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Sabine Delannoy, Corine Hoffer, Mai-Lan Tran, Jean-Yves Madec, Anne Brisabois, et al.. High throughput qPCR analyses suggest that Enterobacterales of French sheep and cow cheese rarely carry genes conferring resistances to critically important antibiotics for human medicine. *International Journal of Food Microbiology*, 2023, 403, pp.110303. 10.1016/j.ijfoodmicro.2023.110303 . anses-04143864

**HAL Id: anses-04143864**

**<https://anses.hal.science/anses-04143864>**

Submitted on 28 Jun 2023

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# High throughput qPCR analyses suggest that Enterobacterales of French sheep and cow cheese rarely carry genes conferring resistances to critically important antibiotics for human medicine

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## ARTICLE INFO

### Keywords:

Resistome  
Raw milk cheese  
Dairy products  
Antimicrobial resistance genes  
Enterobacterales  
High-throughput qPCR  
Metabarcoding

## ABSTRACT

Bacteria present in raw milk can carry acquired or intrinsic antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs). However, only a few studies have evaluated raw milk cheese as a potential reservoir of ARGs. This study thus aimed at providing new data regarding resistance markers present in raw milk cheese. Sheep (n = 360) and cow (n = 360) cheese samples produced in France were incubated in buffered peptone water supplemented with acriflavin or novobiocin; as corroborated by 16S metabarcoding, samples were enriched in Gram-negative bacteria since *Escherichia coli* and *Hafnia alvei* respectively accounted for 40 % and 20 % of the samples' microbiota. Screening of the samples for the presence of 30 ARGs and 16 MGEs by high throughput qPCR array showed that nine ARGs conferring resistances to 1st-generation beta-lactams, aminoglycosides, trimethoprim/sulfonamides and tetracyclines occurred in >75 % of both sheep and cow samples. This is neither surprising nor alarming since these resistance genes are widely spread across the One Health human, animal and environmental sectors. Conversely, genes conferring resistances to last-generations cephalosporins were rarely identified, while those conferring resistances to carbapenems or amikacin, which are restricted to human use, were never detected. Multiple MGEs were detected, the most frequent ones being IncF plasmids, confirming the potential transmission of ARGs. Our results are in line with the few studies of the resistome of milk or milk cheese showing that genes conferring resistances to 1st-generation beta-lactams, aminoglycosides and tetracyclines families are widespread, while those conferring resistances to critically important antibiotics are rare or absent.

## 1. Introduction

Milk is an integral part of the human diet, either directly or as derived products such as cheese. Raw milk is a complex non-sterile matrix whose rich bacterial composition is dominated by Firmicutes (Quigley et al., 2013). Some of these bacteria, such as the Gram-positive *Lactobacillus* spp., *Lactococcus* spp. or *Streptococcus* spp. favor dairy fermentation. In addition, raw milk could be potentially contaminated by pathogenic bacteria like *Listeria* spp., *Salmonella* spp., Shiga toxin-producing *E. coli* or *Staphylococcus aureus* when hygienic practices are not respected enough (Jones et al., 2019; Pineda et al., 2021; Praça et al., 2023; Quigley et al., 2013). Raw milk cheeses owe their complex taste to

the diversity of their microflora that has not been heated above 40 °C. Compliance with good hygiene practices is essential to guarantee the sanitary quality of raw milk cheeses.

Bacteria present in raw milk can carry antimicrobial resistance genes (ARGs), either acquired or intrinsic. Due to the large abundance of Firmicutes, antimicrobial resistance (AMR) in milk and cheese thereof has long been studied principally from the perspective of Gram-positive bacteria, most of the time selectively looking for *mecA*-positive *Staphylococcus aureus* (El Garch et al., 2020; Parco et al., 2021). The presence of Gram-negative bacteria has also been reported, again using culture-based approaches and selectively looking for resistances to critically important antibiotics such as extended-spectrum cephalosporins (ESC)

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<https://doi.org/10.1016/j.ijfoodmicro.2023.110303>

Received 27 April 2023; Received in revised form 19 June 2023; Accepted 22 June 2023

