/dalfopristin	100	100
Linezolid	96.8	33.3
Trimethoprim	98.0	95.0
Ciprofloxacin	94.2	85.0
Rifampicin	100	100
Total	92.4	65.0

[a]Genotype and phenotype matched when the susceptible or resistant phenotype reflected the absence or presence of (candidate) ARG(s) or mutation(s), respectively.
[b]The proportion of exceeded minimum inhibitory concentrations (phenotypic resistance) validated by genetic analyses.

negative (91.3%) predictive values and specificity (99.6%) were high, sensitivity was lower (64.3%).

Genetic environment of the ARGs

The mobility of ARGs was estimated with the aid of the MGE analysis. Importantly, genomic island, a region of foreign origin indicative of horizontal gene transfer (27), was found in the genetic environment of acquired ARGs and one candidate ARG (Fig S1). In general, intrinsic ARGs were devoid of MGEs. This implies that the risk of horizontal transmission of intrinsic antimicrobial resistance can be considered minimal.

Genetic organisation of the discovered MGEs is depicted in Fig 5. Analysis demonstrated that enterococci frequently carry MGEs. For example, *tet*(*M*) is encoded on an integrative and conjugative element, Tn*916* (24.6 kbp) (Fig 5A), whereas *tet*(*L*), which is associated with the mobility genes *pre*/*mob* and *repB*, resides on an incomplete element that shows sequence similarity to a segment of Tn*6079* (Fig 5B). *ANT*(*6*)-*Ia*, *SAT-4*, *APH*(*3′*)-*IIIa*, *cat*, and *erm*(*B*) genes are located on an element similar to the enterococcal plasmid pRE25 (Fig 5C). The full-length plasmid was not recovered. Moreover, we discovered that *dfrG* is associated with a MGE that showed strong homology to a short segment of ICESauTW20-2 from *Staphylococcus aureus* and *tet*(*U*) with a putative novel plasmid (Fig 5D).

Our data show that small genomic islands can be found in bifidobacterial genomes, including in probiotics *B. animalis* subsp. *lactis* (Fig 5E). A novel genomic island containing *erm*(*49*) and three genes of unknown function was discovered in *B. longum* strain from a dietary supplement (Fig 5F), whereas the probiotic *B. breve* strain carried a genomic island consisting of three coding sequences (*tnpV*, *tet*(*O*), and the *RNA polymerase sigma factor*) (Fig 5G) and is also present in ICESsuLP081102 from *Streptococcus suis*.

The probiotic *Limosilactobacillus reuteri* strain has a tetracycline (pLR581, 12.2 kbp; Fig 5H) and a lincomycin (pLR585, 14.2 kbp; Fig 5I) resistance plasmid typical of the widely used probiotic strain *Limosilactobacillus reuteri* SD2112 (28). Similarly, *tet*(*S*) and *ANT*(*6*)-*Ia* reside on a 49,741-bp contig that is a putative plasmid (carries *repA*) (Fig 5J). Interestingly, the results indicate that *ANT*(*6*)-*Ia* is actually located within a novel integrative and mobilisable element (harbours an integrase and a relaxase, but lacks type IV secretion system genes) with partial homology to ICESsu(SC84) from *S. suis*. The integrative and mobilisable element is delimited by the potential *attL* (2098968..2098982, cgatttttgattttt) and *attR* (2117014..2117028) site-specific attachment sites. The genetic environment of *tet*(*S*) on the contrary resembles a composite transposon bounded at both ends by an insertion sequence IS1216, which is also present on plasmid pLraf_19_4S_1 in *L. raffinolactis*.

Surprisingly, only one candidate ARG (*arr-4*) was carried by a putative MGE (Fig 5K). Its genomic island consists of 26 coding sequences that share significant sequence similarity with a putative phage-inducible chromosomal island bIL310. A homologous integrase, an accessory gene, a regulatory region, and hypothetical genes were found. The element is flanked by the putative *attL* (818..835, cgctttttactacgtt) and *attR* (18120..18137) sequences.

