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Shotgun metagenomic sequencing of bulk tank milk filters reveals the role of Moraxellaceae and Enterobacteriaceae as carriers of antimicrobial resistance genes.

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1. Introduction

The rise in antimicrobial resistance has recently been defined as the most likely cause of the next pandemic (FAO, 2021). Antimicrobial resistance is recognized globally as one of the most serious threats to public health, with significant adverse outcomes for human and animal health and for the agriculture and food sectors (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2021). Addressing this serious threat requires continuous and deep-level surveillance of antimicrobial usage and emergence of resistance through a one-health approach, whilst considering human, animal, and environmental reservoirs (Lammie & Hughes, 2016). In this context, the high use of antimicrobial compounds in food-producing animals has attracted particular attention, as it is known that the use and the misuse of antibiotics can exert a selective pressure on bacteria driving the emergence of resistance in both pathogens and commensal bacteria alike (Call et al., 2008; Cuccato et al., 2021). The genetic reservoirs of antimicrobial resistance (AMR) genes include both commensal and pathogenic bacteria;

the potential transmission of resistant isolates from food-producing animals to humans *via* direct contact or *via* the food chain is therefore considered a risk of the utmost importance (Call et al., 2008).

Antibiotics are extensively used to treat bacterial infections in cattle production systems; bovine mastitis, in particular, is recognized as the root cause of antimicrobial compound use in dairy farms worldwide, leading to considerable financial losses for farmers and the potential selection and introduction of AMR-encoding genes and associated bacteria in milk production environments and dairy products (Nobrega et al., 2018). Among the indicators of intramammary gland infection, an increased somatic cell count (SCC) in milk is considered a prognostic indicator. According to the International Dairy Federation guidelines, the optimal SCC value to distinguish between infected and uninfected cows is around 200,000 cells/ml, while the Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 has established 400,000 SCC as the cut-off point to consider the milk suitable for human consumption. In the context of growing AMR use, the interest in raw milk, which has already proven to pose a risk for the consumer due to the possible presence of foodborne pathogens, is a cause for concern (Claeys et al., 2013).

Although AMR is not limited to pathogenic bacteria, AMR surveillance is usually concentrated on a modest number of bacterial genera of importance to public health (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2021). As resistance can be intrinsic or acquired, emergence and transmission of AMR may occur through different mechanisms, including point mutations in target genes and the horizontal movement of resistance genes. Among the strategies underpinning its occurrence, horizontal gene transfer (HGT) can lead to the lateral exchange of genetic elements between different bacteria, thereby amplifying the risk of AMR gene dissemination (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2021).

Among the different techniques applied to characterize the so called resistome, defined as the totality of all genes encoding AMR in a given sample, Next Generation Sequencing (NGS) technologies are regarded as useful tools to facilitate characterization; as for functional and resistome diversity, shotgun metagenomics enables effective, timesaving and culture-independent exploration of microbial communities in complex samples, thereby overcoming the limitations posed by more traditional culture-dependent methods and targeted genomics (Quince et al., 2017). However, shotgun metagenomic sequencing has some limitations, especially once applied to host-derived samples. Indeed, the advantage of sequencing all DNA present in a sample will also include a large percentage of the host genome, and this is observed in the case of most of the food-related matrices, such as milk and dairy products, containing DNA of bovine origin (Alexa (Oniciuc) et al., 2020; McHugh et al., 2020; Rubiola et al., 2020; Yap et al., 2020). Although a greater sequencing depth can partially solve this problem, it leads to an increase in cost. As our recent pilot study reported, in-line milk filters constitute a cheap and valuable tool to overcome this issue when the target matrix is represented by raw milk, providing an alternative for characterizing the microbiome, the resistome and for the detection of foodborne pathogens at farm level (Čížek et al., 2008; Rubiola et al., 2020). Despite the growing concern related to AMR, few attempts have been made thus far to characterize the resistome associated with dairy cattle production systems through the application of NGS strategies (Alexa (Oniciuc) et al., 2020; McHugh et al., 2020; Nikoloudaki et al., 2021). Nevertheless, investigation of samples for the presence of AMR-encoding genes in milk and milk production environments could provide data to estimate the public health risk associated with antimicrobial compound use in the dairy industry, raw milk consumption and raw milk cheese production; indeed, along with potential pathogenic bacteria, raw milk may contain AMR bacteria which can be disseminated to the human gastrointestinal tract (Liu et al., 2020).

Given the current knowledge gap relating to the resistome of milk and its production environment, the aim of the present study was to investigate the resistome of dairy farms with history of different bulk tank SCCs through a shotgun metagenomic sequencing approach, taking advantage of in-line milk filters as promising though yet unexplored tools.

2. Materials and methods

2.1 Farms selection and collection of samples

Farms were selected based on their historical bulk tank SCC data recorded by ARAP (Associazione Regionale Allevatori del Piemonte) reference laboratories on milk collected from dairy farms located in Piedmont (North-West Italy) from January 1st, 2017, to December 31st, 2019. Milk SCCs were analyzed by the fluoro-opto-electronic method according to ISO 13366-2/2006 (European Regulation No. 2019/627). The SCC geometric average was calculated over the three-year period for farms having at least two SCC recorded data per month, thereby identifying three categories of farms - defined as farms with low historical SCC (geometric average ≤ 150,000 cells/mL), farms with mid-level historical SCC (geometric average >150,000 and ≤300,000) and farms with high historical SCC (geometric average > 300,000 cells/mL). The low- and the high-SCC herd groups were used to select 5 dairy farms from each, representing a total of 10 dairy farms. Data on herds size, number of lactating cows, milk production trends and welfare assessment based on ClassyFarm integrated monitoring system (www.classyfarm.it) of the Italian Ministry of Health, including farm management, housing and animal-based measures (ABMs), were also collected.

The samples were collected in May 2020 and 2021 and the sampling procedure included the use of disposable in-line milk filters that were taken from the bulk tank of each selected dairy farm. Milk filters were collected directly from the tank under aseptic conditions, then inserted in sterile

plastic sampling bags (Whirl-Pack, NASCO) and transported in controlled temperature to the Laboratory of Food Inspection - Department of Veterinary Science, University of Turin - where DNA extraction was performed immediately.

2.2 DNA extraction and shotgun metagenomic sequencing

Upon arrival at the laboratory, 10 g of each milk filter were added to 90 ml of sterile buffered saline solution (Ringer's solution, Oxoid, Basingstoke, UK) in a sterile stomacher bag and homogenized for 2 min at 230 rpm in a stomacher (Seward Stomacher Blender 400, London, UK). Total DNA was then extracted from filter homogenates using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), with minor adjustments. Samples were centrifuged for 10 min at 100 × *g* to pellet and discard eukaryotic cells; milk serum was then centrifuged at 13,000 × g for 15 min at 4°C to pellet prokaryotic cells and pellets recovered resuspended in phosphate-buffered saline [PBS] (Oxoid Basingstoke, UK). Isolation of genomic DNA was then performed following the manufacturer's protocol; DNA was eluted in 50 µl 10 mM Tris-HCl buffer (pH 8.5) and frozen at −20°C until analyzed. Template DNA of each sample was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) with the Qubit double-stranded DNA (dsDNA) high-sensitivity assay kit. DNA integrity and purity were verified by conventional 2% agarose gel electrophoresis and also using a NanoDrop spectrophotometer (ThermoFisher Scientific, Belgium). Samples meeting quality criteria were submitted for library preparation and subsequent shotgun metagenomic sequencing. DNA library preparation and shotgun metagenomic sequencing steps were performed by Genewiz (Leipzig, Germany). DNA library preparation was conducted according to the NEBnext Ultra II DNA library preparation guide and four PCR cycles were applied to generate libraries (New England Biolabs, Ipswich, MA); samples were sequenced on the Illumina NovaSeq 6000 platform to generate

2 × 150 bp paired-end (PE) reads. The required sequencing depth for each sample was 50 million PE reads.