Available online 24 June 2023

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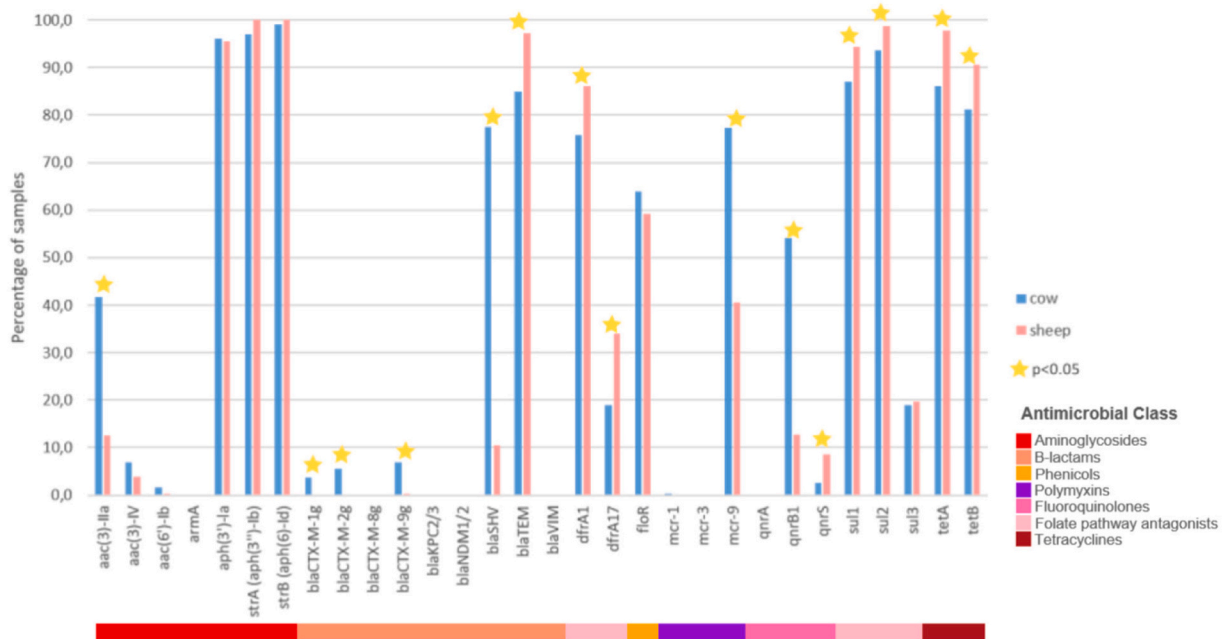
(Geser et al., 2012; Kaesbohrer et al., 2019) or carbapenems (Bonardi et al., 2023; Yaici et al., 2016). However, AMR is not restricted to pathogens, and the presence of resistance genes can be expected in milk both because of the complexity of this matrix in which multiple bacteria are competing for a same niche, and because antibiotics are used to treat livestock thus imposing a selective pressure.

Only a few studies reported a more global analysis. One study reports the PCR analysis of 40 genes in DNA extracted from 35 raw Brazilian cheese (De Paula et al., 2018). Specifically designed on Gram-positive genes, this study detected the *blaZ* gene in 100 % of the samples, and *tetB*, *bla<sub>TEM</sub>*, *sul1* and *sul2* in 80 % of them. Rubiola et al. (2022) using shotgun metagenomics on bulk tank milk filters, detected a total of 160 ARGs among the 10 tested farms, mostly conferring resistances to aminoglycosides, beta-lactams, tetracyclines and macrolides-lincosamides.

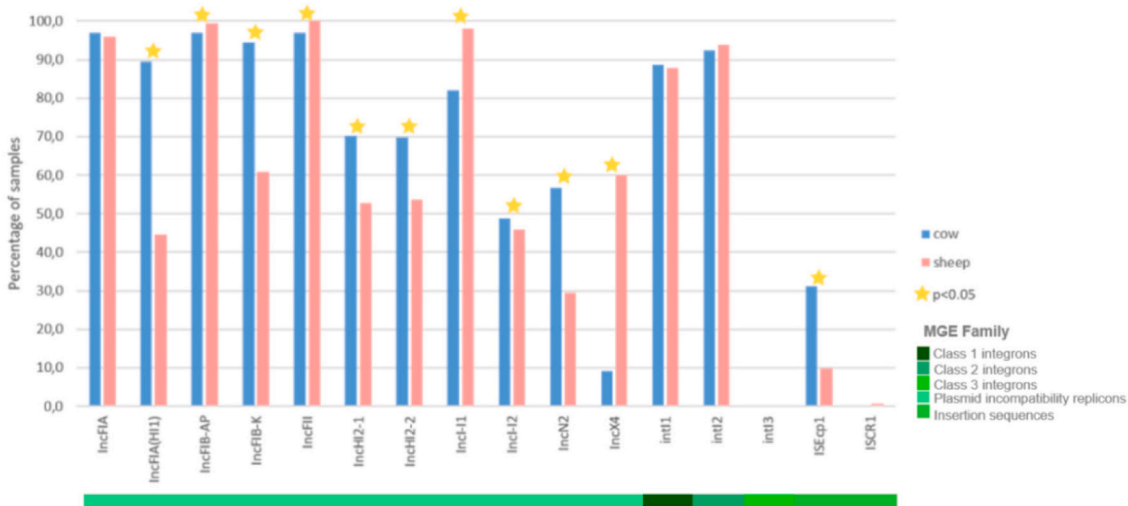
Also using shotgun metagenomics on milk samples, Liu et al. (2020) proved that raw milk contains much more resistance genes than pasteurized milk, in which 174 ARGs conferring resistances to 12 classes of antibiotics were identified.