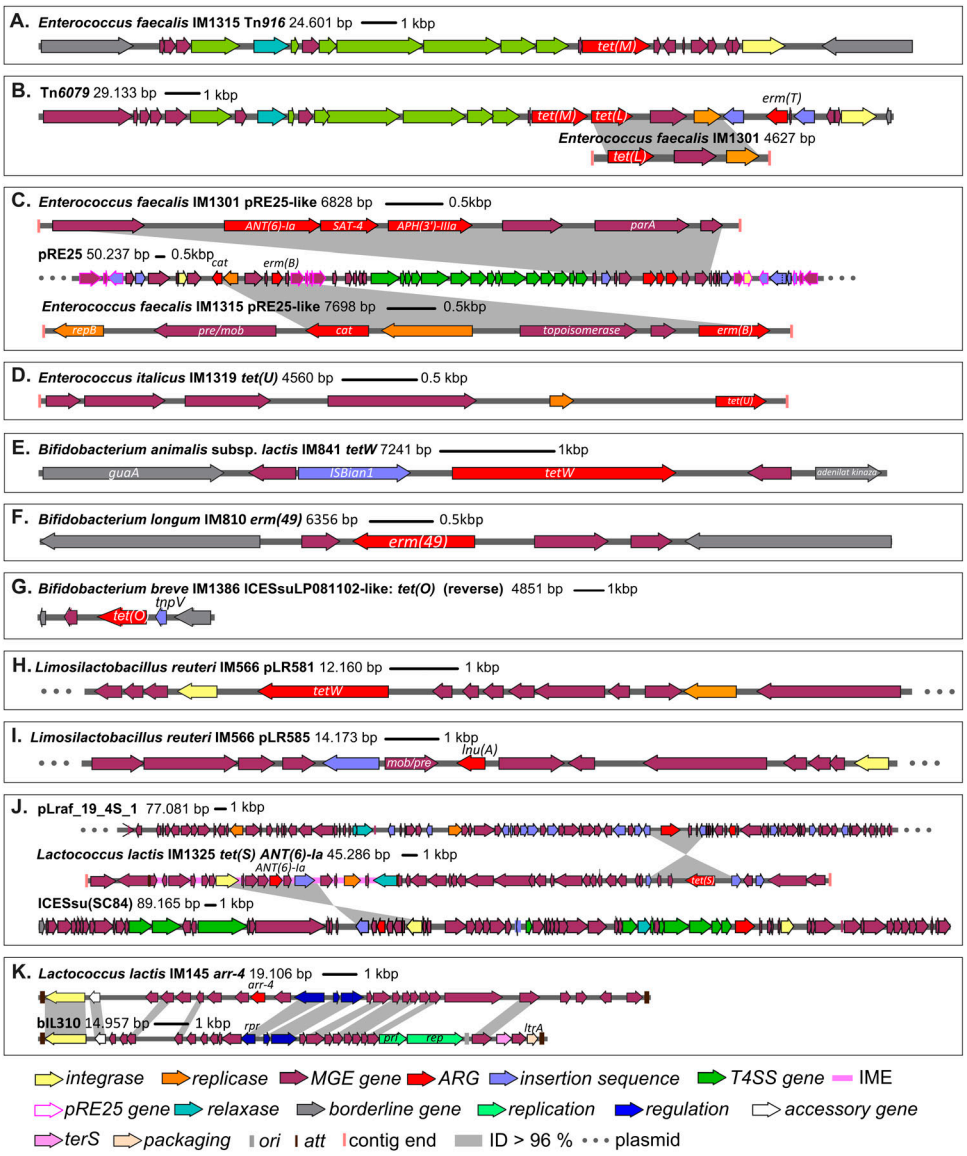

Figure 5. Genetic organisation of the detected mobile genetic elements.
(A) Gene *tet*(*M*) resides on Tn*916*. **(B)** Gene *tet*(*L*) is located on an incomplete element that shows sequence similarity to a segment of Tn*6079*. **(C)** *ANT*(*6*)-*Ia*, *SAT-4*, *APH*(*3′*)-*IIIa*, *cat*, and/or *erm*(*B*) are located on elements similar to the enterococcal plasmid pRE25. **(D)** Gene *tet*(*U*) was associated with a putative novel plasmid. Small genomic islands were found in **(E)** strains of *B. animalis* subsp. *lactis*, **(F)** *B. longum* IM810, and **(G)** *B. breve* IM1386. Probiotic bacterium *Limosilactobacillus reuteri* IM566 carries plasmids **(H)** pLR581 and **(I)** pLR585. **(J)** Genes *tet*(*S*) and *ANT*(*6*)-*Ia* reside on a putative plasmid. **(K)** Candidate *arr-4* is on a putative phage-inducible chromosomal island. Gene function was determined using BLAST and HMMER3, whereas genetic organisation was prepared using snapgene-viewer. ARG, antimicrobial resistance gene; ID, BLAST identity; IME, integrative and mobilisable element; T4SS, type IV secretion system.

Discussion

Because LAB and bifidobacteria are a potential source of antibiotic resistance for gut bacteria, including pathogens, commercial strains should not carry mobile ARGs (6). However, data on resistance mechanisms, especially intrinsic and mutational resistance, are lacking. The main objective of our study was to identify the potential underlying mechanisms of the observed phenotypic resistance in 103 LAB and bifidobacteria and to assess the transferability potential using comparative genomics.

We confirmed that phenotypic resistance is a common trait in LAB and bifidobacteria, which has been described by numerous authors (2, 3, 4, 29, 30, 31). However, genetic analyses revealed that intrinsic resistance in LAB and bifidobacteria was more prevalent than acquired resistance. In accordance with the Qualified Presumption of Safety status requirement (6), acquired ARGs were not common in strains intentionally added to the agro-food chain.

Nevertheless, several probiotic bifidobacteria harboured tetracycline or erythromycin resistance genes, the presence of which on mobile elements raises the possibility of horizontal spread. The *tetW*, *tet*(*O*), and *erm*(*49*) genes were reported in *Bifidobacterium* sp. before (32). In our recent study, we reported limited mobility of *tetW* and *erm*(*49*) in the metagenomic sequences of the human gut microbiota, as they were not widely disseminated and were not found outside the species of origin (33), suggesting that these two genomic islands do not pose a serious threat to food safety. The *tet*(*O*) genomic island, on the contrary, had a high transmission potential (33) and thus poses a risk if consumed. In accordance, Martínez et al reported the rare occurrence of *erm*(*49*) in the microbiomes of adults and infants (34), whereas *tet*(*O*) was frequently detected (35).

Our results suggest that foodborne *E. faecalis* strains play an important role in the spread of resistance. For example, *E. faecalis* strains harboured Tn*916*, which is responsible for much of the tetracycline resistance in the gut microbiota, even in pathogenic strains (36, 37), and thus poses a risk of transfer. Similarly, the pRE25 multidrug resistance plasmid was conjugated into the chromosomes of *E. faecalis*, *L. lactis*, and *Listeria innocua* (38). In concordance with our results, *tet*(*U*) was reportedly located on a small plasmid in *E. faecium* (39) and *tet*(*S*) near the transposase(s) IS1216 on a plasmid of *L. lactis*, *E. faecium*, and *S. dysgalactiae* (40). Unlike acquired ARGs, intrinsic ARGs are considered to have minimal potential for horizontal spread (12), which we confirmed by the MGE analysis.

Resistance data for many genera of LAB and bifidobacteria are not as extensive as for pathogenic bacteria; thus, fewer ARGs are available in the databases. Consequently, lower BLAST similarities are expected to be found. We uncovered numerous candidate ARGs (Fig 2 and Table S1), but their effect on the resistant phenotype needs to be verified in vitro. To the best of our knowledge, this is the first report of these genes in LAB and bifidobacteria. Surprisingly, a candidate *arr-4* gene in *L. lactis* IM145, which we presume to encode rifampicin resistance, resides on the putative phage-inducible chromosomal island (Fig 5K). Compared with a typical prophage genome, a phage-inducible chromosomal island is smaller in size given that it does not code for capsid and lytic proteins, which we did not detect. These elements were reported in staphylococci, lactococci, pneumococci, streptococci, and enterococci and may contain genes for diverse metabolic activities or resistance genes (41).