2.3 Bioinformatics pipelines

Raw read sequencing data quality control was carried out using FastQC v.0.11.9 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and MultiQC v1.11 (Ewels et al., 2016) with default parameters. Raw reads were quality-trimmed using Trimmomatic version 0.39 (Bolger et al., 2014) (leading, 3; trailing, 3; slidingwindow, 4:20; minlen, 36), removing low-quality regions, adaptor sequences and sequencing primers. After the quality filtering step, clean reads were aligned using Bowtie2 v.2.4.4 (Langmead & Salzberg, 2012) against the *Bos taurus* ARS-UCD1.2 bovine reference genome (NCBI genome database), to remove host DNA sequences. Unmapped reads were then used for the downstream analysis.

Host-filtered reads were aligned against the AMR databases MEGARes v.1.0.1 (Lakin et al., 2017), the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020), ARG-ANNOT (Gupta et al., 2014), and ResFinder 4.1 (Zankari et al., 2012) using the Burrows Wheeler Aligner (BWA-MEM) (Li, 2013) with default settings to perform reads-based resistome characterization; bam-files containing mapped reads were generated with SAMtools v1.10 (Li et al., 2009) and paired reads were extracted from the bam-files with the Coverage Sampler tool (https://github.com/cdeanj/coveragesampler) using an 80% gene fraction threshold (Zaheer et al., 2018). Counts of short reads aligned to the AMR genes were recorded and redundant genes were manually removed. Read counts originating from alignments to housekeeping genes associated with AMR requiring single nucleotide polymorphism (SNP) confirmation were filtered out before further analyses. Reads predicted to encode AMR were taxonomically classified using Kraken2 v2.1.2 (Wood

et al., 2019). Taxonomic classification of host-filtered reads was assigned by mapping the reads against the MiniKraken2 v1 reference database using Kraken2; the package Bracken was then used on Kraken's reports to re-estimate species abundance (threshold=10) (Lu et al., 2017). AMR and microbial count tables were normalized using the cumulative sum scaling (CSS) method (Paulson et al., 2013). The pairwise Spearman's correlation test was applied to investigate correlations between detected AMR determinants and identified microbial species with a relative abundance > 0.99 % (R software, version 3.5.1). Additionally, Spearman's correlation test was performed in order to assess any correlation between the presence of AMR genes, the SCCs herd group and the sampling year ($p < 0.05$).

Host-filtered reads were assembled *de novo* using IDBA-UD v1.1.3 (Peng et al., 2012); the generated scaffolds were quality-checked using CheckM v1.1.3 (Parks et al., 2015) and used for screening for AMR genes using ABRicate tool (https://github.com/tseemann/abricate) against MEGARes v1.0.1, CARD, ARG-ANNOT, NCBI and ResFinder (Camacho et al., 2009) databases with a coverage > 80% and a minimum identity threshold set to 80%; multiple hits against the same AMR gene were regarded as one hit due to the fragmented nature of the metagenome assemblies, and redundant genes were manually removed (Esaiassen et al., 2018). MOB-typer and MOB-recon tool from MOB-suite v.1.4.9 (Robertson & Nash, 2018) were used on scaffolds longer than 500 bp to predict plasmid sequences and mobility from single-sample assemblies and each plasmid FASTA file generated by MOB-suite was screened for the presence of AMR genes using ABRicate tool as previously described. HGT events were identified in metagenomic contigs using WAAFLE (https://huttenhower.sph.harvard.edu/waafle/). Single-sample metagenomic assemblies generated with IDBA-UD were used for the reconstruction of microbial genomes. Contigs longer than 1,500 nt were binned with MetaBAT2 v2.12.1 (Kang et al., 2019). Coverage information was generated by mapping quality trimmed reads from individual samples to the contigs from each assembly using

Bowtie2 v2.3.5 and SAMtools v1.7. Multiple bins recovered were quality-assessed with CheckM in order to assess completeness and the presence of contamination; low-quality metagenome-assembled genomes (MAGs) (<50% completeness and/or >10% contamination) were excluded from further analysis. Taxonomic assignment of MAGs was performed with CAT/BAT tool v5.2.3 (von Meijenfeldt et al., 2019). Retained MAGs were used for screening for AMR genes, plasmids and HGT events using ABRicate, MOB-suite and WAAFLE tools as previously described. The overall workflow is shown in Figure 1.

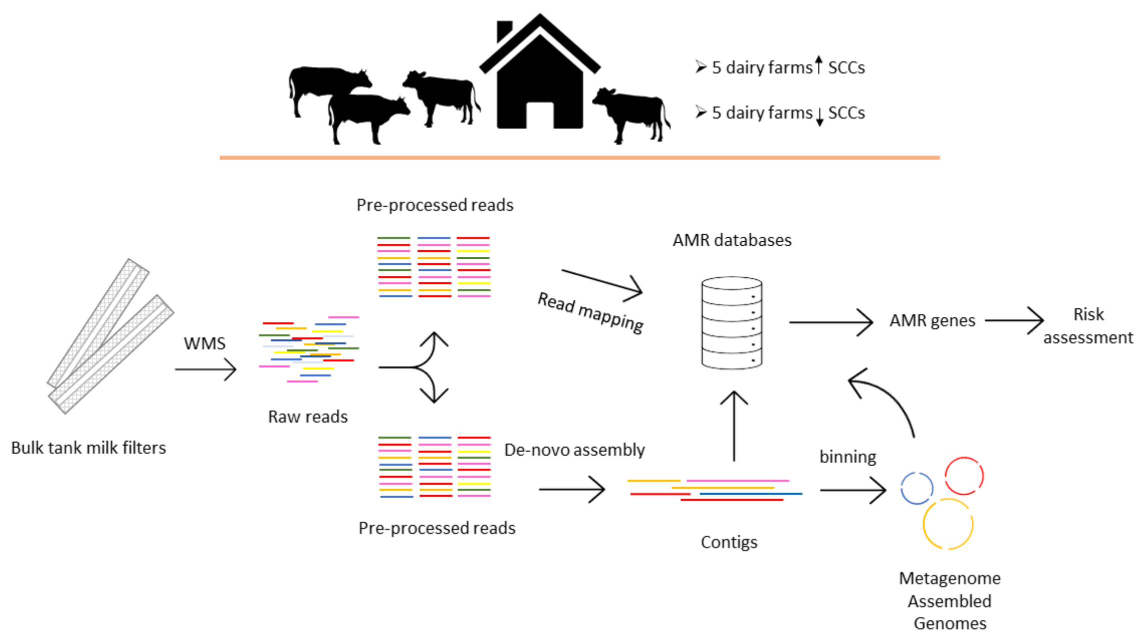


Figure 1. Illustration showing the overall workflow of this study.

