

Artisanal cheesemaking environment as a potential reservoir of antimicrobial-resistant Enterobacteriaceae

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Abstract: The family Enterobacteriaceae is a group of undesirable bacteria in dairy plants. Their presence in cheese-making could affect the food safety of the final products. The objective of this study was to detect, identify, and characterize representatives of the family Enterobacteriaceae in artisanal cheesemaking dairy plants in Slovakia. The susceptibility to different groups of antibiotics was tested using the disc diffusion method. Genes conferring resistance to beta-lactam antibiotics were detected by PCR with gene-specific primers. Of the 86 isolates, *Enterobacter* spp. was the most frequently identified genus (72.1%), followed by *Klebsiella* spp. (8.1%) and *Leclercia* spp. (8.1%). According to the CLSI criteria, the majority of isolates (77.9%) were resistant to cefazolin, followed by those resistant to ceftiofur (76.7%). According to the EUCAST criteria, the highest rates of resistance were to ceftiofur (84.9%) and ampicillin (66.3%). The presence of the *bla*_{TEM} gene was confirmed in 42 (48.8%) Enterobacteriaceae isolates, the *ampC* gene in 9 (10.5%), and the *bla*_{CTX-M} gene in 6 (6.9%). Our findings highlight the presence of Enterobacteriaceae in the artisanal cheese-making environment, showing high levels of resistance to beta-lactam antibiotics and harboring the resistance genes.

Keywords: dairy plants; environmental samples; undesirable bacteria; MALDI-TOF MS; resistance genes

The family Enterobacteriaceae belongs phylogenetically to the order Enterobacterales, the class Gammaproteobacteria, and the phylum Proteobacteria, and has undergone several significant taxonomic revisions.

Significant changes occurred when some genera originally classified within Enterobacteriaceae were reassigned to separate families. These changes were driven by new data obtained through gene sequencing

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technologies (Resendiz-Nava et al. 2022). Enterobacteriaceae are a family of bacteria that indicate hygiene levels in milk production on farms. In addition to their negative impact on dairy product technology, they can also pose a health risk to humans as causative agents of alimentary enterotoxicoeses (Dudriková et al. 2022).

Within the food industry, biofilm formation poses a significant challenge to food safety and hygiene, as it forms on equipment surfaces and has been identified as a cause of foodborne illnesses. Biofilms form in food processing facilities and can be released during production or packaging, resulting in product contamination. Microbial contamination of food reduces its shelf life and increases the risk of bacterial infection transmission (Capita et al. 2020). A study by Cherif-Antar et al. (2016) recorded the presence of biofilm produced by representatives of the family Enterobacteriaceae (*Klebsiella pneumoniae*, *Serratia marcescens*, and *Enterobacter* spp.) on the surface of a stainless-steel pipe in a dairy plant. They detected *Enterobacter* spp. in samples collected after equipment disinfection.

The use of antibiotics can affect the food industry because antibiotic-resistant bacteria in animals can be transferred to food products. There is also an indirect risk of horizontal transfer of resistance genes to pathogenic microorganisms at various points in the food chain (Capita et al. 2020). Antimicrobial resistance (AMR) is a global health challenge involving the transfer of bacteria and genes among humans, animals, and the environment (Larsson and Flach 2021).

The ability of Enterobacteriaceae to produce broad-spectrum betalactamases is a major concern for the scientific community. Many researchers have highlighted the potential risk of transmission of ESBLs (extended-spectrum beta-lactamases), AmpC (aminopenicillin-inactivating cephalosporinases), and CP (carbapenemases) produced by Enterobacteriaceae [such as *Escherichia coli* and *K. pneumoniae*, which provide resistance to many commonly used beta-lactam antibiotics, to consumers through milk and dairy products sold directly from farms (Ntuli et al. 2016). The *bla*_{TEM} genes encode variants of beta-lactamases in the TEM family, which confer resistance to beta-lactam antibiotics, primarily ampicillin and other penicillins. The *bla*_{CTX-M} genes code for various CTX-M family beta-lactamase variants that are associated with resistance to cephalosporins (third and fourth generation). Furthermore, the beta-lactamases encoded by *ampC* genes are important in conferring resistance to methoxy-beta-lactam antibiotics, extended-spectrum cephalosporins, and monobactams. One of the

essential mechanisms observed among Enterobacteriaceae is the production of ESBLs associated with *bla*_{TEM} and *bla*_{CTX-M} (Dey et al. 2023). Genes encoding ESBLs are often found in transposons or plasmid insertion sequences. Consequently, animal production has become a major concern in recent decades due to its close connection with the food chain and potential role as a reservoir, vehicle, or transmission route for the dissemination of ESBLs. In the past, the presence of these genes was investigated primarily in isolates exhibiting phenotypic resistance. However, it was later discovered that their presence could also be detected in isolates that were not phenotypically resistant (Dokuta et al. 2025).