Our study highlights that mutations of chromosomal genes (Fig 3 and Table S2) that are not considered a hazard (12) may be a frequent mechanism of resistance in LAB and bifidobacteria. The use of probiotic strains with mutational resistance may be beneficial, as they are known to help restore the natural microbiota after antibiotic therapy and reduce the severity of antibiotic-associated diarrhoea (42). Consistent with reports on fitness cost (43), polymorphisms in the active site of *16S* or *23S rRNA* were not common (Fig 3B and C). To our surprise, probiotic *Lactobacillus paragasseri* IM216A carried multiple mutations in *16S* and *23S rRNA*, which were not previously reported in this species. The A1408G mutation in *16S rRNA* causes aminoglycoside resistance (44) and does not result in a significant fitness cost compared with the lethal A1408C and A1408U mutations (43). We believe that a novel A1197T polymorphism leads to tetracycline resistance because this nucleotide is involved in hydrogen bonding with the drug (45). This strain also carried *23S rRNA* mutations (G2057T, C2610T, and A2062T) that were found in *Legionella pneumophila* (46), *Streptococcus pneumoniae* (47), and *Mycoplasma hominis* (48), respectively. Furthermore, strains of *Lacticaseibacillus rhamnosus* also had SNPs in *16S* (C1054T) or *23S rRNA* (A2058G), which is in agreement with reports for *S. pneumoniae* and *Lacticaseibacillus rhamnosus* (49, 50). The A986T SNP in *16S rRNA* has been described in the tetracycline-resistant mutant of *Mycoplasma pneumoniae* FH (51) and may therefore be linked to the tetracycline-resistant phenotypes observed in our strains. Observed S12 mutations were reported in *M. tuberculosis* (52) and *B. breve* Yakult (53), but not in lactobacilli. Similarly, the *rsmG* mutations were reported in other species (8, 54, 55), but not in LAB or bifidobacteria.

Overall, the correspondence of genotypes and phenotypes in our study was high (in 92.4%) (Fig 4 and Table 3), but further genetic studies are needed to determine the unexplained phenotypic resistance. In accordance with our findings, Duranti et al (2017) reported a good correspondence between phenotype and genotype for type strains of bifidobacteria (2), whereas for type strains of lactobacilli, the agreement was lower (67%) (4). On the contrary, high agreement is usually reported for enterococci (9) as a result of more thoroughly characterised resistance mechanisms. Different methods for detecting ARGs and similarity cut-off values chosen, and additional screening for mutations, may also explain the observed discrepancies. Technical recommendations and requirements for whole-genome sequencing and analysis recently published by EFSA (56) are indeed an important step towards harmonisation of future studies.

In conclusion, our findings improve our understanding of the resistance mechanisms in LAB and bifidobacteria. We identified several mobile ARGs that pose a risk of transfer to pathogenic bacteria when ingested, but the prevalence of intrinsic ARGs was greater. Because intrinsic ARGs are free of MGEs, their risk of horizontal transmission can be considered minimal. We also observed that mutations may be a common mechanism of resistance. Overall, the analyses revealed high agreement between genotype and phenotype, but further genetic studies are needed to determine the unexplained phenotypic resistance. Our study presents a basis for risk assessment analyses that will ultimately ensure the safety of products used in human and animal nutrition in terms of antimicrobial resistance.

Materials and Methods

Bacterial strains

LAB and bifidobacteria were isolated from dietary supplements, starter and protective cultures, feed additives, human milk or colostrum, and fermented products (n = 66) or were obtained from the manufacturer or from a culture collection (n = 17). In addition, 20 probiotic and starter strains examined in our previous study (11) were reanalysed to provide additional data on candidate ARGs, mutations, and genotype–phenotype agreement. Collectively, 103 isolates were analysed (Table S2).

Serially diluted samples were cultured on the selective agar media (MRS, M17, Rogosa [Merck], and/or TOS-MUP [Yakult Honsha]) as indicated in Table S4. Strains derived from human milk or colostrum were isolated as described by Tušar et al (57) and obtained from the culture collection of the Institute of Dairy Science and Probiotics (Biotechnical faculty, University of Ljubljana) and ZIM culture collection (https://www.zim-collection.si/), which is a member of the World Federation of Culture Collections (#810). The strains were stored at −80°C, propagated under the conditions indicated in Table S4, and subcultured twice in broth medium (1% vol/vol) before all experiments.

Isolation of genomic DNA and identification of isolates at the species level

Genomic DNA was extracted from pure overnight cultures (1 ml) using a commercial kit (ISOLATE II Genomic DNA Kit [Bioline] or

Wizard Genomic DNA Purification Kit [Promega]). Cultures were centrifuged (3 min, 12,000*g*) (Hettich), and the pellet was resuspended in 500 μl of TE buffer containing mutanolysin (25 U/ml) and lysozyme (10 mg/ml) and incubated for 2 h at 37°C. Further steps were performed according to the manufacturer's instructions.

Strains were initially identified at the species level either by PCR using species-specific primers and protocols (Table S5) or by sequencing of the 16S rDNA genes (Microsynth). Using BLAST (58), the 16S rDNA sequences were classified to species level. The taxonomic affiliation of the strains was verified by calculating the average nucleotide identity (ANI) to the WGS of a type (or selected) strain (ANI > 95% (59)) using pyani 0.2.10 (60).

Antimicrobial susceptibility testing

MICs of the antimicrobials (see Table S2), covering almost all major classes (7), were determined by the broth microdilution method in the LSM medium (pH = 6.7) according to the standard guidelines ISO 10932 (61). We used the precoated plates VetMIC Lact-1 and Lact-2 (Statens Veterinärmedicinska Anstalt), and Sensititre AST plates EU Surveillance *Staphylococcus* EUST, EU Surveillance *Enterococcus* EUVENC, and/or NARMS Gram Positive CMV3AGPF (Thermo Fisher Scientific). In some cases, the microtitre plates for testing tylosin, vancomycin, and/or ampicillin were prepared in-house (61). After a 48-h incubation under anaerobic conditions (bifidobacteria 72 h and enterococci and staphylococci 24 h, aerobic incubation) at the temperatures listed in Table S4, the MICs were read visually as the concentration at which growth inhibition occurred. Breakpoint values were adopted from the EFSA guidelines (7) or other published guidelines (e.g., CLSI M100-ED31, EUCAST 2021) or data (see Table S6) for antibiotics not covered by EFSA. *Lacticaseibacillus paracasei* ATCC 334, *Lactiplantibacillus plantarum* ATCC 14917, *B. longum* ATCC 15707, *E. faecalis* ATCC 29212, *E. faecalis* ATCC 51299, and *L. lactis* ATCC 19435 were used as quality control strains.