3. Results

The study area selected to enroll the dairy farms comprised the neighboring Provinces of Turin and Cuneo, located in the southwest and in the northeast part of the Piedmont region (North-West Italy);

each dairy farm was separated by up to ∼100 km from others. The mean herd size was 237 cows (range 143-382 cows), while lactating herds ranged between 70 and 166 cows, with a mean lactating herd size of 106 cows at the times of sampling and a mean herd level production of 30,3 kg milk/cow (range 22-39 kg). All selected farms raised Friesian cattle and had a high level of animal welfare based on the ClassyFarm integrated monitoring system (www.classyfarm.it) of the Italian Ministry of Health (Ventura et al., 2021).

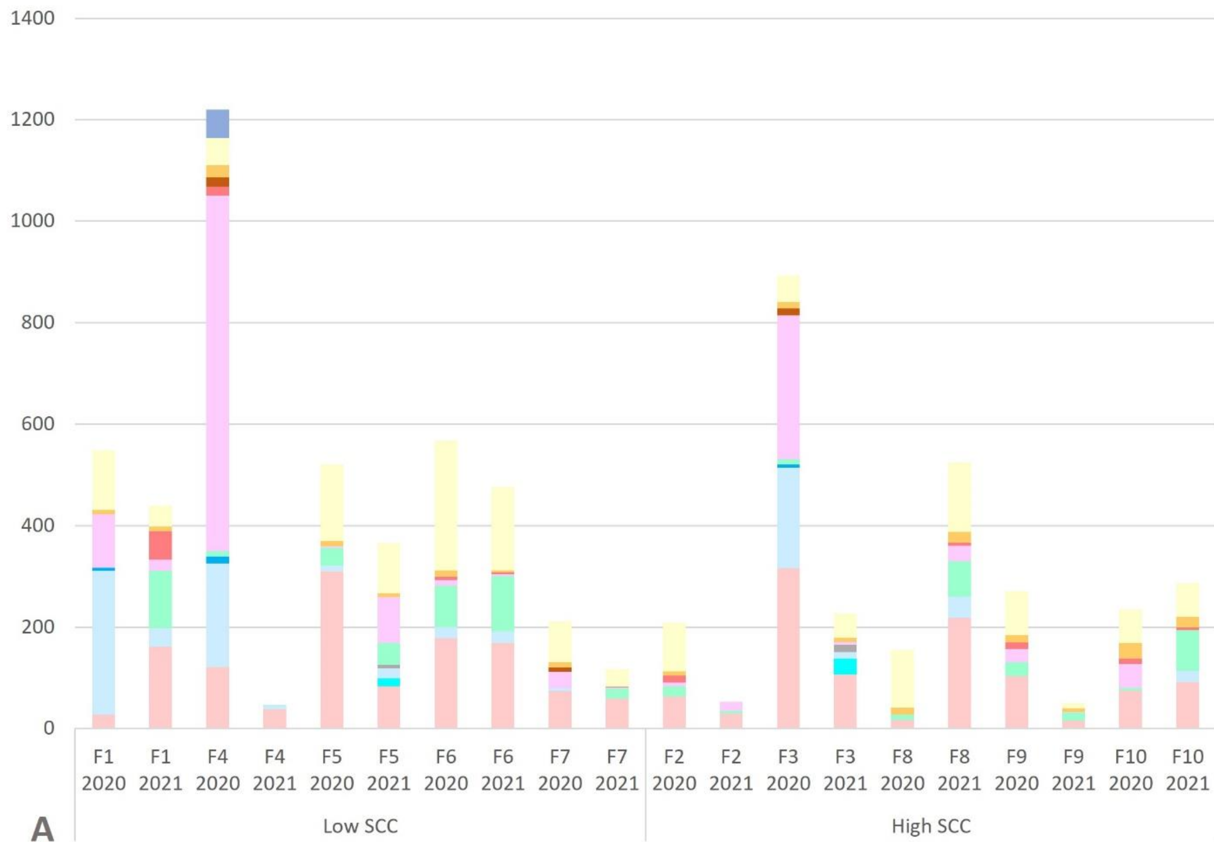
3.1 Shotgun metagenomic sequencing

Shotgun metagenomic sequencing yielded 1.06 billion reads, with an average of 53.1 million reads per sample (range 44.8-76.8 M, Supplementary File S1). The mean read quality score for samples ranged from 35 to 36. Trimming resulted in removal of 2.4-3.5% reads per sample, while the percentage of reads associated with the bovine reference genome ranged from 91.3% to 98.8% across all samples (Supplementary File S1). Out of 29.9 million remaining reads, 6.2 million were identified at the bacterial and archaeal phyla level (20.7%).

3.2 Resistome characterization: reads-based approach

Across all sample datasets, 29,162 reads (0,47% of microbial reads, 0,003% of total reads) were successfully aligned to the manually curated AMR databases including MEGARes, CARD, ARG-ANNOT and ResFinder, with an average count per sample of 1,458 reads (range 74 – 4,828). A total of 160 individual AMR genes were identified and assigned to 39 AMR mechanisms and 12 AMR classes; the median number of unique AMR genes identified per sample was 20 across all sample datasets (range 5 - 51), 25 across the low SCCs farm group samples (range 5 - 51) and 16 across the high SCCs farm group samples (range 5 - 35). The resistome profiles were broadly similar across all samples with

aminoglycoside (n=29), β-lactam (n=27), tetracycline (n=20), multidrug (n=47) and macrolide-lincosamide-streptogramin (MLS) (n=14) being the most abundant classes in both high- and low-SCCs farm groups, followed by phenicol (n=9), antimicrobial peptides (AMPs) (n=4), fosfomycin (n=3) and sulfonamide (n=2) classes. Two AMR genes conferring resistance to triclosan (*triA* and *triC*) were identified in the low-SCCs farm group, while two more AMR classes were identified in the high SCCs farm group, corresponding to rifampin (n=1) and fluoroquinolone (n=2) classes (Figure 2). Aminoglycoside O-phosphotransferases, aminoglycoside O-nucleotidyltransferases, tetracycline resistance ribosomal protection proteins, tetracycline resistance major facilitator superfamily MFS efflux pumps, class A beta-lactamases, lincosamide nucleotidyltransferases, and drug and biocide RND efflux pumps were the most abundant AMR mechanisms (Supplementary File S2). The Spearman's correlation test did not highlight any significative correlation between the presence of AMR determinants and the SCCs herd group or the sampling year.