This study investigated the presence of Enterobacteriaceae in the artisanal cheesemaking environment of small dairy plants in Slovakia, whose products are available on local markets. Given the ongoing debate over the interpretation of antibiotic susceptibility under the CLSI and EUCAST systems, the antibiotic test results were evaluated separately using both criteria. Subsequently, the presence of selected *bla* resistance genes (*bla*_{TEM}, *bla*_{CTX-M}, *ampC*) was assessed in all identified isolates, with or without phenotypic resistance to beta-lactam antibiotics.

MATERIAL AND METHODS

Isolation and identification of the isolates. A total of 75 environmental samples were collected on a single sampling occasion from four dairy plants (West, North, East, and Southeast) in Slovakia, which focus on artisanal cheesemaking. Our visits to the dairy were announced in advance to each dairy plant. The environmental samples were taken after cleaning and disinfection. Swab samples collected throughout the cheesemaking process included surfaces that were either in direct contact with or not in contact with food. A total of 75 samples were tested, namely apron ($n = 1$), shelves in the ripening rooms ($n = 4$), tables for draining ($n = 10$), vats for cheesemaking ($n = 6$), walls, floors ($n = 12$), pasteurisation equipment ($n = 6$), equipment in contact with food ($n = 13$), cheesemaking molds ($n = 9$), and workers' hands ($n = 14$).

Swabs from aprons, shelves in the ripening rooms, tables for draining, vats for cheesemaking, walls, floors, and pasteurisation equipment were collected using sterile swab templates with a capacity of 100 cm², in accordance with ISO 18593:2018. Swabs from cheesemaking molds, knives, and workers' hands were taken

from an unmeasured area. Sterile cotton swabs, moistened before sampling, were used to collect the samples and then were immersed in 10 mL of 0.1% (w/v) peptone water. The cotton swabs and swab templates were prepared and sterilised on-site in the laboratory. The samples were transported at 4 °C without preservatives to the laboratory at the University of Veterinary Medicine and Pharmacy in Košice for analysis.

Within 4 h after collection, all samples taken were subjected to microbiological analysis. Cotton swabs were used for precise and intensive vortexing. All the samples underwent decimal dilution in accordance with ISO 6887-1:2017. The volume of 1.0 mL of single dilutions, in duplicate, was poured onto Violet Red Bile Glucose agar (Merck, Germany) and incubated at 37 °C for 24 h. Based on their characteristic appearance, three to five colonies of different morphologies were taken from the plate and purified three times on Brain Heart Infusion agar (Merck, Germany). For further identification, the isolates were stored in Brain Heart Infusion broth (Merck, Germany) supplemented with glycerol at –18 °C.

The DNA was extracted from the isolates grown overnight in Brain Heart Infusion broth (Merck, Germany) as follows: 1 mL of culture was centrifuged at 10 000 rpm for 10 min. The sediment obtained was resuspended in 200 µL of 20% Chelex 100 (Bio-Rad, USA) and thoroughly vortexed. The prepared samples were incubated at 95 °C for 10 min and centrifuged again at 13 000 rpm for 3 min. The supernatant obtained contained total bacterial DNA.

PCR was used to identify members of the family Enterobacteriaceae, as described by Ke et al. (1999). To provide independent molecular confirmation of isolate identity, a PCR assay targeting a conserved region of the 16S rRNA gene, commonly used to detect members historically classified within the family Enterobacteriaceae, was performed in parallel with MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) identification. The target region (259 bp) was amplified using primers EntF (5'CGT TAC YCG CAG AAG AAG CA3') and EntR (5'CTG AGC GTC AGT CTT YGT CC3'), which are reported to detect most genera traditionally assigned to Enterobacteriaceae, although they may also capture closely related taxa (Resendiz-Nava et al. 2022). The primers were synthesised by Metabion (Germany). The PCR protocol was optimised as follows: initial denaturation at 95 °C for 13 min, followed by 30 cycles (denaturation at 95 °C for 20 s, annealing at 52 °C for 30 s, and extension at 72 °C for 2 min).

The final extension was carried out at 72 °C for 10 min. PCR reactions were performed in a final volume of 20 µL, containing 4 µL of 2 × HotFirePol® Master Mix (Solis BioDyne, Estonia), 0.5 µL of each primer, 2.5 µL of template DNA, and nucleasefree water to the final volume. The PCR products were visualised in a 1.5% agarose gel with Goldview™ nucleic acid staining (Beijing SBS Genetech Co., Ltd., China). The *E. coli* strain (CNCTC-7374) was used as a positive control to identify the isolates at the family level, Enterobacteriaceae, and to detect *bla*_{TEM} and *bla*_{CTX-M}.

