Antimicrobial resistance of bacterial isolates from different dairy products and their emergence in the food chain

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Abstract. The antibiotic resistance of foodborne pathogens represents a healthcare concern globally. This phenomenon has an increasing impact on medicine and economy. A total of 26 spoilage and pathogenic bacterial isolates originating from different dairy products have been screened against eight different antibiotics. Based on the type of the selective agar medium used for their isolation, the isolates were: five staphylococci isolates, six *Vibrio* isolates, two *Pseudomonas* sp. isolates, three *Salmonella* isolates, five *E. coli* isolates, and five coliform isolates. The overall resistance to the tested antimicrobials of the bacterial isolates was 31.73%, the majority being susceptible. Based on the results, there are isolates with multiple antibiotic patterns that can be possible risk factors and may call for preventive measures.

1 Introduction

Food may harbour antibiotic-resistant bacteria. The source of these microorganisms is the soil, the environment, or it can originate from humans or animals. The prevalence of these microbes can also result from cross-contamination. Meats are contaminated during slaughter or plants during irrigation, while processed food during manufacture (*Verraes et al.*, 2013).

The antibiotic resistance of foodborne pathogens entering the food chain has a growing impact on human health and the economy, too. The adequate use of antibiotics in agriculture, the spread of bacteria and their genetic determinants, and also the possible infection capacity result in the appearance of these bacteria in the food chain (*Founou et al.*, 2016).

The European Union has introduced different directives, requirements, and calls for monitoring the antibiotic resistance of bacterial species with public health concern (*EFSA*, 2018).

According to *EFSA*, 2018, antibiotic resistance is defined as "the ability of the bacteria to become increasingly resistant to an antimicrobial to which they have been previously susceptible". Natural selection or genetic mutation can lead to this phenomenon. The routine use of medicines increases the survival of bacteria in antibiotic environments acquiring antibiotic resistance genes (*Cole & Singh*, 2017).

Watkins and *Bonomo* (2016) reported different factors that contribute to the development of antibiotic resistance such as the number of bacteria in hospitals and their transfer, unsatisfactory disinfection, increase of high-risk patient populations, tourism, unsafe water, overdoses of antibiotics, inadequate diagnosis and the treatment with antibiotics, as well as lack of different authorized vaccines.

Bacteria with different biochemical mechanisms inactivate the antibiotic compound. These strategies are the alteration of antibiotic-bound protein, enzymatic inactivation of the antibiotic, prevention of the access into the cell, or elimination from the cell with (ATP)-powered drug efflux pump (*Cole & Singh*, 2017; *O'Bryan et al.*, 2018). *Friedman* (2015) reported foodborne antibiotic-resistant pathogenic bacteria such as *Campylobacter jejuni*, *Bacillus cereus*, *Clostridium perfringens*, *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Vibrio cholerae*, or *Vibrio parahemolyticus*. *Friedman* (2015) also referred to the occurrence of antibiotic-resistant bacteria in dairy products, mainly from raw milk.

Phenotypically, the antibiotic resistance of a bacterial strain can be measured by the determination of inhibition zones or minimal inhibitory concen-

tration (MIC) for antibiotics (*Butaye et al.*, 2015). Even though antibiotic resistance is a health concern, there are few studies on antibiotic resistance in food-related bacteria in our region. Our study aimed to determine the antibiotic resistance of different spoilage and pathogenic bacterial isolates originating from different dairy products.

2   Materials and methods

The antibiotic resistance of the bacterial isolates originating from different dairy products has been determined using the disk diffusion method. The tested isolates were recovered from different dairy samples on selective mediums: Pseudomonas Selective Agar, ChromoBio^R Coliform, TBX Chromo-Agar, Brilliance^{TM} Salmonella Agar Base, Mannitol Salt Agar, ChromoBio^R Cereus Base, and Vibrio Selective Agar TCBS Agar. The presumptive isolates were tested from each selective medium.

Isolates have been inoculated into a physiological solution, and a suspension of 1 OD has been prepared. 0.1 ml of bacterial suspensions has been spread on Nutrient agar (Sigma-Aldrich) plates. From the eight different antimicrobial sensitivity discs (Oxoid Ltd. are used for antibiotic sensitivity testing), two and four discs (in two replicates) have been placed on Nutrient agar plate and have been incubated at 37 °C for 24 h. The applied antibiotics disks (belong to different classes of antibiotics) were: Ampicillin 10µg (Am), Amikacin 30µg (A), Tobramycin 10µg (T), Streptomycin 10µg (Str), Cefoperazone 75µg (Cef), Ofloxacin 5µg (O), Levofloxacin 1µg (L), and Ceftriaxone 30µg (C). After incubation, the diameters of the inhibition zones have been measured in millimetres. The results of the inhibition zones have been interpreted as sensitive, intermediate, or resistant, according to a Zone-Size Interpretation Chart (http://www.oxoid.com/UK/blue/techsupport/its.asp?itsp=faq&faq=tsfaq020 &cat=antibiotic+sensitivity+testing&lang=EN&c=UK, HiMediaCatalogue 2017–18).

The Multiple Antibiotic Resistance (MAR) index has been calculated with the formula x/y, where x stands for the number of antibiotics to which the isolate exhibited resistance, and y is the number of total antibiotics for which the isolate was tested (*Adenaike et al.*, 2016).

3   Results and discussion

A total of 26 bacterial isolates originating from different dairy products have been tested for the resistance to eight antibiotics (*Table 1*).