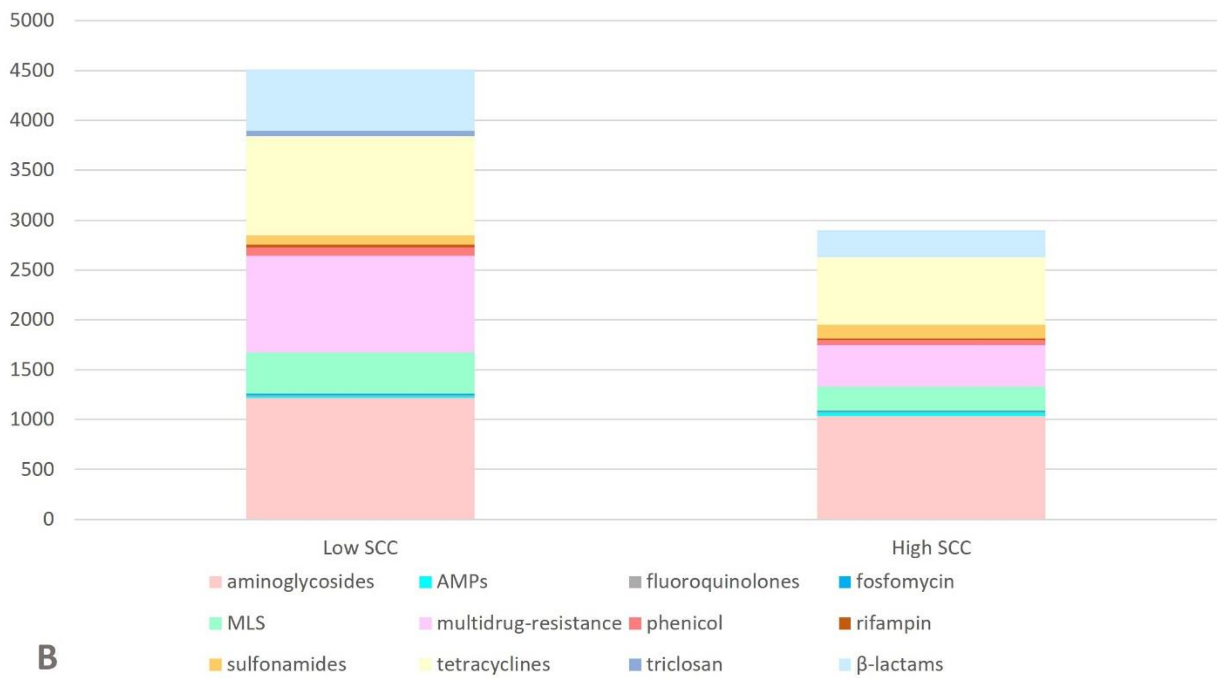


Figure 2. Actual abundance of AMR classes across filter samples corresponding to high and low SCCs farm groups separated (A) and merged (B) after normalization; the y-axis shows the reads per million (RPM).

Of a total 1.06 billion reads generated, 6.19 million reads were identified at the bacterial, archeal and viral phyla level, with an average number of 307,730 reads aligning to phyla per sample (range 61,101 – 726,995). Across all datasets, 37 phyla, 74 classes, 169 orders, 371 families, 1,152 genera and 3,744 species were identified. Proteobacteria (18,2% - 81,4%), Actinobacteria (9,3% - 38,6 %) and Firmicutes (3,1% - 44,6%) were the most abundant phyla, followed by Bacteroidetes (0,7% - 6,7%) with Euryarcheota (0,1% - 3,3%). *Moraxellaceae* (4,3% - 50,3 %), *Enterobacteriaceae* (0,7% - 33,7%), *Streptococcaceae* (0,1% - 31,2 %), *Bifidobacteriaceae* (0,2% - 12,9%), *Corynebacteriaceae* (0,9% - 8%), *Burkholderiaceae* (0,1% - 12,2%), *Pseudomonadaceae* (1% - 13,1%), *Microbacteriaceae* (0,1% - 7%), *Staphylococcaceae* (0,1% - 12,3%) and *Xanthomonadaceae* (0,5% - 7,3%) among the ten most abundant families across all sample datasets. *Acinetobacter* (3% - 49,8%), *Enterobacter* (0,1% - 28,5%), *Bifidobacterium* (0,2% - 12,9%), *Corynebacterium* (0,9% - 8%), *Pseudomonas* (0,8% - 5,6%), *Escherichia* (0,4% - 11,5%), *Lactococcus* (0,1% - 11,2%), *Ralstonia* (0,1% - 8,6%), *Staphylococcus* (0,1% - 11,3%) and *Bradyrhizobium* (0,1% - 6,2%) were the most abundant genera detected.

The associations between the relative abundance of the 160 AMR genes and the 20 most abundant bacterial genera (> 0.99 %) were assessed by Spearman's correlation, revealing the presence of 19 significant interactions ($p<0.05$) among β-lactam, tetracycline, MLS and aminoglycoside resistance-encoding genes and bacteria belonging to the genera *Aerococcus*, *Corynebacterium*, *Ralstonia*, *Bifidobacterium*, *Microbacterium*, *Bradyrhizobium* and *Sphingomonas* (Supplementary File S3).

Some 40,7% of reads predicted to encode AMR were taxonomically assigned at the phylum level, the most abundant originating from Proteobacteria, followed by Firmicutes, Actinobacteria and Bacteroidetes (Figure 3). Only 30,1% of identified resistance determinants could be taxonomically assigned to the genus level, with most originating from Gram-negative bacteria, namely *Enterobacter, Acinetobacter, Escherichia* and *Pseudomonas,* and Gram-positive bacteria, namely *Clostridioides, Staphylococcus* and *Bifidobacterium* (Figure 3).