Independent of PCR results, all isolates were streaked on Columbia Blood agar (Merck, Germany) to prepare for MALDI-TOF MS examination and incubated at 37 °C for 24 h. For sample preparation, extraction was performed using ethanol and formic acid. Two inoculation loops containing 1 µL of bacterial cells were resuspended in an Eppendorf tube *in vitro* with 300 µL of distilled water, to which 900 µL of absolute ethanol was added, and the mixture was then centrifuged at 15 000 rpm for 2 min. To the sediment, 50 µL of 70% formic acid and 50 µL of acetonitrile were added before analysis. The mixed solution was then centrifuged again. Subsequently, 1.0 µL of the supernatant was applied to a MALDI plate and after drying, it was overlaid with a saturated solution of HCCA (α-cyano-4-hydroxycinnamic acid) in 50% acetonitrile and 2.5% trifluoroacetic acid MS (Bruker Daltonics, USA). The results were analysed with an Ultraflex III instrument (Bruker, USA) using flexAnalysis software (version 2.0, Bruker, USA) and were evaluated using MALDI Biotyper software (version 1.1., Bruker, USA).

Antimicrobial susceptibility testing. The identified Enterobacteriaceae isolates were tested for antibiotic susceptibility using the disc diffusion method (DDM) according to the CLSI M100-S33 (2023) procedure. Ten antibiotics were tested: ampicillin (AMP, 10 µg), cefoxitin (FOX, 30 µg), ceftizoxime (ZOX, 30 µg), cefazolin (CZ, 30 µg), kanamycin (K, 30 µg), streptomycin (S, 10 µg), tetracycline (TE, 30 µg), gentamicin (CN, 10 µg), ofloxacin (OFX, 5 µg), and chloramphenicol (C, 30 µg). Antibiotics tested were selected based on the most commonly used agents in human and veterinary medicine in Slovakia during 2019–2023 (ESAC-Net 2023, ESVAC 2023). According to the CLSI criteria, ten antibiotics were evaluated, whereas only five antibiotics could be evaluated according to the EUCAST, namely: ampicillin (AMP, 10 µg), cefoxitin (FOX, 30 µg), ofloxacin (OFX, 5 µg), gentamicin (CN, 10 µg), and chloramphenicol (C, 30 µg). The discs used for susceptibility testing were manufactured by HiMe-

Table 1. Selected criteria for disc diffusion method (mm) according to CLSI (2023) and EUCAST (2023) in order Enterobacterales

Antimicrobial agent (disc content in µg)	Zone diameter breakpoints according to CLSI			Zone diameter breakpoints according to EUCAST		
	R	I	S	R	I	S
Ampicillin (10)	≤ 13	14–16	≥ 17	< 14	–	≥ 14
Cefoxitin (30)	≤ 14	15–17	≥ 18	< 19	–	≥ 19
Ceftizoxime (30)	≤ 21	22–24	≥ 25	–	–	–
Cefazolin (30)	≤ 14	–	≥ 15	–	–	–
Kanamycin (30)	≤ 13	14–17	≥ 18	–	–	–
Streptomycin (10)	≤ 11	12–14	≥ 15	–	–	–
Tetracycline (30)	≤ 11	12–14	≥ 15	–	–	–
Gentamicin (10)	≤ 12	13–14	≥ 15	< 14	–	≥ 17
Ofloxacin (5)	≤ 12	13–15	≥ 16	< 22	–	≥ 24
Chloramphenicol (30)	≤ 12	13–17	≥ 18	< 17	–	≥ 17

R – resistant; I – intermediate susceptible; S – susceptible; – zones not determined

dia (India). *E. coli* ATCC 25922 was used as a reference strain. The results were evaluated according to the CLSI M100-S33 (2023) and EUCAST (2023) guidelines. The diameters of the inhibition zones were recorded in millimeters (mm) and interpreted as susceptible, intermediate-susceptible, or resistant (Table 1).

Detection of antibiotic resistance genes. Genes conferring resistance to beta-lactam antibiotics were detected by PCR using the gene-specific primers listed in Table 2. The PCR protocol was the same as that for PCR identification of the family of Enterobacteriaceae except for the annealing temperature (Table 2).

Data analysis. The results obtained were processed in MS Office Excel® 2021 (Microsoft Corp., USA). The occurrence and distribution of bacterial genera were determined by calculating the percentage representation of each genus from the total number of Enterobacteriaceae isolates identified at the genus level. The MS Office 2021 programme was used for graph visualisation. The evaluation of isolate susceptibility to selected antibiotics was performed using simple methods to calculate

the percentage of resistant isolates in the entire group of identified bacteria and in individual genera. PCRs were used as reference variables in univariate analyses based on the percentages of PCR products detected.

RESULTS AND DISCUSSION

Occurrence and distribution of Enterobacteriaceae isolates. Enterobacteriaceae were present in 31 (41.3%) of the 75 samples taken from the cheese-making environment in four different dairy plants. The results of their detection across nine types of environmental samples are shown in Figure 1. The highest number of positive samples was obtained from walls and floors in 8 (66.7%), from cheesemaking vats in 4 (66.7%), and from workers' hands in 8 (57.1%). Only one swab was collected from the work apron because workers at the examined dairy plants wear washable work clothing and do not wear aprons. The important finding was that no Enterobacteriaceae were detected in the swabs from the pasteurisation equipment.