Whole-genome sequencing and assembly

The genomes of 75 bacterial strains (Table S7) were sequenced on the Illumina MiSeq platform (v3) using the Illumina TruSeq Nano library (300-bp paired-end module, Microsynth) or the Nextera XT DNA library (250-bp paired-end module, National Laboratory of Health, Environment and Food), whereas others were retrieved from public databases (GenBank accession numbers are listed in Table S2).

Quality control and trimming and filtering of raw reads were done using FastQC 0.11.9 (Babraham Bioinformatics) and TrimmomaticPE 0.39 (parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:28 MINLEN:21) (62), respectively, whereas paired-end reads were merged using FLASh 1.2.11 (parameters: –min-overlap=15) (63). The resulting high-quality reads were assembled de novo using SPAdes 3.14.0 (64) with the –careful command option. To improve genome assembly, the protocol was adjusted for some strains as indicated in Table S7. QUAST 5.0.2 (65) was used to inspect the assembly statistics. Genomes were annotated using Prokka 1.14.6 (66), the level of contamination was determined using Mash Screen 2.0 (67), and plasmids were reconstructed using MOB-suite 1.4.9 (68). A total of 2 × 66,019,708 reads were obtained. On average, 2 × 785,691 reads of 300 bp length and 2 × 1,179,891 reads of 250 bp length were retrieved per genome, giving an average genome coverage of 148× and 172×, respectively.

The whole-genome sequencing data generated in this study have been submitted to the European Molecular Biology Laboratory under the project accession PRJEB49530.

Sequence analysis

The ARG database consisted of five publicly available databases (CARD 3.0.8 (69), ResFinder v. 2020-02-11 (70), ARG-ANNOT V6 (71), KEGG (v. November 2017) (72), and NCBI's Bacterial Antimicrobial Resistance Reference Gene Database (73)). Redundancy was removed using CD-HIT 4.7 (parameter -c 0.99) (74). In addition, the following ARG sequences were added: EfrB (accession number WP_172504673.1), bifidobacterial aminoglycoside phosphotransferases (ABE95342.1, ABE96255.1), EfmM (ADI87521.1), LmrC (WP_166668045.1), and CAD-1 (AAV65950.1).

Genome sequences were employed to query the joint ARG database with the local version of the BLAST tool (v. 2.10.0+, parameters -evalue 1e-10, -max_target_seqs 10, query coverage ≥ 60%) using a custom script. *E. faecium* DO (accession number NZ_ACIY01000000) was used as a positive control. A gene was annotated as an ARG on the basis of the best BLAST hit with a sequence similarity threshold greater than 70%. The BLAST search criterion was selected in the way to minimise the detection rate of false positives at the expense of the true positives with lower similarities based on the BLAST alignment of the ARGs database against a test dataset SwissProt (EMBL-EBI) (Table S8) as described by Hu et al (75). The ARGs discovered by BLAST were also validated with hmmsearch (HMMER3 3.1b2, parameter -E 1e-70) (76) and hidden Markov models (v. 2020-05-13) (77). The intrinsic and acquired nature of ARGs was determined with the aid of MGE prediction and pan-genome analyses. The pan- and core-genomes were computed using Roary 3.13.0 (78). In addition to the sequenced genomes, WGS were obtained from public databases and quality-checked before the analyses. QUAST was used to extract genome statistics, Mash Screen to estimate contamination, and pyani to verify taxonomic affiliation.

Genes that had a BLAST similarity threshold between 40% and 70% (BLAST data) or an E-value less than 1E-70 (HMMER data) and matched with the observed phenotype were considered as candidate ARGs. Phylogenetic analyses of these genes were conducted using RAxML-HPC v.8 (parameters: -f a -N 100 -m PROTGAMMAAUTO -p 12345 -× 12345) (79) and CompareM 0.1.1 (80) was used to calculate average amino acid identity. All-to-all BLAST results of the discovered (candidate) ARGs were filtered and clustered into groups that indicate similar functions using a custom script and mcl (v. 14-137) (The University of Utrecht). Multiple genome alignments were constructed by progressiveMauve (81), whereas protein domain analysis was performed using the Pfam database 33.1 and HMMER3 (hmmsearch, -E 1e-10). To examine mutations in proteins previously reported to be involved in resistance (n = 24), multiple sequence alignments were generated with Clustal Ω (EMBL-EBI). Subsequently, mutations were examined manually. To validate coverage and variances of SNPs in the *16S* and *23S rRNA* genes, we have

Table 4. Statistical parameters of genotype–phenotype agreement analysis.

Statistical parameter	Definition
True positive	Phenotypic resistance validated by the genetic analyses (presence of antibiotic resistance gene (ARG) or mutation).
False positive	Phenotypic susceptibility not validated by the genetic analyses (presence of acquired ARG).
True negative	Phenotypic susceptibility validated by the genetic analyses (absence of acquired ARG).
False negative	Phenotypic resistance not validated by the genetic analyses (absence of acquired ARG or mutation).
Positive predictive value	TP/(TP+FP)
Negative predictive value	TN/(TN+FN)
Sensitivity	TP/(TP+FN)
Specificity	TN/(TN+FP)

mapped the sequenced reads to the assembled sequences using Bowtie2 (82).

Phenotype–genotype agreement

A total of 1,314 MICs were considered for phenotype–genotype agreement analysis (Table S2). Genotype and phenotype matched when the susceptible or resistant phenotype reflected the absence or presence of (candidate) ARG(s) or mutation(s), respectively. Sensitivity, specificity, and predictive values of phenotype prediction based on genotypic data were calculated as indicated in Table 4.