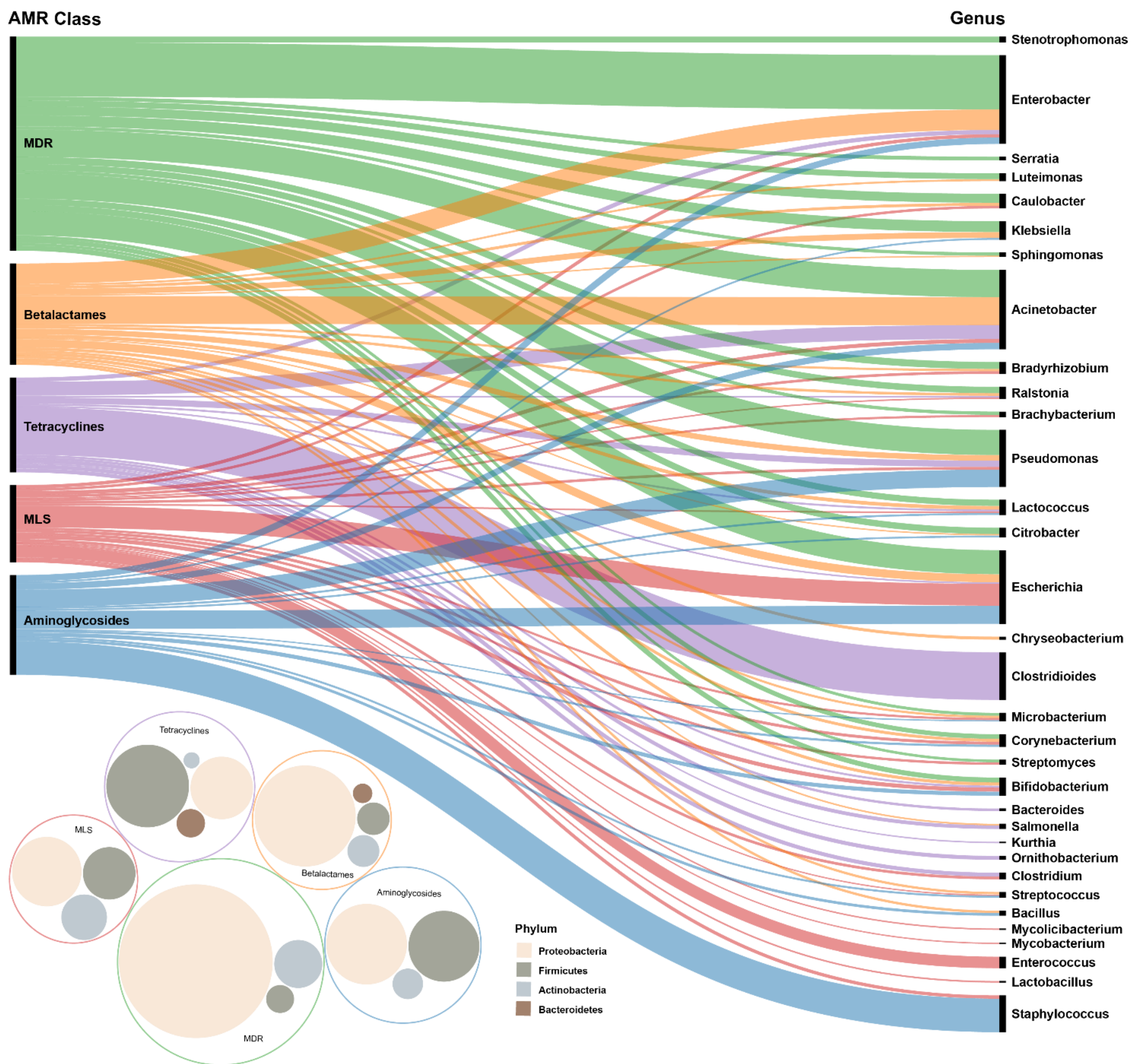


Figure 3. Illustration showing phylum (circle packing) and genus-level (alluvial diagram) classification of AMR genes per AMR class based on kraken2 classification.

3.3 Resistome characterization: assembly-based approach

Single-sample *de novo* assembly of trimmed and host-DNA cleaned reads produced up to 85,118 contigs in each sample; quality parameters of generated assemblies are reported in Supplementary File S4.

The scaffold-based approach allowed the identification of 71 individual AMR-encoding genes across all single-sample assemblies, corresponding to 27 AMR mechanisms and 9 AMR classes, namely aminoglycoside (n=19), multidrug (n=19), β-lactam (n=9), tetracycline (n=9), MLS (n=8), sulfonamide (n=2), fosfomycin (n=2), phenicol (n=2) and AMPs (n=1) classes. All single-sample assemblies harbored at least one AMR gene (range 1 - 30 genes), except for one sample which did not show any AMR determinant. Aminoglycoside O-nucleotidyltransferases (n=12) were the main AMR mechanism detected in most single-sample assemblies, followed by aminoglycoside O-phosphotransferases, tetracycline resistance MFS efflux pumps, lincosamide nucleotidyltransferases, multidrug RND efflux regulator and multidrug RND efflux pumps. The resistome profiles were broadly similar to the annotated AMR genes derived from the reads-based approach (shown above), despite the lower number of AMR genes detected due to the loss of data resulting from *de novo* assembly. As shown by Venn diagrams (Figure 4), 9 AMR classes, 26 AMR mechanisms and 62 AMR genes were identified through both the reads-based and the scaffolds-based approach, while 3 additional AMR classes, 13 AMR mechanisms and 99 AMR genes were identified through the reads-based approach.

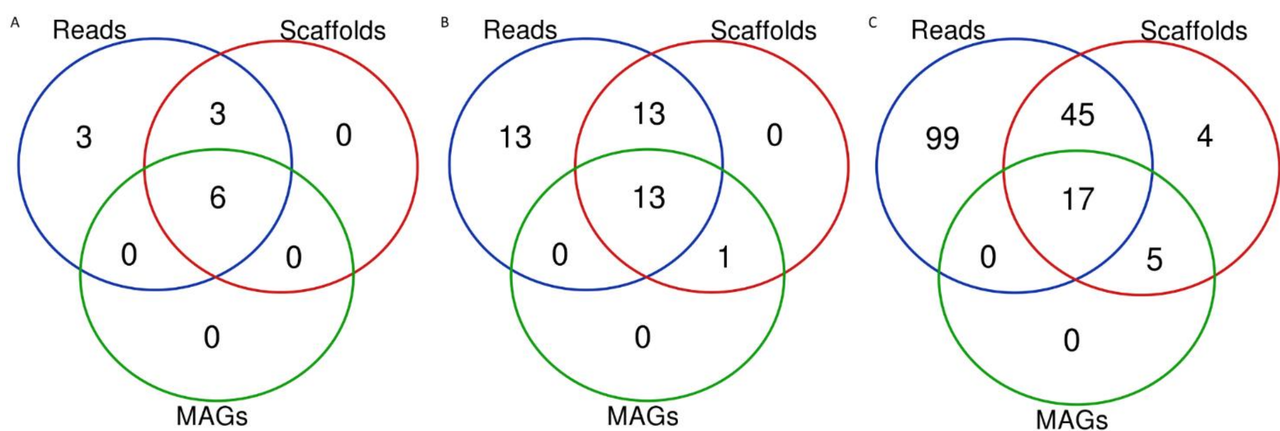


Figure 4. Venn diagrams showing the number of **a)** AMR classes, **b)** AMR mechanisms and **c)** AMR genes shared or unique among the reads-based approach, the scaffolds-based approach and the high-quality MAGs based approach.

Thirty-seven plasmids carrying between 1 and up to 6 AMR-encoding genes were detected across 14 of 20 single-sample assemblies; among them, 14 plasmids were classified as mobilizable by MOB-suite tools (Supplementary File S5). Mobilizable plasmids carried 10 AMR genes belonging to 5 AMR classes, namely aminoglycoside (n=5), tetracycline (n=2), β-lactam (n=1), MLS (n=1) and sulfonamide (n=1) classes. Among mobilizable plasmids, the small plasmid pALWED1.8 (4,135 bp) carrying the streptomycin/spectinomycin resistance gene *aadA27*, whose observed host range includes *Acinetobacter* spp., was detected in nine different samples; plasmid pDJ91S (3,928 bp), carrying the aminoglycoside nucleotidyltransferase *spd* gene, plasmid paadD (7,190 bp), carrying the macrolide phosphotransferase *mphB* gene conferring resistance to erythromycin, spiramycin and telithromycin, plasmid pvSw1 (13,186), carrying the aminoglycoside nucleotidyltransferase *ant6* gene, the multiresistance plasmid p2012N21 (73,223), carrying two streptomycin resistance genes (*strA* and *strB*), the *tet(X3)* and *tetM* genes, conferring resistance to tetracyclines, the *oxa-58* beta-lactamase and the sulfonamide resistant dihydropteroate synthase *sul2* gene, and the multiresistance plasmid pKLH80 (14,835 bp), carrying two streptomycin resistance genes (*strA* and *strB*), were detected in one sample each. MOB-typer output values for mobilizable plasmids, including observed host range and AMR determinants, are reported in Supplementary File S6.

Across 19 of the 20 single-sample assemblies, 144 occurrences of HGT events were identified, most of which occurred between members of the same phylum (Figure 5). The HGT direction could be determined for 7 instances. HGTs were most frequent between members of the phylum Proteobacteria, especially among *Psychrobacter* and *Acinetobacter*. Out of the 144 instances, 26

were identified as transposases, of which one had a determined HGT direction (UniRef90_C8Q1E5), while one was identified as a site-specific recombinase, phage integrase family (UniRef50_E2XJZ7), two were identified as ABC-type transporters (UniRef50_B3DSG6; UniRef90_A0A011TBE8), one was identified as a macrolide 2'-phosphotransferase II (UniRef90_O32553) and one was identified as a beta-lactamase (UniRef90_P30897).