Table 2. The primers used in this study for the detection of resistance genes using a PCR-based method

Gene	Primer name	Product size (base pairs)	Annealing temperature (°C)	References
<i>bla_{TEM}</i>	TCGCCGCATACACTATTCTCAGAATGA	445	61	Amador et al. (2011)
	ACGCTCACC GGCTCCAGATTTAT			
<i>amp^C</i>	CGCCTCTTGCTCCACAT	491	51	Kuang et al. (2024)
	CGCCGAACAAACCGATA			
<i>bla_{CTX-M}</i>	ATGTGCAGYACCAGTAARGTKATGGC TGGGTRAARTARGTSACCAGAAAYCAGCGG	593	65	Amador et al. (2011)

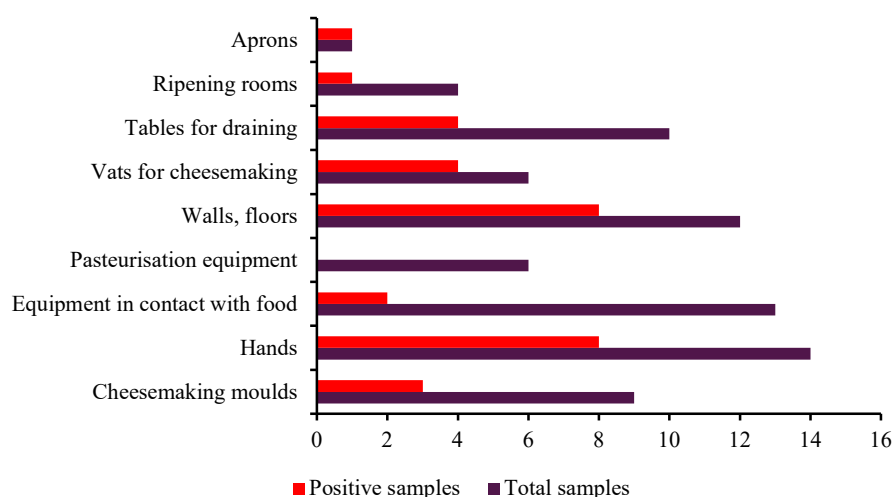


Figure 1. The origin of samples from the cheesemaking environment and the number of positive samples of Enterobacteriaceae in four dairy plants in Slovakia

However, as Mladenović et al. (2021) note, inadequate employee hygiene can affect the presence of Enterobacteriaceae in final products. Representatives of the family Enterobacteriaceae were present on workers' hands (44%) and on their work aprons (16%) in retail environments. *Klebsiella* spp., *Citrobacter* spp., *Raoultella* spp., and *Escherichia* spp. were found on the hands of meat workers. In a Czech study (Gelbíčová et al. 2021), the authors investigated the presence of the genus *Klebsiella* in the processing environment, personal, and raw material. The presence of *Klebsiella* spp. was detected in 49% of the swabs collected from the cheese processing plant. In our study, Enterobacteriaceae were detected on surfaces that were in contact with food (workers' hands, cheesemaking molds, cheesemaking vats, tables for draining, shelves in ripening rooms, and chopping boards). Nonetheless, it should be noted that the pasteurisation equipment was used without investigation of bacteria.

Because the Ent-F/Ent-R primer set used in this study is reported to detect most genera traditionally assigned to the family Enterobacteriaceae, and may also amplify

closely related taxa, a higher number of PCR-positive isolates was expected. Of the 151 isolates examined, 145 were PCR-positive using primers reported to cover 89% of genera within the family Enterobacteriaceae (Resendiz-Nava et al. 2022). All isolates were simultaneously identified by MALDI-TOF MS. As several genera historically included in Enterobacteriaceae have been reclassified into other families under the current taxonomy, isolates identified by MALDI-TOF MS as belonging to these genera were excluded from further analysis. Only isolates with a MALDI-TOF MS score above 2.000, indicating high confidence in genus-level identification, were retained. Based on MALDI-TOF MS, all isolates belonged to the order Enterobacterales, but only 86 were classified within the family Enterobacteriaceae according to the current taxonomy.

In environmental samples, the experimental analysis confirmed the presence of seven Enterobacteriaceae genera (Figure 2). Overall, the most common genus was *Enterobacter* (72.1%), followed by *Klebsiella* (8.1%) and *Leclercia* (8.1%). Only one isolate was identified as *Citrobacter* spp.