These studies also showed that the milk/cheese resistome was brand- or region-specific. Differences might rely, among other factors, on antimicrobial use. In France, aminoglycosides, polymyxins and tetracyclines are the most frequently used antibiotics to treat cow mastitis intramammary during lactation while aminoglycosides, 1st and 2nd generations cephalosporins and penicillins are mostly used at drying off (ANSES, 2021). In order to document the type and variety of mobile ARGs in raw sheep and cow cheese produced in France, the goal of this study was to study the transferable resistome of Enterobacterales using high-throughput real-time PCR.

**A.**



**B.**



**Fig. 1.** Occurrence of antimicrobial resistance genes (ARGs) (A) and mobile genetic elements (MGE) (B) in sheep cheese (n = 360) and cow cheese (n = 360). \*The null hypothesis was rejected when p-values were < 0.05.

## 2. Materials and methods

### 2.1. Sheep cheese and cow cheese samples

Raw milk products collected in France from the sheep and cow dairy sectors are routinely tested for the presence of pathogenic *E. coli* according to the ISO/TS 13136:2012. In a previous study, the CNIEL (French Dairy Inter-branch Organization), ANICAP (Association Nationale Inter-professionnelle Caprine) and Confédération Générale de Roquefort provided us with DNA extracts issued from routine testing of raw milk products for screening pathogenic *E. coli* markers by PCR (Delannoy et al., 2022a). Sheep and cow cheese samples tested in this study were enriched overnight at 37 °C in Buffered Peptone Water (BPW) supplemented either with acriflavine or novobiocin to favor the growth of Gram-negative bacteria (ISO, 2012) and the DNA from these enriched cultures were extracted as described in Delannoy et al. (2022a). For the present study, 720 DNA extracts from sheep cheese (n = 360) and cow cheese (n = 360) provided by local routine laboratories were used. Sheep cheese originated from three different production sites, which provided respectively 272, 73 and 15 samples, while cow cheese came from two production sites, which provided 204 and 156 samples, respectively.

### 2.2. High-throughput real-time PCR

A pre-amplification step was applied to all samples prior to high-throughput PCR in order to guaranty the sensitivity of the method (Michelet et al., 2014). For DNA pre-amplification, the TaqMan PreAmp Master Mix (Fluidigm, USA) was used according to the manufacturer's instructions and as described in Delannoy et al. (2022a). Pre-amplified DNAs were stored at -20 °C until needed for high throughput PCR amplification. The Biomark™ real-time PCR system (Fluidigm, USA) was used for high-throughput microfluidic real-time PCR amplification using 48.48 dynamic arrays (Fluidigm) as described by Delannoy et al. (2022a). One negative water control was included per dynamic array, as well as positive controls for each assay (in pools). A 16S assay was included as loading/inhibition control. The sensitivity of the Fluidigm microfluidic real-time PCR with a pre-amplification step is equivalent to that of conventional real-time PCR (Delannoy et al., 2022a; Olwagen et al., 2019) and is equivalent to 10e3 to 10e4 targets/mL culture. The 720 samples were screened for the presence of a set of antimicrobial resistance genes (ARG) and mobile genetic elements (MGE) mainly carried by Gram-negative bacteria. All primers and probes targeting the ARGs and MGEs sequences were previously described by Delannoy et al. (2022b) and are reported in Table S1. The primers and probes used for the 16S assay were described in Patel et al. (2012).

### 2.3. Statistical analysis of high-throughput real-time PCR data

Statistical analyses were performed on R Studio version 1.2.5019. Two-proportions z-tests (two-tailed, with or without continuity correction – a continuity correction was applied when both counts were <10) were used to compare proportions of PCR positive for each genetic marker (ARG and MGE markers) between the sheep cheese and the cow cheese. The statistical tests were performed with  $\alpha$  of 5 %. The null hypothesis was rejected when p-values were <0.05.

Hierarchical clustering of the PCR data was performed on R Studio version 4.1.2 using the R package ComplexHeatmap (version 2.13.1) (Gu et al., 2016). The distance matrix was computed using the binary method and the hierarchical clustering was performed using the ward.D2 method.