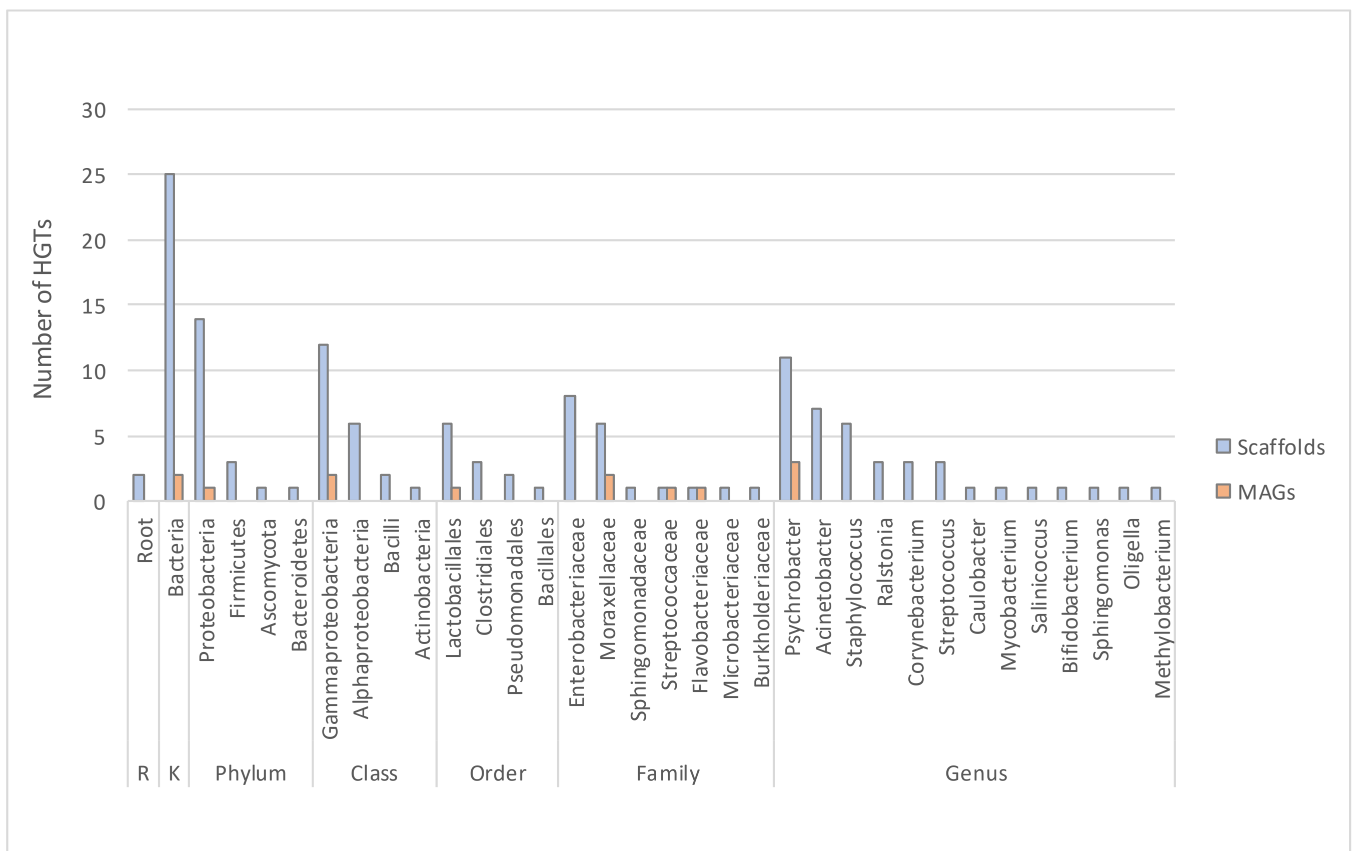


Figure 5. HGTs detected in single-sample assemblies and MAGs; the taxa within which HGTs were detected are reported on the x-axis.

Scaffolds longer than 1,500 bp underwent binning and the *de novo* assembly resulted in 8 high-quality MAGs, whose genome sizes varied from 1.3 to 4.2 Mb. Quality parameters of the generated MAGs are reported in Table 1. Seven MAGs of eight were identified at the genus or family level, including

one *Psychrobacter* sp., two *Acinetobacter* spp., one *Lactococcus* sp., one member of the *Moraxellaceae* family, one member of the *Enterobacteriaceae* family and one member of the *Weeksellaceae* family. The remaining MAG belonged to phylum Gammaproteobacteria (Table 2).

Table 1. Quality parameters of the 8 high quality MAGs gained after binning and single-sample *de novo* assembly.

MAG ID	Sample ID	Genome size	Phylum	GC (%)	N. of scaffolds	Longest scaffold (bp)	N50	Completeness	Contamination	Coding density	Predicted genes
MAG 1	F3 2020	3,018,221	Proteobacteria	38.51	414	80193	11542	91.85	1.05	0.881	3208
MAG 2	F4 2020	1,833,608	Proteobacteria	41.83	522	17074	3934	68.63	4.31	0.854	2062
MAG 3	F4 2020	2,314,343	Firmicutes	34.82	468	26466	6785	91.76	1.35	0.858	2656
MAG 4	F4 2020	4,265,693	Proteobacteria	55.3	562	90260	12840	94.22	1.16	0.902	4412
MAG 5	F5 2020	1,265,479	Bacteroidetes	36.92	505	9344	2491	54.17	1.35	0.89	1597
MAG 6	F10 2021	2,415,712	Proteobacteria	41.45	515	28906	5881	87.34	6.33	0.855	2597
MAG 7	F10 2021	2,390,653	Proteobacteria	44.19	360	35354	9388	88.35	2.44	0.862	2479
MAG 8	F10 2021	2,324,675	Proteobacteria	42.89	354	50554	9367	92.75	0.84	0.833	2155

The MAGs-based approach allowed the identification of 22 AMR genes across seven of eight MAGs, corresponding to 14 AMR mechanisms and 6 AMR classes, including multidrug, β-lactam, aminoglycoside, tetracycline, AMPs and fosfomycin classes. Three matches, related to genes

conferring resistance to β-lactams (*bla*$_{ACT-52}$ and CARB-16) and tetracyclines (*tetH*) had perfect nucleotide coverages and identity, while 20 AMR genes had strict nucleotide matches, with sequences coverage ranging between 90.54% and 100 % and sequences identity ranging between 80.20 and 98.97 %. MAG 4 (highlighted in grey shading) was identified as a member of the *Enterobacteriaceae* family; this genome harbored 11 multidrug-resistance genes, most of which were complete, conferring resistance to diaminopyrimidine, fluoroquinolone, glycylcycline, nitrofuran, tetracycline, carbapenem, cephalosporin, cephamycin, monobactam, penam, penem, phenicol, rifamycin, triclosan, aminocoumarin, aminoglycoside, macrolide and peptide antibiotics, together with three class C beta-lactamases (ACT-9, ACT-28 and ACT-52), one penicillin binding protein (PBP2), one gene conferring resistance to bacitracin (*bacA*) and one gene conferring resistance to Fosfomycin (*fosA2*). While MAG 1 was not annotated to contain any AMR gene, MAG 2 and MAG 7 harbored the multidrug-resistance gene MexT, a transcriptional regulator conferring resistance to phenicol, diaminopyrimidine and fluoroquinolone antimicrobial compounds; MAG 5 and MAG 3 harbored a tetracycline efflux protein linked to the resistance genes *sul2* and *strAB* and a chromosomally-encoded efflux pump that confers resistance to lincosamides, respectively. The gene CARB-16, an IMP beta-lactamase conferring resistance to penems, was annotated in MAG 8. Finally, MAG 6 harbored the complete streptomycin/spectinomycin resistance gene aadA27, a variant of the *aadA* gene identified on the small mobilizable plasmid pALWED1.8 of *Acinetobacter* spp.