Genetic environment of the ARGs

The genetic environment upstream and downstream (15 coding sequences) of the (candidate) ARGs extracted with SeqKit (83) was surveyed for the presence of MGEs by performing a BLAST alignment (query coverage ≥ 80%, similarity cut-off ≥ 80%) of the flanking regions with the MGE database. A custom, comprehensive, non-redundant database of MGEs (285,059 MGE genes) consisted of integrative and conjugative/mobilisable elements, transposons, insertion sequences, plasmids, integrons, prophages, and phage-inducible chromosomal islands retrieved from public databases, including those carrying ARGs in LAB and bifidobacteria. In addition, publicly available specialised databases of MGEs were included: MobilomeDB (insertion sequences, v. September 2016) (84), PlasmidFinder (v. February 2020) (85), ICEBERG 2.0 (v. May 2018) (86), PHASTER (v. August 2019) (87), and SecReT4 (v. September 2019) (88). Additional analyses were performed using tools progressiveMauve, ICEBerg 2.0, PHASTER, and hmmsearch against the Pfam database. Genetic organisation of MGEs was visualised using the snapgene-viewer 5.2.4 (SnapGene) and/or BRIG 0.95 (89).

Data Availability

The whole-genome sequencing data from this publication have been deposited to the European Nucleotide Archive database (https://www.ebi.ac.uk/ena/browser/home) under the project accession PRJEB49530. Databases and codes are available in Figshare at https://doi.org/10.6084/m9.figshare.c.6063839.v2

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/lsa.202201637

Acknowledgements

This research was supported by the Slovenian Research Agency (Ljubljana, Slovenia) through the Young Researchers' Program (grant numbers 6316-1/2017-273 and 603-1/2017-13), the Research Project J4-1769, and the Research Program P4-0097.

Author Contributions

V Rozman: conceptualisation, investigation, methodology, and writing—original draft.
P Mohar Lorbeg: investigation and writing—review and editing.
P Treven: visualisation and writing—review and editing.
T Accetto: software and writing—review and editing.
S Janežič: investigation and writing—review and editing.
M Rupnik: conceptualisation, funding acquisition, and writing—review and editing.
B Bogovič Matijašić: conceptualisation, funding acquisition, and writing—review and editing.

Conflict of Interest Statement

The authors declare that they have no conflict of interest.

References

1. World Health Organisation (2011) *Tackling Antibiotic Resistance from a Food Safety Perspective in Europe*. Copenhagen: WHO Regional Office for Europe.

2. Duranti S, Lugli GA, Mancabelli L, Turroni F, Milani C, Mangifesta M, Ferrario C, Anzalone R, Viappiani A, van Sinderen D, et al (2017) Prevalence of antibiotic resistance genes among human gut-derived bifidobacteria. *Appl Environ Microbiol* 83: e02894-16. doi:10.1128/AEM.02894-16

3. Zaheer R, Cook SR, Barbieri R, Goji N, Cameron A, Petkau A, Polo RO, Tymensen L, Stamm C, Song J, et al (2020) Surveillance of *Enterococcus* spp. reveals distinct species and antimicrobial resistance diversity across a One-Health continuum. *Sci Rep* 10: 3937. doi:10.1038/s41598-020-61002-5

4. Campedelli I, Mathur H, Salvetti E, Clarke S, Rea MC, Torriani S, Ross RP, Hill C, O'Toole PW (2018) Genus-wide assessment of antibiotic resistance in *Lactobacillus* spp. *Appl Environ Microbiol* 85: e01738-18. doi:10.1128/aem.01738-18

5. Liu L, Chen X, Skogerbø G, Zhang P, Chen R, He S, Huang DW (2012) The human microbiome: A hot spot of microbial horizontal gene transfer. *Genomics* 100: 265–270. doi:10.1016/j.ygeno.2012.07.012

6. EFSA (2007) Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *EFSA J* 5: 587. doi:10.2903/j.efsa.2007.587

7. EFSA Panel on Additives and Products or Substances used in Animal Feed FEEDAP, Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos MdL, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, et al (2018) Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA J* 16: e05206. doi:10.2903/j.efsa.2018.5206

8. Flórez AB, Mayo B (2017) Antibiotic resistance-susceptibility profiles of *Streptococcus thermophilus* isolated from raw milk and genome analysis of the genetic basis of acquired resistances. *Front Microbiol* 8: 2608. doi:10.3389/fmicb.2017.02608

9. Tyson GH, Sabo JL, Rice-Trujillo C, Hernandez J, McDermott PF (2018) Whole-genome sequencing based characterization of antimicrobial resistance in *Enterococcus*. *Pathog Dis* 76: fty018. doi:10.1093/femspd/fty018

10. Deng F, Chen Y, Zhou X, Xiao H, Sun T, Deng Y, Wen J (2021) New insights into the virulence traits and antibiotic resistance of enterococci isolated from diverse probiotic products. *Microorganisms* 9: 726. doi:10.3390/microorganisms9040726

11. Rozman V, Mohar Lorbeg P, Accetto T, Bogovič Matijašić B (2020) Characterization of antimicrobial resistance in lactobacilli and bifidobacteria used as probiotics or starter cultures based on integration of phenotypic and *in silico* data. *Int J Food Microbiol* 314: 108388. doi:10.1016/j.ijfoodmicro.2019.108388

12. EFSA-FEEDAP (2012) Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J* 10: 2740. doi:10.2903/j.efsa.2012.2740

13. Werner G, Coque TM, Franz CMAP, Grohmann E, Hegstad K, Jensen L, van Schaik W, Weaver K (2013) Antibiotic resistant enterococci - tales of a drug resistance gene trafficker. *Int J Med Microbiol* 303: 360–379. doi:10.1016/j.ijmm.2013.03.001

14. Oh TG, Kwon AR, Choi EC (1998) Induction of *ermAMR* from a clinical strain of *Enterococcus faecalis* by 16-membered-ring macrolide antibiotics. *J Bacteriol* 180: 5788–5791. doi:10.1128/jb.180.21.5788-5791.1998

15. Stallings CL, Chu L, Li LX, Glickman MS (2011) Catalytic and non-catalytic roles for the mono-ADP-ribosyltransferase Arr in the mycobacterial DNA damage response. *PLoS One* 6: e21807. doi:10.1371/journal.pone.0021807

16. Allocati N, Federici L, Masulli M, Di Ilio C (2012) Distribution of glutathione transferases in Gram-positive bacteria and *Archaea*. *Biochimie* 94: 588–596. doi:10.1016/j.biochi.2011.09.008