Co-occurrence analysis was performed using the coccur package (v. 1.3) (Griffith et al., 2016) in order to identify statistically significant pairwise co-occurrences between ARGs and MGEs. Only ARGs with at least 20 total occurrences (equal to the number of ARGs investigated) were considered in order to be significant.

**Table 1**

ARGs positively associated with MGE.

AMR gene	Positively associated plasmids (p < 0.05)	Positively associated MGE (p < 0.05)
<i>aac3_Ila</i>	IncF, IncHI1, IncHI2, IncI1	<i>int11</i> , <i>ISEcp1</i>
<i>aac3_IV</i>	None	None
<i>aph3_la</i>	IncF, IncHI1, IncHI2, IncI2, IncN, IncX4	<i>int11</i> , <i>int12</i>
<i>strA</i>	IncF, IncI1	IncI2, <i>int11</i>
<i>strB</i>	IncI1	None
<i>bla<sub>CTX-M-2</sub>group</i>	IncF, IncHI1, IncN	<i>ISEcp1</i>
<i>bla<sub>CTX-M-9</sub>group</i>	IncF, IncHI1, IncHI2	<i>ISEcp1</i>
<i>bla<sub>SHV</sub></i>	IncF, IncHI1, IncHI2, IncN	<i>ISEcp1</i>
<i>bla<sub>TEM</sub></i>	IncF, IncHI1, IncHI2, IncN, IncI1, IncI2, IncX4	<i>int11</i> , <i>int12</i>
<i>dfrA1</i>	IncF, IncHI1, IncHI2, IncN, IncI1, IncI2, IncX4	<i>int11</i> , <i>int12</i> , <i>ISEcp1</i>
<i>dfrA17</i>	IncF, IncHI1, IncHI2, IncI1, IncI2, IncX4	<i>int11</i> , <i>int12</i>
<i>sul1</i>	IncF, IncHI1, IncHI2, IncN, IncI1, IncI2, IncX4	<i>int11</i> , <i>ISEcp1</i>
<i>sul2</i>	IncF, IncHI2, IncN, IncI1, IncI2, IncX4	<i>int11</i> , <i>int12</i>
<i>sul3</i>	IncF, IncHI1, IncHI2, IncN, IncI1, IncI2, IncX4	<i>int11</i> , <i>ISEcp1</i>
<i>floR</i>	IncF, IncHI1, IncHI2, IncI1, IncI2, IncX4	<i>int11</i> , <i>int12</i> , <i>ISEcp1</i>
<i>mcr-9</i>	IncF, IncHI1, IncHI2, IncN	<i>ISEcp1</i>
<i>qnrB1</i>	IncF, IncHI1, IncHI2, IncN	<i>ISCR1</i> , <i>ISEcp1</i>
<i>qnrS</i>	IncI2, IncX4	<i>int11</i>
<i>tetA</i>	IncF, IncHI1, IncHI2, IncI1, IncI2, IncX4	<i>int11</i>
<i>tetB</i>	IncF, IncHI1, IncN, IncI1, IncI2, IncX4	<i>int11</i> , <i>ISEcp1</i>

### 2.4. 16S rRNA sequencing and analysis

A subset of 96 samples (48 sheep cheese and 48 cow cheese) were selected for 16S rRNA sequencing. The 16S rRNA gene library was prepared using Nextera XT DNA Library Preparation Kit (Illumina, USA) from amplicons (approx. 550 bp) generated from the V3–V4 region of the 16S rRNA gene using the forward primer: 5'-TCGTCGGCAGCGT-CAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and reverse primer: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTA CHVGGGTATCTAATCC-3' (Klindworth et al., 2013). Sequencing was performed on an Illumina MiSeq platform using the MiSeq Reagent Kit v3 (2 × 300 bp) (Illumina Inc.).

Raw Illumina reads were analyzed using the R shiny application SHAMAN available at <https://shaman.pasteur.fr/> (Volant et al., 2020). Taxonomic classification was performed using the Silva SSU database.

## 3. Results

### 3.1. Detection of antimicrobial resistance genes in raw milk cheese

A total of 720 DNA extracts deriving from sheep (n = 360) and cow cheese (n = 360) were screened for the presence of 30 ARGs (Table S1). These samples were from routine testing of raw milk cheeses for screening pathogenic *E. coli* markers (Delannoy et al., 2022a). Among these 30 investigated genes, seven were found in none of the cow cheese samples (*armA*, *bla<sub>CTX-M-8</sub>group*, *bla<sub>KPC2/3</sub>*, *bla<sub>NDM1/2</sub>*, *bla<sub>VIM</sub>*, *mcr-3*, and *qnrA*). These same genes, as well as three additional ones (*bla<sub>CTX-M-1</sub>group*, *bla<sub>CTX-M-2</sub>group*, *mcr-1*), were also not found in the sheep samples.