The mobilizable plasmid pALWED1.8 carrying the resistance gene *aadA27*, conferring resistance to streptomycin and spectinomycin, was the only plasmid detected on high-quality MAGs (Table 2); the same plasmid gene was previously detected on the corresponding single-sample assembly. The detection of HGTs on MAGs was consistent with the annotation of HGTs from scaffolds (Figure 5). Thirteen occurrences of HGTs were identified across 5 of the 8 MAGs; the direction could be determined for 4 instances. Four instances were identified as transposases (UniRef90_Q1Q7T2,

UniRef50_E6MV57, UniRef90_UPI00029B00C6 and UniRef90_A5EVZ2) and one was identified as a beta-lactamase (UniRef90_P30897).

Table 2. A table showing the taxonomy and identification of AMR genes, plasmids and HGT events in the 8 high-quality MAGs.

MAG id	Taxonomy	AMR genes	Plasmids	HGTs
MAG 1	*Acinetobacter sp.*	0	0	1
MAG 2	Gammaproteobacteria	1	0	1
MAG 3	*Lactococcus sp.*	1	0	3
MAG 4	*Enterobacteriaceae sp.*	17	0	0
MAG 5	*Weeksellaceae sp.*	1	0	1
MAG 6	*Acinetobacter sp.*	1	1	0
MAG 7	*Moraxellaceae sp.*	1	0	0
MAG 8	*Psychrobacter sp.*	1	0	7

4. Discussion

In the context of growing concerns about AMR, a comprehensive resistome characterization of different niches, including food and food-related matrices, has been identified as a knowledge gap. The recent advances in high throughput sequencing technologies are now enabling the fast and untargeted exploration of microbial communities in a wide range of environments, including food. However, as highlighted by some recent studies using host-derived samples, a detailed resistome investigation of food matrices might be challenging due to the large concentration of host DNA encountered, which can be up to 99% of the nucleic acid content for milk and dairy products (Alexa (Oniciuc) et al., 2020; Liu et al., 2020; McHugh et al., 2020; Rubiola et al., 2020; Tóth et al., 2020;

Vasquez et al., 2022; Warder et al., 2021). The microbiome of bulk tank milk is strongly related to the microbiome of the in-line bulk tank milk filter, whose pores, which reach a size of 100–150 µm, are designed to retain debris and large particles of organic material while allowing bacteria to enter the bulk milk. Therefore, the microbiome and the related AMR determinants detected on milk filters can be considered representative of the microbiome and the resistome of milk and milk production environments (Murphy et al., 2005; Rubiola et al., 2020). Here, the use of milk filters has provided the opportunity to overcome the host DNA issue related to the shotgun metagenomic sequencing techniques. In the present study, the resistome of 5 dairy farms with high historical SCCs and 5 dairy farms with low historical SCCs were characterized through the use of 20 disposable milk filters subjected to the reads- and the assembly-based resistome profiling. In this study different AMR determinants were detected in each analyzed sample, showing the absence of a significative correlation between the presence of AMR determinants and the SCCs herd group and demonstrating similar resistomes despite their origin from different farms. The use of historical SCCs as indicators of recurrent mastitis and resultant high antimicrobial compound use has already been investigated (Ruegg & Tabone, 2000); our findings, although focused on a different matrix, are consistent with other studies evaluating the faecal resistome of cattle from conventional and “raised without antibiotics” farms and in cattle experimentally treated and untreated with antibiotics (Doster et al., 2018; Rovira et al., 2019), suggesting that other factors, together with the antimicrobial use, may influence the resistome composition, including environmental factors. In this context, however, it is worth noting that any comparisons between different milk filter samples should be carefully interpreted, even after normalization, due to the different proportion of host-related reads recovered from each milk filter, as a higher number of microbial reads may result in a greater probability of finding AMR genes (Rovira et al., 2019).

As previously mentioned, for detection of AMR genes in the metagenomic datasets obtained from milk filters, both the unassembled reads and the *de novo* assembled reads were queried, with the aim of achieving a more comprehensive resistome characterization. Indeed, despite the limitations of the reads-based method, this approach has gained attention in recent years thanks to the speed and relatively low computational demand, now becoming the favored technical approach; besides, the reads-based approach allows the detection of AMR genes from low abundance bacteria that might otherwise be undetectable by assembly-based approaches with incomplete assemblies (Boolchandani et al., 2019; Lal Gupta et al., 2020). However, the direct analysis of unassembled reads can result in a high false-positive prediction arising from artificial alignment of reads to AMR gene datasets due to local sequence homology (Lal Gupta et al., 2020). On the other hand, characterizing the resistome from contigs or MAGs after *de novo* assembly, despite being time consuming, computationally demanding and requiring a higher genome coverage, allows a more accurate detection of valid coding DNA sequences and a better exploration of the surrounding genomic context, which can then be used to study co-linked genes, given sufficient coverage (Boolchandani et al., 2019; Lal Gupta et al., 2020). In the present study, the application of both approaches facilitated a comprehensive resolution of the resistome while confirming the loss of data resulting from the assembly-based method. Through the reads-based approach, 160 AMR genes were detected, mostly belonging to the aminoglycoside, β-lactam, tetracycline, multidrug and MLS classes; notably most of the species harboring AMR genes were predicted to be Gram-negative genera, namely *Enterobacter, Acinetobacter, Escherichia,* and *Pseudomonas.* This prediction was further confirmed by the assembly-based approach, which allowed the identification of different MAGs belonging to the *Moraxellaceae* and to the *Enterobacteriaceae* families harboring AMR genes and the subsequent detection of 71 AMR-encoding genes mostly belonging to the multidrug, aminoglycoside, β-lactam, tetracycline and MLS classes. While the high relative abundance of *Enterobacteriaceae* detected in

some milk filters, including bacteria commonly isolated from bulk tank milk, can be related to faecal contamination of udder surfaces or dairy farm environments, *Acinetobacter* and *Pseudomonas* spp. have been identified as the dominant bacterial communities in the bulk tank milk by some recent studies reporting on the high abundance of psychrotolerant bacteria in milk samples prior to thermal treatments (McHugh et al., 2020; Nikoloudaki et al., 2021). The role of these bacterial families as important reservoirs of AMR genes is consistent with a recent study which predicted the presence of four main families harboring AMR genes in raw milk maintained at room temperature for 24 h, namely *Pseudomonadaceae*, *Enterobacteriaceae*, *Yersiniaceae* and *Moraxellaceae* families (Liu et al., 2020); another recent paper emphasized the role of *Pseudomonas* as the main bacterial genus harboring AMR genes in raw milk (Nikoloudaki et al., 2021). Both *Acinetobacter* spp. and *Pseudomonas* spp. are ubiquitous Gram-negative bacteria widely distributed in nature which can acclimate to different ecological habitats and temperatures; thereby, their detection in bulk tank milk can be due to their frequent presence on udder surfaces, milking machines and in contaminated water used for cleaning purposes. Although these bacterial genera are avirulent for healthy humans, nevertheless some strains including opportunistic pathogens such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Pseudomonas* spp. have proven to be potential reservoirs of AMR genes; their ability to survive in the aquatic environments for long periods forming biofilms and to resist heat (Jain & Danziger, 2004; Meng et al., 2020; Panebianco et al., 2022), together with the possible exchange of genetic material with other non-pathogenic or pathogenic bacteria, points to the fact that their presence in milk filters, hence in the corresponding bulk tank milk, should be considered a matter of public health concern. In addition to the presence of AMR psychrotolerant bacteria, the detection of multidrug-resistant *Enterobacteriaceae* in milk filters, including *Enterobacter* spp. and *Escherichia* spp., as previously reported in poorly handled dairy chains, has been linked to many food safety and spoilage issues and should be considered yet another

public health challenge due to the potential to transmit AMR genes from milk to humans through the food supply (Ntuli et al., 2016).