17. Aslangul E, Massias L, Meulemans A, Chau F, Andremont A, Courvalin P, Fantin B, Ruimy R (2006) Acquired gentamicin resistance by permeability impairment in *Enterococcus faecalis*. *Antimicrob Agents Chemother* 50: 3615–3621. doi:10.1128/aac.00390-06

18. Isnard C, Malbruny B, Leclercq R, Cattoir V (2013) Genetic basis for *in vitro* and *in vivo* resistance to lincosamides, streptogramins A, and pleuromutilins (LSAP Phenotype) in *Enterococcus faecium*. *Antimicrob Agents Chemother* 57: 4463–4469. doi:10.1128/aac.01030-13

19. Singh KV, Murray BE (2005) Differences in the *Enterococcus faecalis lsa* locus that influence susceptibility to quinupristin-dalfopristin and clindamycin. *Antimicrob Agents Chemother* 49: 32–39. doi:10.1128/aac.49.1.32-39.2005

20. Montealegre MC, Roh JH, Rae M, Davlieva MG, Singh KV, Shamoo Y, Murray BE (2017) Differential Penicillin-Binding Protein 5 (PBP5) levels in the *Enterococcus faecium* clades with different levels of ampicillin resistance. *Antimicrob Agents Chemother* 61: e02034-16. doi:10.1128/aac.02034-16

21. Pietta E, Montealegre MC, Roh JH, Cocconcelli PS, Murray BE (2014) *Enterococcus faecium* PBP5-S/R, the missing link between PBP5-S and PBP5-R. *Antimicrob Agents Chemother* 58: 6978–6981. doi:10.1128/aac.03648-14

22. Dale GE, Broger C, D'Arcy A, Hartman PG, DeHoogt R, Jolidon S, Kompis I, Labhardt AM, Langen H, Locher H, et al (1997) A single amino acid substitution in *Staphylococcus aureus* dihydrofolate reductase determines trimethoprim resistance. *J Mol Biol* 266: 23–30. doi:10.1006/jmbi.1996.0770

23. Cammarata M, Thyer R, Lombardo M, Anderson A, Wright D, Ellington A, Brodbelt JS (2017) Characterization of trimethoprim resistant: *E. coli* dihydrofolate reductase mutants by mass spectrometry and inhibition by propargyl-linked antifolates. *Chem Sci* 8: 4062–4072. doi:10.1039/c6sc05235e

24. Correia S, Poeta P, Hébraud M, Capelo JL, Igrejas G (2017) Mechanisms of quinolone action and resistance: Where do we stand? *J Med Microbiol* 66: 551–559. doi:10.1099/jmm.0.000475

25. Castanheira M, Watters AA, Mendes RE, Farrell DJ, Jones RN (2010) Occurrence and molecular characterization of fusidic acid resistance mechanisms among *Staphylococcus* spp. from European countries (2008). *J Antimicrob Chemother* 65: 1353–1358. doi:10.1093/jac/dkq094

26. Nakata N, Kai M, Makino M (2011) Mutation analysis of the *Mycobacterium leprae folP1* gene and dapsone resistance. *Antimicrob Agents Chemother* 55: 762–766. doi:10.1128/aac.01212-10

27. Delavat F, Miyazaki R, Carraro N, Pradervand N, van der Meer JR (2017) The hidden life of integrative and conjugative elements. *FEMS Microbiol Rev* 41: 512–537. doi:10.1093/femsre/fux008

28. Rosander A, Connolly E, Roos S (2008) Removal of antibiotic resistance gene-carrying plasmids from *Lactobacillus reuteri* ATCC 55730 and characterization of the resulting daughter strain, *L. reuteri* DSM 17938. *Appl Environ Microbiol* 74: 6032–6040. doi:10.1128/aem.00991-08

29. Klare I, Konstabel C, Werner G, Huys G, Vankerckhoven V, Kahlmeter G, Hildebrandt B, Muller-Bertling S, Witte W, Goossens H (2007) Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J Antimicrob Chemother* 59: 900–912. doi:10.1093/jac/dkm035

30. Ma Q, Fu Y, Sun H, Huang Y, Li L, Yu Q, Dinnyes A, Sun Q (2017) Antimicrobial resistance of *Lactobacillus* spp. from fermented foods and human gut. *LWT* 86: 201–208. doi:10.1016/j.lwt.2017.07.059

31. Shani N, Oberhaensli S, Arias-Roth E (2021) Antibiotic susceptibility profiles of *Pediococcus pentosaceus* from various origins and their implications for the safety assessment of strains with food-technology applications. *J Food Prot* 84: 1160–1168. doi:10.4315/jfp-20-363

32. Cao L, Chen H, Wang Q, Li B, Hu Y, Zhao C, Hu Y, Yin Y (2020) Literature-based phenotype survey and *in silico* genotype investigation of antibiotic resistance in the genus *Bifidobacterium*. *Curr Microbiol* 77: 4104–4113. doi:10.1007/s00284-020-02230-w

33. Rozman V, Mohar Lorbeg P, Treven P, Accetto T, Golob M, Zdovc I, Bogovic Matijasic B (2022) Lactic acid bacteria and bifidobacteria deliberately

introduced into the agro-food chain do not significantly increase the antimicrobial resistance gene pool. *Gut Microbes* 14: 2127438. doi:10.1080/19490976.2022.2127438