Among the 23 detected ARGs, nine were found in >75 % of both sheep and cow samples (*aph(3')-la*, *bla<sub>TEM</sub>*, *dfrA1*, *strA* (*aph(3')-Ib*), *strB* (*aph(6)-Id*), *sul1*, *sul2*, *tetA* and *tetB*), while two additional ones were found in >75 % of cow samples only (*mcr-9*, *bla<sub>SHV</sub>*) (Fig. 1A). The *floR* and *qnrB1* genes were found in more than half of the cow samples. The proportions of the different ARGs were significantly different between cow and sheep samples for 15/23 of the detected genes. The most

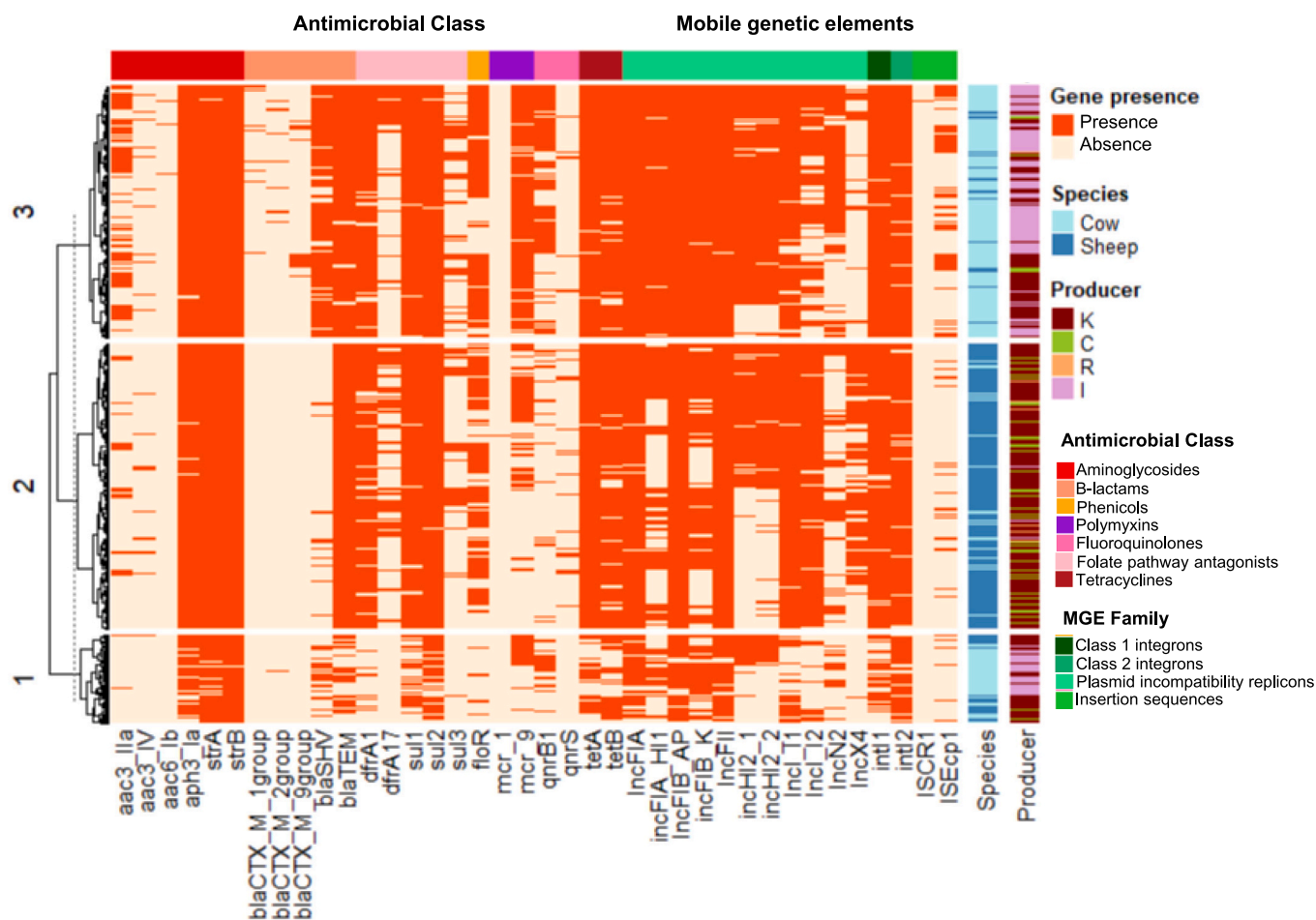


Fig. 2. Hierarchical clustering of the samples according to the presence/absence of 30 ARGs and 16 MGE.

striking differences concerned the *bla<sub>SHV</sub>*, *mcr-9* and *qnrB1* genes, which were respectively found in 78 % (n = 279), 77 % (n = 278) and 54 % (n = 195) of the cow samples, while they were only found in 11 % (n = 38), 41 % (n = 146) and 13 % (n = 46) of the sheep samples (Fig. 1A).