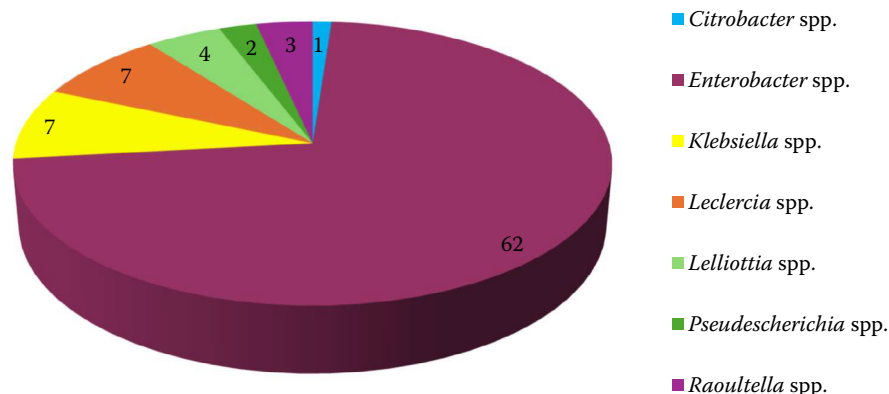


Figure 2. Distribution and number of isolates among the family Enterobacteriaceae

Table 3. Numbers and percentages of antimicrobial-resistant and intermediate-susceptible phenotypes among Enterobacteriaceae isolates, evaluated according to CLSI (2023) and EUCAST (2023)

System	Resistant									
	ZOX	FOX	AMP	K	CZ	OFX	S	CN	C	TE
CLSI	25 (29.1%)	66 (76.7%)	57 (66.3%)	11 (12.8%)	67 (77.9%)	0 (0%)	13 (15.1%)	5 (5.8%)	1 (1.2%)	4 (4.7%)
EUCAST	nda	73 (84.9%)	57 (66.3%)	nda	nda	41 (47.7%)	nda	17 (19.8%)	4 (4.6%)	nda
System	Intermediate susceptible									
	ZOX	FOX	AMP	K	CZ	OFX	S	CN	C	TE
CLSI	20 (23.3%)	4 (4.7%)	15 (17.4%)	46 (53.5%)	nda	0 (0%)	39 (45.4%)	21 (24.4%)	7 (8.1%)	2 (2.3%)
EUCAST	nda	nda	nda	nda	nda	nda	nda	nda	nda	nda

nda – no data available; ZOX – ceftizoxime; FOX – ceftiofloxacin; AMP – ampicillin; K – kanamycin; CZ – cefazolin; OFX – ofloxacin; S – streptomycin; CN – gentamicin; C – chloramphenicol; TE – tetracycline

Pasquali et al. (2022) focused on Enterobacteriaceae in artisanal cheesemaking. All samples were taken during cheese processing (the largest part of the environment), and 166 isolates belonged to the family Enterobacteriaceae, with the majority of *Klebsiella* spp. (26.5%), followed by *Enterobacter* spp. (23.5%), *Citrobacter* spp. (12.7%), *Escherichia* spp. (4.8%), *Raoultella* spp. (4.8%), and *Leclercia* spp. (0.6%). All *Klebsiella oxytoca* were collected from the warm and maturation room and the packaging area in the food and environment (data not shown). In our work, the representation of the identified genera was very similar, but *Enterobacter* was the dominant genus. A study from Korea examined the dynamics of the microbiota in milk, the environment, and in cheeses; out of the 59 isolates, 89.8% belonged to the family Enterobacteriaceae, with *Enterobacter* spp. being the most represented. The published list of selected bacterial isolates showed that representatives of the family Enterobacteriaceae, specifically the genera *Enterobacter* and *Lelliottia*, were found on the dairy plant floor. *Raoultella terrigena* was detected in the rinsing water of dairy equipment (Yi et al. 2024). The representation of Enterobacteriaceae isolates was similar to our findings.

Milk is a suitable medium for the growth of various microorganisms because it is rich in nutrients, has a high-water content, and maintains a neutral pH. Biofilms form very rapidly on equipment used in milk processing. A study by Cherif-Antar et al. (2016) indicated the presence of biofilm produced by Enterobacteriaceae (*K. pneumoniae*, *Enterobacter* spp.) on the surface of stainless-steel pipes in a dairy plant. Although our work did not focus on investigating the biofilm, the presence of *Klebsiella* spp. and *Enterobacter* spp. isolates was also confirmed.

Antimicrobial susceptibility test results for Enterobacteriaceae. The antimicrobial susceptibility results were evaluated separately according to the CLSI M100-S33 (2023) and EUCAST 2024 documents (Table 3).

Despite methodological differences, evaluations using both the CLSI and EUCAST systems showed similar resistance trends (e.g. ceftiofloxacin and ampicillin were highly resistant in both systems). Among all investigated isolates, the highest resistance to cephalosporins, according to EUCAST, was observed for ceftiofloxacin, with 84.9% of isolates resistant. According to CLSI, resistance to cefazolin was observed in 77.9% of isolates, followed by ceftiofloxacin in 76.7%. A higher intermediate susceptibility was found in 53.5% of isolates to kanamycin and in 45.4% to streptomycin. The largest difference was observed for ofloxacin: no isolates were classified as resistant by CLSI, whereas 47.7% were classified as resistant by EUCAST.

The number and distribution of antimicrobial-resistant phenotypes in Enterobacteriaceae by genus, according to CLSI (Figure 3), showed that among all *Enterobacter* spp. isolates, the highest resistance was observed to cefazolin (96.7%) and ceftiofloxacin (87.1%). A high level of resistance to ceftiofloxacin was found in *Klebsiella* spp. isolates (85.7%). No resistance was observed in the two isolates of *Pseudomonas* spp. to any of the antibiotics tested.