34. Martínez N, Luque R, Milani C, Ventura M, Banuelos O, Margolles A (2018) A gene homologous to rRNA methylase genes confers erythromycin and clindamycin resistance in *Bifidobacterium breve*. *Appl Environ Microbiol* 84: e02888-17. doi:10.1128/aem.02888-17
35. de Vries LE, Vallès Y, Agersø Y, Vaishampayan PA, Garcia-Montaner A, Kuehl JV, Christensen H, Barlow M, Francino MP (2011) The gut as reservoir of antibiotic resistance: Microbial diversity of tetracycline resistance in mother and infant. *PLoS One* 6: e21644. doi:10.1371/journal.pone.0021644
36. Roberts AP, Mullany P (2011) Tn*916*-like genetic elements: A diverse group of modular mobile elements conferring antibiotic resistance. *FEMS Microbiol Rev* 35: 856–871. doi:10.1111/j.1574-6976.2011.00283.x
37. Pärnänen K, Karkman A, Hultman J, Lyra C, Bengtsson-Palme J, Larsson DGJ, Rautava S, Isolauri E, Salminen S, Kumar H, et al (2018) Maternal gut and breast milk microbiota affect infant gut antibiotic resistome and mobile genetic elements. *Nat Commun* 9: 3891. doi:10.1038/s41467-018-06393-w
38. Teuber M, Schwarz F, Perreten V (2003) Molecular structure and evolution of the conjugative multiresistance plasmid pRE25 of *Enterococcus faecalis* isolated from a raw-fermented sausage. *Int J Food Microbiol* 88: 325–329. doi:10.1016/s0168-1605(03)00195-8
39. Ridenhour MB, Fletcher HM, Mortensen JE, Daneo-Moore L (1996) A novel tetracycline-resistant determinant, *tet(U)*, is encoded on the plasmid pKQ10 in *Enterococcus faecium*. *Plasmid* 35: 71–80. doi:10.1006/plas.1996.0009
40. Novais C, Freitas AR, Silveira E, Baquero F, Peixe L, Roberts AP, Coque TM (2012) Different genetic supports for the *tet(S)* gene in enterococci. *Antimicrob Agents Chemother* 56: 6014–6018. doi:10.1128/aac.00758-12
41. Martínez-Rubio R, Quiles-Puchalt N, Martí M, Humphrey S, Ram G, Smyth D, Chen J, Novick RP, Penades JR (2017) Phage-inducible islands in the Gram-positive cocci. *ISME J* 11: 1029–1042. doi:10.1038/ismej.2016.163
42. Zommiti M, Feuilloley MGJ, Connil N (2020) Update of probiotics in human world: A nonstop source of benefactions till the end of time. *Microorganisms* 8: 1907. doi:10.3390/microorganisms8121907
43. Kondo J, Koganei M (2020) Structural bases for the fitness cost of the antibiotic-resistance and lethal mutations at position 1408 of 16S rRNA. *Molecules* 25: 159. doi:10.3390/molecules25010159
44. Recht MI, Fourmy D, Blanchard SC, Dahlquist KD, Puglisi JD (1996) RNA sequence determinants for aminoglycoside binding to an A-site rRNA model oligonucleotide. *J Mol Biol* 262: 421–436. doi:10.1006/jmbi.1996.0526
45. Brodersen DE, Clemons WM, Carter AP, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V (2000) The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B, on the 30S ribosomal subunit. *Cell* 103: 1143–1154. doi:10.1016/s0092-8674(00)00216-6
46. Descours G, Ginevra C, Jacotin N, Forey F, Chastang J, Kay E, Etienne J, Lina G, Doublet P, Jarraud S (2017) Ribosomal mutations conferring macrolide resistance in *Legionella pneumophila*. *Antimicrob Agents Chemother* 61: e02188-16. doi:10.1128/aac.02188-16
47. Canu A, Malbruny B, Coquemont M, Davies TA, Appelbaum PC, Leclercq R (2002) Diversity of ribosomal mutations conferring resistance to macrolides, clindamycin, streptogramin, and telithromycin in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 46: 125–131. doi:10.1128/aac.46.1.125-131.2002
48. Furneri PM, Rappazzo G, Musumarra MP, Di Pietro P, Catania LS, Roccasalva LS (2001) Two new point mutations at A2062 associated with resistance to 16-membered macrolide antibiotics in mutant strains of *Mycoplasma hominis*. *Antimicrob Agents Chemother* 45: 2958–2960. doi:10.1128/aac.45.10.2958-2960.2001
49. Flórez AB, Ladero V, Álvarez-Martín P, Ammor MS, Alvarez MA, Mayo B (2007) Acquired macrolide resistance in the human intestinal strain *Lactobacillus rhamnosus* E41 associated with a transition mutation in *23S rRNA* genes. *Int J Antimicrob Agents* 30: 341–344. doi:10.1016/j.ijantimicag.2007.06.002
50. Grossman TH (2016) Tetracycline antibiotics and resistance. *Cold Spring Harb Perspect Med* 6: a025387. doi:10.1101/cshperspect.a025387
51. Dégrange S, Renaudin H, Charron A, Pereyre S, Bebear C, Bebear CM (2008) Reduced susceptibility to tetracyclines is associated *in vitro* with the presence of 16S rRNA mutations in *Mycoplasma hominis* and *Mycoplasma pneumoniae*. *J Antimicrob Chemother* 61: 1390–1392. doi:10.1093/jac/dkn118
52. Ballif M, Harino P, Ley S, Coscolla M, Niemann S, Carter R, Coulter C, Borrell S, Siba P, Phuanukoonnon S, et al (2012) Drug resistance-conferring mutations in *Mycobacterium tuberculosis* from Madang, Papua New Guinea. *BMC Microbiol* 12: 191. doi:10.1186/1471-2180-12-191
53. Kiwaki M, Sato T (2009) Antimicrobial susceptibility of *Bifidobacterium breve* strains and genetic analysis of streptomycin resistance of probiotic *B. breve* strain Yakult. *Int J Food Microbiol* 134: 211–215. doi:10.1016/j.ijfoodmicro.2009.06.011
54. Nishimura K, Hosaka T, Tokuyama S, Okamoto S, Ochi K (2007) Mutations in *rsmG*, encoding a 16S rRNA methyltransferase, result in low-level streptomycin resistance and antibiotic overproduction in *Streptomyces coelicolor* A3(2). *J Bacteriol* 189: 3876–3883. doi:10.1128/jb.01776-06
55. Wong SY, Lee JS, Kwak HK, Via LE, Boshoff HIM, Barry CE (2011) Mutations in *gidB* confer low-level streptomycin resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 55: 2515–2522. doi:10.1128/AAC.01814-10
56. European Food Safety Authority EFSA (2021) EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain. *EFSA J* 19: e06506. doi:10.2903/j.efsa.2021.6506
57. Tušar T, Žerdoner K, Bogovič Matijašić B, Paveljšek D, Benedik E, Bratanič B, Fidler Mis N, Rogelj I (2014) Cultivable bacteria from milk from slovenian breastfeeding mothers. *Food Technol Biotechnol* 52: 242–247.
58. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+: Architecture and applications. *BMC Bioinformatics* 10: 421. doi:10.1186/1471-2105-10-421
59. Richter M, Rosselló-Móra R (2009) Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A* 106: 19126–19131. doi:10.1073/pnas.0906412106
60. Pritchard L, Glover RH, Humphris S, Elphinstone JG, Toth IK (2016) Genomics and taxonomy in diagnostics for food security: Soft-rotting enterobacterial plant pathogens. *Anal Methods* 8: 12–24. doi:10.1039/c5ay02550h
61. International Organization for Standardization (2010) *ISO 10932: Milk and Milk Products - Determination of the Minimal Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-enterococcal Lactic Acid Bacteria (LAB)*. Switzerland: International Organization for Standardization and International Dairy Federation: 31.
62. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics* 30: 2114–2120. doi:10.1093/bioinformatics/btu170
63. Magoč T, Salzberg SL (2011) FLASH: Fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27: 2957–2963. doi:10.1093/bioinformatics/btr507
64. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, et al (2012) SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 19: 455–477. doi:10.1089/cmb.2012.0021
65. Gurevich A, Saveliev V, Vyahhi N, Tesler G (2013) QUAST: Quality assessment tool for genome assemblies. *Bioinformatics* 29: 1072–1075. doi:10.1093/bioinformatics/btt086