### 3.2. Detection of mobile genetic elements in raw milk cheese

Since ARGs are often carried by plasmids, probes were designed to detect six major incompatibility groups, namely IncF, IncI1/12, IncHI1, IncHI2, IncN and IncX4. Markers of the presence of two largely spread insertion sequences (*ISEcp1* and *ISCR1*) as well as the three classes of integrons were also included. Most of these MGE were found in more than half of the samples tested (Fig. 1B). The class3 integron (*intI3*) was not found in the samples, while *ISCR1* was only found in two sheep cheese samples. As for the ARGs, proportions of the MGE tested were significantly different between the cow and sheep cheese samples (Fig. 1B). The most striking difference concerned IncX4, which was found in 60 % (n = 216) of the sheep samples but in only 9 % (n = 33) of the cow samples. Co-occurrence of ARGs and MGEs was tested (Table 1), confirming that the detected ARGs can recurrently been found on different MGEs, the most frequent ones being IncF plasmids. These statistical associations indicate co-localization at a rate higher than expected from a random distribution, but are not a proof of physical co-localization of the ARGs and the corresponding MGEs.

### 3.3. Defining AMR/MGE patterns

Hierarchical clustering of the samples according to the presence/absence of the AMR and MGE markers tested allowed to group the samples in three clusters named 1, 2 and 3 (Fig. 2). Clustering was not related to the production site, and only cluster 3 contained significantly more samples from cow than from sheep cheese. This cluster 3 differed from the clusters 1 and 2 by the over-representation of the *aac3-IIa* and *bla<sub>SHV</sub>* genes (and to a lesser extent of the *qnrB1* gene), the IncFIA\_HI1 and IncFIB-K plasmid replicons as well as the *ISEcp1* insertion sequence. On the contrary, the IncX4 plasmid was much more prevalent in cluster 2. Cluster 2 contained most of the sheep samples, while cluster 1 contained the remaining cow and sheep samples. Cluster 1 specificity was the absence of the *dfrA17* gene, as well as the under-representation of the *bla<sub>TEM</sub>*, *dfrA1*, *sul1*, *floR* genes compared to clusters 2 and 3.

### 3.4. Microbiota of raw milk cheese after enrichment

We investigated the bacterial species present in the BPW enriched samples by metabarcoding (Fig. 3). The genus *Escherichia* constituted on average 40 % of the bacterial population in samples from both species (between 1.8 % and 92.5 % in cow samples with an average of 42.5 %, and between 0.8 % and 87.9 % in sheep samples with an average of 39 %) (Fig. S1). The second most prevalent genus in samples from both species was *Hafnia* (between 0.5 % and 89.7 % in cow samples with an average of 28.2 %, and between 1 % and 86.5 % in sheep samples with