The evaluation using the EUCAST system showed high resistance among *Enterobacter* spp. isolates. (90.3%) and *Klebsiella* spp. (85.7%) to ceftiofloxacin. All identified isolates of *Lelliottia* spp. and *Raoultella* spp. were resistant to ceftiofloxacin (Figure 4). As with the overall evaluation, ampicillin resistance across all isolates was the same for CLSI and EUCAST. The most notable differences were found in the evaluation of ofloxacin resistance, as mentioned above, and of gentamicin resistance in *Klebsiella* spp.

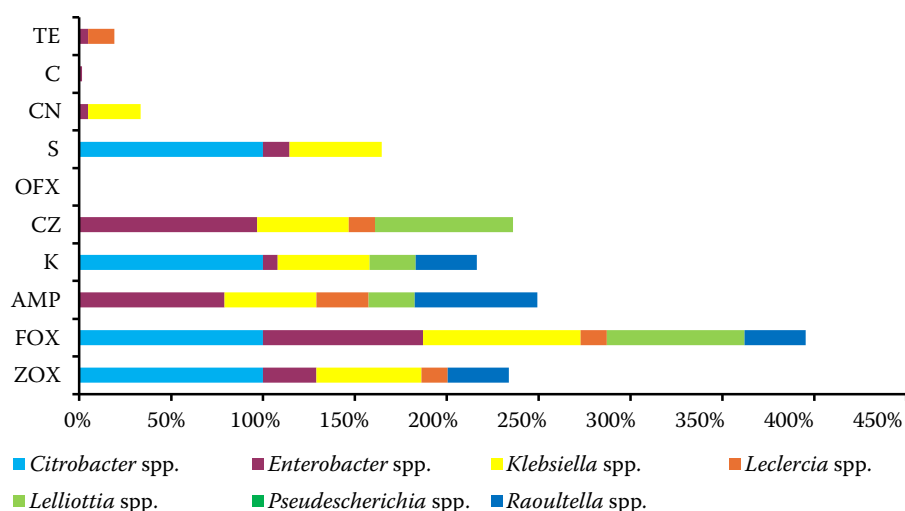


Figure 3. Percentage of resistant isolates among Enterobacteriaceae genera across tested antibiotics (CLSI 2023)

ZOX – ceftizoxime; FOX – cefoxitin; AMP – ampicillin; K – kanamycin; CZ – cefazolin; OFX – ofloxacin; S – streptomycin; CN – gentamicin; C – chloramphenicol; TE – tetracycline

Differences in antimicrobial resistance are influenced by national therapeutic guidelines and practices, antibiotic use in human and veterinary medicine, and their interactions. An important question in the approach to evaluating AMR is whether to use the CLSI or EUCAST criteria. In 2008, the first EUCAST breakpoint table was published, and the relationship between breakpoints and antimicrobial exposure was emphasised from the beginning. Each breakpoint was related to dose, frequency, and mode of administration. Some European scientists evaluating AMR strictly follow the EUCAST criteria. However, there are cases in which applying the CLSI criteria is required because no equivalent EUCAST criteria are available. The study by Naghavi et al. (2024), which examined the global burden of bacterial AMR, provided contextual information for classifying isolates as susceptible or non-susceptible according to the 2023 CLSI breakpoints and for evaluating how resistance proportions would differ when using other CLSI

or EUCAST versions. These relationships were then used to create adjustment factors and correct the data. The scientific and regulatory communities continue to work towards unifying interpretive criteria, taking into account methodological variability and the fact that breakpoint definitions are primarily based on clinical isolates and therapeutic considerations (Aupaix et al. 2024).

Our decision to evaluate AMR separately according to CLSI and EUCAST was not intended to highlight inconsistencies, but rather to show that when interpretative criteria differ, susceptibility test results and their final evaluation may vary.

Resistance to antibiotics in the dynamic microbiota of dairy plants was investigated by Yi et al. (2024), and the results were evaluated according to the EUCAST system. The study includes isolates from raw milk, cheese, and environmental swabs without a separate assessment of AMR. In general, most Enterobacteriaceae isolates were resistant to ampicillin (52.8%) and cefoxitin (43.4%).