66. Seemann T (2014) Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 30: 2068–2069. doi:10.1093/bioinformatics/btu153

67. Ondov BD, Treangen TJ, Melsted P, Mallonee AB, Bergman NH, Koren S, Phillippy AM (2016) Mash: Fast genome and metagenome distance estimation using MinHash. *Genome Biol* 17: 132. doi:10.1186/s13059-016-0997-x

68. Robertson J, Nash JHE (2018) MOB-Suite: Software tools for clustering, reconstruction and typing of plasmids from draft assemblies. *Microb Genomics* 4: e000206. doi:10.1099/mgen.0.000206

69. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma AN, et al (2017) CARD 2017: Expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res* 45: D566–D573. doi:10.1093/nar/gkw1004

70. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV (2012) Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 67: 2640–2644. doi:10.1093/jac/dks261

71. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain JM (2014) ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob Agents Chemother* 58: 212–220. doi:10.1128/aac.01310-13

72. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M (2016) KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Res* 44: D457–D462. doi:10.1093/nar/gkv1070

73. Feldgarden M, Brover V, Haft DH, Prasad AB, Slotta DJ, Tolstoy I, Tyson GH, Zhao S, Hsu CH, McDermott PF, et al (2019) Validating the AMRFINder tool and resistance gene database by using antimicrobial resistance genotype-phenotype correlations in a collection of isolates. *Antimicrob Agents Chemother* 63: e00483-19. doi:10.1128/aac.00483-19

74. Huang Y, Niu B, Gao Y, Fu L, Li W (2010) CD-HIT Suite: A web server for clustering and comparing biological sequences. *Bioinformatics* 26: 680–682. doi:10.1093/bioinformatics/btq003

75. Hu Y, Yang X, Qin J, Lu N, Cheng G, Wu N, Pan Y, Li J, Zhu L, Wang X, et al (2013) Metagenome-wide analysis of antibiotic resistance genes in a large cohort of human gut microbiota. *Nat Commun* 4: 2151. doi:10.1038/ncomms3151

76. Eddy SR (2011) Accelerated profile HMM searches. *PLoS Comput Biol* 7: e1002195. doi:10.1371/journal.pcbi.1002195

77. Sayers EW, Beck J, Bolton EE, Bourexis D, Brister JR, Canese K, Comeau DC, Funk K, Kim S, Klimke W, et al (2021) Database resources of the national center for biotechnology information. *Nucleic Acids Res* 49: D10–D17. doi:10.1093/nar/gkaa892

78. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MT, Fookes M, Falush D, Keane JA, Parkhill J (2015) Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31: 3691–3693. doi:10.1093/bioinformatics/btv421

79. Stamatakis A (2014) RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1312–1313. doi:10.1093/bioinformatics/btu033

80. Parks D (2016) *CompareM*. Github. Available at: https://github.com/donovan-h-parks/CompareM

81. Darling AE, Mau B, Perna NT (2010) progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5: e11147. doi:10.1371/journal.pone.0011147

82. Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. *Nat Methods* 9: 357–359. doi:10.1038/nmeth.1923

83. Shen W, Le S, Li Y, Hu F (2016) SeqKit: A cross-platform and ultrafast toolkit for FASTA/Q file manipulation. *PLoS One* 11: e0163962. doi:10.1371/journal.pone.0163962

84. Li J, Tai C, Deng Z, Zhong W, He Y, Ou HY (2018) VRprofile: Gene-cluster-detection-based profiling of virulence and antibiotic resistance traits encoded within genome sequences of pathogenic bacteria. *Brief Bioinform* 19: 566–574. doi:10.1093/bib/bbw141

85. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Moller Aarestrup F, Hasman H (2014) *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 58: 3895–3903. doi:10.1128/aac.02412-14

86. Liu M, Li X, Xie Y, Bi D, Sun J, Li J, Tai C, Deng Z, Ou HY (2019) ICEberg 2.0: An updated database of bacterial integrative and conjugative elements. *Nucleic Acids Res* 47: D660–D665. doi:10.1093/nar/gky1123

87. Arndt D, Grant JR, Marcu A, Sajed T, Pon A, Liang Y, Wishart DS (2016) PHASTER: A better, faster version of the PHAST phage search tool. *Nucleic Acids Res* 44: W16–W21. doi:10.1093/nar/gkw387

88. Bi D, Liu L, Tai C, Deng Z, Rajakumar K, Ou HY (2013) SecReT4: A web-based bacterial type IV secretion system resource. *Nucleic Acids Res* 41: D660–D665. doi:10.1093/nar/gks1248

89. Alikhan NF, Petty NK, Ben Zakour NL, Beatson SA (2011) BLAST ring image generator (BRIG): Simple prokaryote genome comparisons. *BMC Genomics* 12: 402. doi:10.1186/1471-2164-12-402