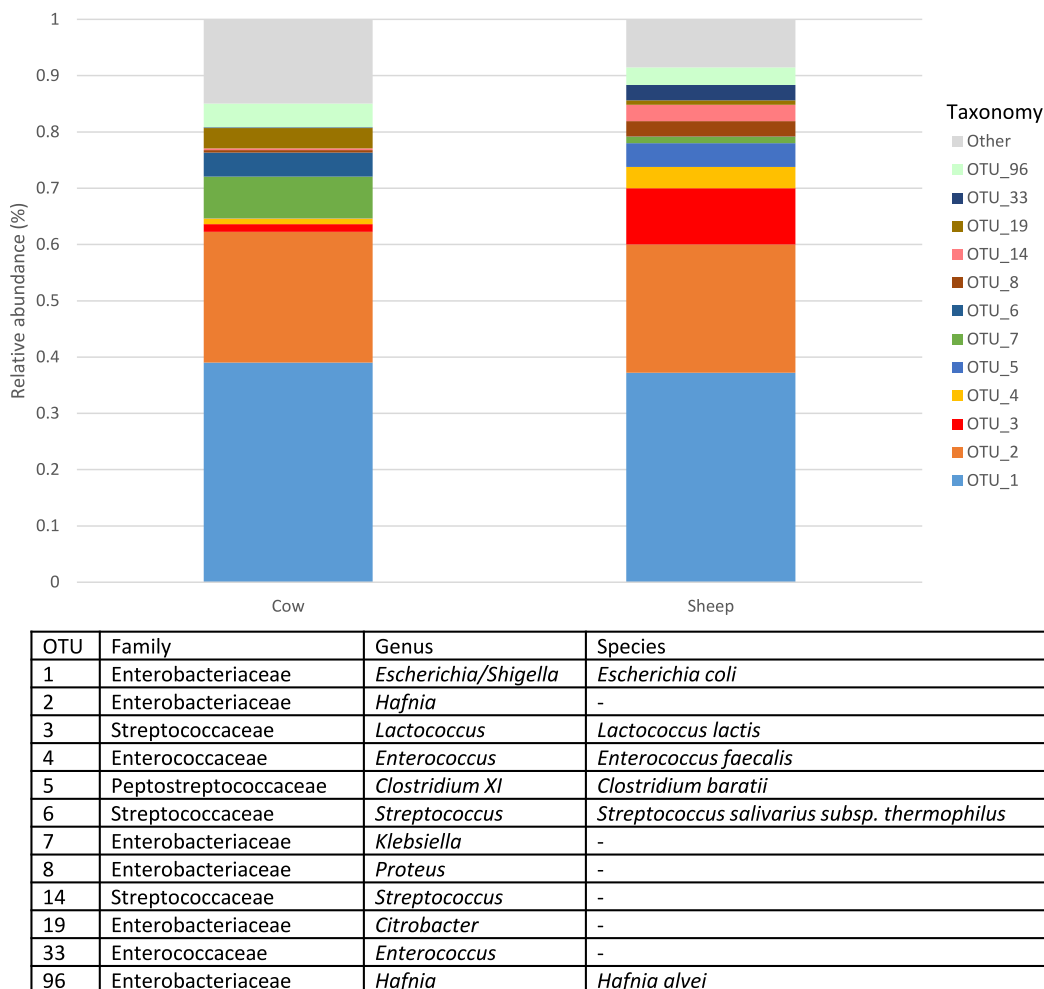


Fig. 3. Average relative abundance of Operational Taxonomic Units (OTU) in enriched cheese samples.

an average of 26 %). The OTUs that contributed the most to the  $\beta$ -diversity were: OTU\_1 (*Escherichia coli*), OTU\_2 (*Hafnia* sp.), OTU\_3 (*Lactococcus lactis*), OTU\_7 (*Klebsiella* sp.), OTU\_96 (*Hafnia alvei*), OTU\_6 (*Streptococcus salivarius* subsp. *thermophilus*) and OTU\_5 (*Clostridium baratii*). There was no major difference between the bacterial populations of the samples from cow milk and sheep milk. Only OTU19 (*Citrobacter* sp.) and OTU14 (*Streptococcus* sp.) showed a significant difference of relative abundance between the samples from cow milk and from sheep milk (Wilcoxon rank sum test with continuity correction, p values 3.031e-07 and 0.0002706 respectively), but the corresponding relative abundances were very low and results should thus be taken with care.

#### 4. Discussion

The goal of our study was to identify the resistance genes and their associated mobile elements that could be found in raw cow and sheep cheese. We investigated samples enriched in Gram negative and Enterobacterales because these bacterial species, with highly plastic genomes and hosts of the diversity of MGEs, are known to carry transmissible ARGs. Among the 23 ARGs searched for, nine were found in >75 % of both sheep and cow samples. These genes conferred resistances to first-generation beta-lactams (*bla<sub>TEM</sub>*), aminoglycosides (*aph(3')-Ia*, *strA*, *strB*), trimethoprim and sulfonamides (*dfrA1*, *sul1*, *sul2*) and tetracyclines (*tetA* and *tetB*). In France, these genes might have been selected by antimicrobial use, since tetracyclines have been widely used in the past in veterinary medicine, while both aminoglycosides and beta-lactams

are now first-line treatments to treat bovine mastitis (ANSES, 2021). The *bla<sub>TEM</sub>*, *tet* and *sul* genes have also been reported in similar proportions (>75 % of the samples) in raw cow's milk cheese from Brazil (De Paula et al., 2018), while metagenomics data identified these three AMR classes as the most frequent ones in dairy herds in Canada (Warder et al., 2021). More globally, these resistance genes are widely dispersed across all sectors of the One Health approach (humans, animals and the environment), so that their presence in raw milk is neither surprising nor alarming. The same conclusion can be drawn from the data on integrons, which were widespread in our cheese samples but also in all human, animal and environmental sectors (Stalder et al., 2014).