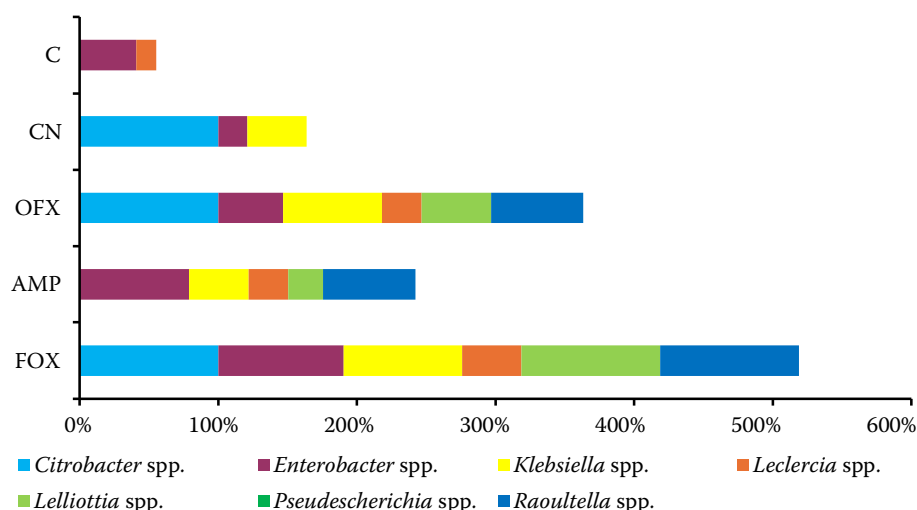


Figure 4. Percentage of resistant isolates among Enterobacteriaceae genera across tested antibiotics, EUCAST (2023)

FOX – cefoxitin; AMP – ampicillin; OFX – ofloxacin; CN – gentamicin; C – chloramphenicol; TE – tetracycline

It was lower than our findings. Another study, which focused on *Klebsiella* isolates, included environmental samples associated with artisanal cheesemaking. Results of antibiotic susceptibility testing were obtained for 72 of the 75 isolates, and 71 showed a naturally antibiotic-susceptible phenotype. All isolates except one showed resistance to ampicillin, as expected for *Klebsiella* spp. due to the presence of an intrinsic chromosomal type A beta-lactamase. It was not possible to identify an association between the source of isolates and antibiotic resistance. Resistance was evaluated according to EUCAST criteria for most antibiotics, and CLSI criteria were applied to tetracycline, azithromycin, and nalidixic acid. This study showed that the EUCAST and CLSI criteria were combined (Crippa et al. 2023). In a study by Beyene et al. (2024) that investigated Enterobacteriaceae in the milk acquisition environment, multidrug resistance across nine antibiotic classes was examined. Overall, 7.7% of the isolates were susceptible to all antibiotics tested, while only one isolate from the genus *Citrobacter* was resistant to all tested antibiotics. The highest percentage of isolates (18.9%) was resistant to five antibiotics simultaneously. The aforementioned research did not evaluate the percentage representation of resistant isolates, but the supplementary material, based on information provided for individual isolates, shows that ampicillin resistance was most frequently detected, which is partially consistent with our findings.

Detection of resistance gene among all isolates of the family Enterobacteriaceae. In our study, the presence of resistance genes *bla*_{TEM}, *bla*_{CTX-M}, and *ampC* was investigated in all identified Enterobacteriaceae isolates with or without phenotypic expression (resistance to ampicillin, cefoxitin, ceftizoxime, and cefazolin).

As documented in Table 4, 42 isolates had confirmed *bla*_{TEM}. Thirty-one isolates carrying the *bla*_{TEM}

gene were identified as *Enterobacter* spp., and 10 of them were resistant to all four beta-lactam antibiotics. In all four isolates belonging to the genus *Lelliottia*, the *bla*_{TEM} gene was confirmed. For two isolates of *Leclercia* spp., there was a correlation between resistance and phenotype, but only according to EUCAST; for the other two, only according to CLSI. The nine isolates identified by PCR as harbouring the *ampC* gene belonged to the genus *Enterobacter*. These isolates were evaluated as phenotypically resistant to cephalosporins and ampicillin simultaneously, according to CLSI and EUCAST criteria. In one isolate belonging to the *Enterobacter* genus and five isolates belonging to the *Klebsiella* genus, the presence of the *bla*_{CTX-M} gene was confirmed. Phenotypic resistance to cephalosporins and ampicillin was detected by both CLSI and EUCAST in all isolates, except for one *Klebsiella* isolate, which showed no resistance to the tested cephalosporins but carried the *bla*_{CTX-M} gene.

Overall, in 51 Enterobacteriaceae isolates, one or two resistance genes were confirmed. Six isolates had two genes simultaneously. In five isolates of *Enterobacter* spp., a combination of the *bla*_{TEM} and *ampC* genes was found and in one isolate belonging to *Klebsiella* spp., a combination of *bla*_{TEM} and *bla*_{CTX-M} was confirmed (Table 4). Thirty-five isolates did not carry the *bla*_{TEM}, *bla*_{CTX-M} or *ampC* genes. It is also essential to recognise that the intrinsic resistance of some Enterobacteriaceae to beta-lactam antibiotics is due to the presence of inducible chromosomal AmpC beta-lactamases, low outer membrane permeability, and active efflux mechanisms. These factors are particularly important in evaluating the phenotypic manifestations of resistance to beta-lactam antibiotics (Nikoloudaki et al. 2021).