Although resulting in a loss of metagenomic data, the single sample assembly-based approach used in this study allowed the reconstruction of different MAGs carrying AMR determinants; notably, out of 8 high quality MAGs, a genome identified as belonging to the *Enterobacteriaceae* family harbored 17 AMR genes, most of which were complete multidrug-resistance genes conferring resistance to diaminopyrimidine, fluoroquinolone, glycylcycline, nitrofuran, tetracycline, carbapenem, cephalosporin, cephamycin, monobactam, penem, phenicol, rifamycin, triclosan, aminocoumarin, aminoglycoside, macrolide and peptide antibiotics. Milk filters have already been used along with traditional bacteriological methods to investigate the presence of *Enterobacteriaceae* which could enter the food chain, such as *Escherichia coli, Salmonella*, *Cronobacter* and *Enterobacter* spp. (Albonico et al., 2017; GIACOMETTI et al., 2012), both as indicators of milk hygiene and as potential foodborne pathogens. Herein, the application of shotgun metagenomic sequencing of milk filters has allowed the concurrent detection of members of the *Enterobacteriaceae* family and the complete characterization of their AMR determinants, highlighting the possible further implication of risk to human health for the consumers of raw milk. Interestingly, in accordance with our findings, the microbial carriage and intake of AMR-expressing *E. coli* through raw milk was recently found to be the primary predictor of AMR prevalence in low-income countries, pointing to the role of raw milk as carrier of AMR reservoirs (Caudell et al., 2018; Liu et al., 2020).

Together with the reconstruction of high quality MAGs, the assembly-based approach allowed the investigation of the mobilome; in this regard, the occurrence of AMR genes in dairy products can be considered less of a risk if those genes are not easily transferred to the gut microbes of the human host following consumption (Walsh et al., 2020). In this study, 14 of 37 detected and reconstructed plasmids carrying AMR genes belonging to the aminoglycoside, tetracycline, β-lactam, MLS and

sulfonamide classes were classified as mobilizable; among them the mobilizable plasmid carrying the gene *aadA27*, conferring resistance to streptomycin and spectinomycin, was detected on one of the high-quality MAGs identified as a member of the *Acinetobacter* genus. Further, HGT occurrences were detected in both scaffolds and MAGs, showing similar results and revealing a higher frequency of HGT events between Gram-negative psychrotolerant bacteria such as *Psychrobacter* and *Acinetobacter*. The evidence of transposon mediated HGT events and mobile genetic elements carrying AMR genes points to the fact that there is potential for transfer of AMR genes between bacteria which can be found in raw milk, mostly among bacteria that are more likely to be introduced from the farm environment. These findings are consistent with previous studies investigating the cheese microbiomes and highlight the need to improve general farm hygiene practices related to the environment and the milking process to reduce the chance of raw milk serving as reservoir of transmissible AMR genes (Walsh et al., 2020). It should be noted that future investigations are required to evaluate the extent to which these findings based on the detection of AMR genes are consistent with an associated resistance phenotype.

Although this study highlights the merits of using high throughput sequencing techniques such as shotgun metagenomic sequencing to characterize the resistome of milk filters as predictors of the raw milk resistome, the NGS approach applied in this study can be improved and further explored to extend the potential of its application together with routine microbiology testing. The high level of bovine DNA contamination of raw milk and dairy products and the resulting high percentage of host DNA in metagenomic libraries remains an issue for the untargeted whole metagenomic sequencing. The resultant lower yields of microbial DNA sequences limits the number of reads recoverable from the untargeted shotgun metagenomics for the microbiome analysis (McHugh et al., 2020). The recently explored use of microbial enrichment methods can decrease the amount of host DNA, though resulting in an increased cost and time spent manipulating the sample, while the enrichment

for targeted microorganisms before DNA extraction can improve the recoverable reads for a specific population (Alexa (Oniciuc) et al., 2020; Rubiola et al., 2020). Developments and standardizations of these laboratory procedures together with further advances in the speed and accuracy of bioinformatics tools can greatly help detecting and tracking potential foodborne pathogens and AMR determinants throughout the food chain, thereby supporting scientifically sound risk reduction decision-making for the protection of public health.

5. Conclusions

Understanding the distribution of AMR genes in a complex, important food matrix such as milk has relevance in terms of protecting consumers and maintaining food safety standards. Our findings suggest that milk filters can successfully be used to investigate the resistome of raw milk through the application of shotgun metagenomic sequencing. This approach facilitates the identification of numerous AMR determinants without the need for culture. In this context, *de novo* assembly allows for a more holistic AMR detection strategy, while the reads-based approach facilitates the detection of AMR genes from low abundance bacteria that might be undetectable by assembly-based methods, with the caveat that it may result in false positive prediction. The application of both reads- and assembly-based approaches, despite being computationally demanding, has facilitated the comprehensive characterization of a food chain resistome, while also allowing the construction of complete MAGs and the investigation of mobile genetic elements. In accordance with our results, raw milk can be considered a source of AMR bacteria and genes; this points out the importance of properly informing food business operators about the risk associated with poor hygiene practices in the dairy production environment and consumers of the potential microbial food safety risks derived from raw milk products consumption. Translating these findings as risk assessment outputs heralds the next generation of food safety controls.

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Authorship contribution

Selene Rubiola: Writing – original draft; formal analysis; investigation; conceptualization. **Guerrino Macori**: Writing – review & editing; resources; validation; conceptualization. **Francesco Chiesa**: Writing – review & editing; visualization; supervision; conceptualization. **Felice Panebianco**: Writing – review & editing; investigation. **Riccardo Moretti**: Writing – review & editing; data curation. **Séamus Fanning**: Writing – review & editing; resources. **Tiziana Civera**: Writing – review & editing; funding acquisition.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA809009.

Supplementary files

- Supplementary File S1. Metagenomic sequencing data before and after trimming, quality filtering, and host DNA removal.
- Supplementary File S2. AMR determinants detected through the reads-based approach including the corresponding class, mechanism, gene and database.
- Supplementary File S3. Significant correlations between detected AMR genes and most abundant bacterial genera (>0.99) assessed by Spearman's correlation.
- Supplementary File S4. Quality parameters of single-sample *de novo* assemblies generated using IDBA-UD v1.1.3.
- Supplementary File S5. Alignments between mobilizable plasmids detected and assembled across single-sample assemblies and MASH nearest neighbor plasmids provided by MOB-Suite. Alignments were visualized using AliTV tool (Ankenbrand et al., 2017).

- Supplementary File S6. MOB-typer output for mobilizable plasmids detected on scaffolds and high-quality MAGs.

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