Conversely, genes conferring resistances to last-generations cephalosporins (*bla<sub>CTX-M</sub>* genes) were rarely identified in our study; likewise, genes conferring resistances to antibiotics restricted to human use such as carbapenems (*bla<sub>KPC2/3</sub>*, *bla<sub>NDM1/2</sub>*, *bla<sub>VIM</sub>*) or amikacin (*armA*) were not identified. In a study where they isolated ESC-resistant Enterobacterales in Germany, Kaesbohrer et al. (2019) showed that 13 % of the raw milk samples and 0.7 % of the raw milk cheese samples presented *bla<sub>CTX-M</sub>* genes. Using the same selective culture-based approach, Tekiner and Özpınar (2016) identified *bla<sub>CTX-M</sub>* genes in 3.7 % of the raw cow milk cheese and 43.6 % of the raw milk samples in Turkey, while Geser et al. (2012) found *bla<sub>CTX-M</sub>* genes in only 0.4 % of the raw milk and 0.3 % of the milk cheese in Switzerland. These studies prove that *bla<sub>CTX-M</sub>* genes can be found in raw milk cheese, even in the sub-dominant flora of a small number of samples, which is in coherence with our findings using high-throughput PCR. The fact that *bla<sub>CTX-M</sub>* genes were only identified in cow cheese samples is also in line with the

fact that ESC-resistance is much more frequent in cows than in small ruminants.

There were several significant differences between the cow and sheep sectors in the proportions of ARGs found; the most striking ones concerned the gentamicin-resistance *aac3\_Ila* gene (more present in cow cheese) and the *mcr-9*, *bla<sub>SVH</sub>* and *qnrB1* gene (largely more frequent in sheep samples). The over-representation of gentamicin-resistance in cow milk cheese could be related to the fact that bovine are more frequently treated than small ruminants, and thus that gentamicin-resistance is more frequent in clinical Enterobacterales from bovine than sheep (<https://shiny-public.anses.fr/resapath2/>). The *mcr-9* and *bla<sub>SVH</sub>* genes are often co-carried by IncHI2 plasmids (Li et al., 2020), justifying that these markers followed the same tendency. However, their over-representation in the sheep sector is more difficult to explain, especially since antibiotic use of ESC and colistin drastically decreased in France following the EcoAntibio action plan from the Ministry of Agriculture (Urban et al., 2023). A recent publication proved that *mcr* genes favor gut colonization by *E. coli* in the absence of antibiotic selection (Dalmaso et al., 2023). This might be a credible role for the promotion of Enterobacterales survival in a complex matrix, which would also elucidate the role of *mcr-9*. Indeed, unlike other *mcr* genes, *mcr-9* does not confer resistance to colistin.

A limitation of this study was that all cheese samples were treated with an enrichment step in buffered peptone water supplemented either with acriflavin or novobiocin. Performed in aerobic conditions, this enrichment step favors the growth of Enterobacterales such as *E. coli*, *Salmonella* or *Klebsiella*, and impair the growth of strict anaerobic species frequently found in milk such as lactic acid bacteria (including *Lactococcus*, *Streptococcus* and *Lactobacillus*), Clostridia (such as *Clostridium perfringens* and *Clostridium sporogenes*) and Propionibacteria. Indeed, 16S metabarcoding showed that about 40 % of the microbiota in the different samples consisted of *E. coli*, and 20 % of *Hafnia alvei*. This pre-treatment has prevented us from getting a global picture of resistance genes and mobile elements that can be present in milk. However, this also gave us the opportunity to identify markers that are associated with Gram-negative bacteria, which are among the major pathogens found in milk.

In conclusion, these results bring consolidated data on AMR-positive Enterobacterales in milk products, in line with the few studies that described the resistome of milk or milk cheese using non culture-based approaches. All studies showed that genes conferring resistances to 1st generation beta-lactams, aminoglycosides and tetracyclines families were widespread, while those conferring resistances to critically important antibiotics were rare or absent. This is encouraging, especially at a time when there is strong pressure on the veterinary sector to reduce antibiotic use, which points to a future decline in AMR in food and food-producing animals.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2023.110303>.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

#### Acknowledgments

This work was funded by the French Agency for Food, Environmental and Occupational Health & Safety (Anses) supported by the French Ministry of Agriculture and Food. We are grateful to the CNIEL and confederation Générale de Roquefort for providing DNA extracts for the

realization of this study. We are grateful to Baudoin Lesueur for his help with the metabarcoding analysis, and Sandra Jaudou for generating the Heat Map of gene presence/absence.

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