Raw milk is the main raw material in dairy plants, and during processing, it can serve as a reservoir of antibiot-

Table 4. Number and percentage distribution of resistance genes *bla*_{TEM}, *bla*_{CTX-M}, and *ampC* in isolates of the Enterobacteriaceae family

Isolates	Resistance genes			Number of genes		
	<i>bla</i> _{TEM}	<i>ampC</i>	<i>bla</i> _{CTX-M}	0 gene	1 gene	2 genes
<i>Citrobacter</i> spp.	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)
<i>Enterobacter</i> spp.	31 (50.0%)	9 (14.5%)	1 (1.6%)	26 (41.9%)	31 (50.0%)	5 (8.1%)
<i>Klebsiella</i> spp.	2 (28.6%)	0 (0%)	5 (71.4%)	1 (14.3%)	5 (71.4%)	1 (14.3%)
<i>Leclercia</i> spp.	4 (57.1%)	0 (0%)	0 (0%)	3 (42.9%)	4 (57.1%)	0 (0%)
<i>Lelliottia</i> spp.	4 (100%)	0 (0%)	0 (0%)	0 (0%)	4 (100%)	0 (0%)
<i>Pseudeshcherichia</i> spp.	0 (0%)	0 (0%)	0 (0%)	2 (100%)	0 (0%)	0 (0%)
<i>Raoultella</i> spp.	0 (0%)	0 (0%)	0 (0%)	3 (100%)	0 (0%)	0 (0%)

ic resistance genes (ARGs). The study by Yi et al. (2024) identified the presence of ARGs, specifically *bla*_{CTX-M} and *bla*_{TEM}, in Enterobacteriaceae isolates. Metagenomic analysis in this work revealed that these ARGs were present at all stages of cheese production, from raw milk to final products. A study from Ethiopia investigated the presence of the resistance genes *bla*_{TEM}, *bla*_{SHV}, and *bla*_{CTX-M} in ESBL-producing isolates obtained from environmental swabs collected in milk production facilities. Most of these isolates were confirmed to carry the *bla*_{TEM} (85.0%) and *bla*_{CTX-M} (78.8%) genes (Beyene et al. 2024). The proportion of isolates with confirmed resistance genes reported in the Ethiopian study was substantially higher than that observed in our investigation. However, this comparison must be interpreted with caution, as that study assessed resistance genes only in ESBL-producing isolates. In our investigation of the *bla*_{TEM} and *bla*_{CTX-M} genes in isolates from the cheesemaking environment, *bla*_{TEM} was predominant.

Moreover, our study examined three resistance genes, and resistance to beta-lactam antibiotics may also be mediated by other genes not included in our study. A further weakness of our work is the absence of sequencing-based confirmation, which would provide stronger evidence for the presence of resistance genes.

CONCLUSION

Our study found that Enterobacteriaceae can be present in artisanal cheesemaking environments. They were detected on various food-contact surfaces (workers' hands, cheesemaking molds, cheesemaking vats, tables for draining, shelves in ripening rooms, and a chopping board). The positive finding across all four dairy plants was that the pasteurisation equipment contained none of the investigated bacteria. However, the studied bacteria were detected on food-contact surfaces after milk pasteurisation. In general, the most common genus was *Enterobacter* (72.1%), followed by *Klebsiella* and *Leclercia* (8.1%).

Evaluation of antimicrobial resistance according to the CLSI and EUCAST criteria indicated that the most noticeable difference in the number of resistant isolates was observed for ofloxacin. Most isolates were resistant to cephalosporins (cefazolin in 77.9% and cefoxitin in 76.4% according to CLSI) and ampicillin (according to CLSI and EUCAST). In general, 51 Enterobacteriaceae isolates carried one or two investigated resistance genes. Six isolates harbored two genes simultaneously. Four isolates (4.7%) without confirmed phenotypic resistance to any of the antibiotics tested in our study carried the resistance genes *bla*_{TEM} or *bla*_{CTX-M}.

Our study has several limitations. The sampling was carried out as a single cross-sectional event and included four artisanal dairy plants in Slovakia, which may limit the generalisability of the results.

Differences in susceptibility classifications between the CLSI and EUCAST systems, resulting from their distinct breakpoint systems, underline the need for cautious interpretation of resistance data. Although our study does not statistically assess these differences, the descriptive patterns we observed illustrate how the selection of an interpretive system can influence the classification of isolates. More comprehensive analyses, including statistical evaluation, would be valuable in future studies to clarify the practical implications of these system-dependent variations. For completeness and for a precise assessment of the risk associated with the dissemination of ARGs through artisanal cheesemaking, it is essential to detect resistance genes directly in the final products intended for consumption (e.g. cheeses in our study), which would enable the determination of the frequency with which antibiotic resistant strains are transferred from the production environment. Furthermore, only three beta-lactam resistance genes were investigated, and additional resistance mechanisms may therefore remain undetected.

Despite these limitations, we consider that our study could provide valuable evidence that environmental surfaces in artisanal cheesemaking plants can serve as reservoirs for Enterobacteriaceae and their associated resistance genes. Strengthening hygiene practices and incorporating environmental monitoring into food safety management systems should be regarded as essential steps in artisanal cheesemaking.

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