Table 1: Antibiotic susceptibility pattern of the bacterial isolates originating from dairy products

Bacterial isolate		Ampicillin	Amikacin	Tobramycin	Streptomycin	Cefopera-zone	Ofloxacin	Levoflo-xacin	Ceftria-xone
Staphylococcus isolates	S1	R	S	S	S	S	S	S	S
	S4	S	S	R	R	S	R	R	S
	S6	S	S	S	S	S	S	S	S
	S11	R	S	S	S	I	S	S	I
	MOMAN	S	S	S	S	S	S	S	S
Vibrio sp.	V2	S	R	R	R	R	R	R	R
	V8	S	R	R	R	I	S	R	I
	V13	S	R	I	R	S	S	I	S
	V17	S	R	R	R	S	I	R	I
	V18	S	R	R	R	I	S	I	I
	V15	S	R	R	R	R	R	R	I
Pseudomonas sp.	P5	R	S	S	R	S	R	R	S
	P14	R	I	R	S	S	S	S	S
	Sa7	R	S	S	S	S	R	R	S
Salmonella sp.	OSXLD	S	R	I	S	R	R	R	R
	Sa10	R	S	S	S	S	S	S	S
	E.C3	R	R	R	R	S	R	R	S
	EC12	R	R	R	S	R	R	R	R
E. coli	PTKOK	S	S	R	S	S	S	S	I
	EC16	S	R	R	R	R	R	R	R
	PTBX	S	S	S	R	S	S	S	S
	MAKO	S	S	I	S	S	S	S	S
	TKO	S	S	R	R	S	S	S	I
Coliforms	OSKO	S	S	R	R	S	S	S	S
	MOKO1	R	S	R	I	S	S	S	R
	MOKO2	S	S	S	S	S	S	S	S

R = Resistant, S = Susceptible, I = Intermediate

Bacterial isolates have been grouped into six groups based on the type of the selective agar medium used for their isolation. Five were staphylococci isolates (S1, S4, S6, S11, MOMAN), six *Vibrio* isolates (V2, V8, V13, V15, V17, V18), two *Pseudomonas* sp. isolates (P14, P5), three *Salmonella* isolates (Sa10, Sa7, OSXLD), five *E. coli* isolates (E.C3, EC12, EC16, PTKOK, PTBX), and five coliform isolates (MAKO, TKO, OSKO, MOKO1, MOKO2). The overall resistance to the tested antimicrobials of the bacterial isolates was 31.73% (66/208).

From the 26 bacterial isolates, 34.62% have shown resistance to Ampicillin, whereas most of the tested isolates exhibited susceptibility (*Table 2*).

Table 2: Percentage of antibiotic resistance, intermediate and susceptible isolates

Antibiotics	Resistant isolates (%)	Intermediate isolates (%)	Sensitive isolates (%)
Ampicillin	34.62	0	65.38
Amikacin	38.46	3.85	57.69
Tobramycin	53.85	11.54	34.62
Streptomycin	50.00	3.85	46.15
Cefoperazone	19.23	11.54	69.23
Ofloxacin	34.62	3.85	61.54
Levofloxacin	42.30	7.69	50
Ceftriaxone	19.23	23.08	57.69

The following isolates have been resistant to Ampicillin: two of the staphylococci isolates (S1, S11), two of the *Salmonella* isolates (Sa7, Sa10), two of the *E. coli* isolates (EC3, EC12), one of the coliforms (MOKO1), and the *Pseudomonas* isolates (P5, P14). All the tested *Vibrio* isolates resulted as sensitive to this antimicrobial. None of the isolates has shown intermediate susceptibility.

The majority of the studied bacterial isolates showed susceptibility to Amikacin (57.69%). None of the *Staphylococcus*, coliform, and *Pseudomonas* isolates exhibited resistance to this antimicrobial compound. One *Pseudomonas* isolate (P14) represents an exception as it has been detected to show an intermediate resistance. The other isolates have been sensitive to this antibiotic. *Vibrio* isolates showed resistance to Amikacin. Only one *Salmonella* (OS-

XLD) and three *E. coli* isolates (E.C3, EC12, EC16) have been resistant to this antibiotic.

14 out of 26 bacterial isolates exhibited resistance to Tobramycin. This phenomenon has been detected in the case of a *Staphylococcus* isolate (S4), five *Vibrio* isolates, one *Pseudomonas* isolate (P14), four *E. coli* isolates (E.C3, EC12, PTKOK, EC16), and three coliforms (TKO, OSKO, MOKO1). None of the *Salmonella* isolates has been resistant to this studied antimicrobial. Intermediate resistance has been detected in the case of three isolates.

Resistance to Streptomycin has been exhibited by half of the studied isolates. One *Staphylococcus* (S4) and one *Pseudomonas* isolate (P5), three *E. coli* (EC12, EC16, PTBX), and two coliforms (TKO, OSKO) were resistant to this antimicrobial agent. All the *Vibrio* isolates exhibited resistance. Intermediate susceptibility has been detected in the case of one bacterial isolate (MOKO1) obtained from selective agar medium for coliforms.

The highest level of susceptibility has been detected in the case of Cefoperazone. The majority of the tested isolates, 69.23%, exhibited sensitivity to this antibiotic. None of the *Staphylococcus* sp., *Pseudomonas*, and coliform isolates showed resistance against this agent. In the case of one *Staphylococcus* (S11) and two *Vibrio* isolates (V8, V18), intermediate resistance was detected, while the other isolates were susceptible to this antibiotic.

34.62% of the studied isolates obtained on selective agar media from different dairy products showed resistance to Ofloxacin. None of the coliforms showed resistance against it; they all have been susceptible. One isolate from the staphylococci group (S4), two *Vibrio* isolates (V2, V15), one *Pseudomonas* isolate (P5), two *Salmonella* isolates (Sa7, OSXLD), and three *E. coli* isolates (E.C3, EC12, EC16) showed resistance. One *Vibrio* isolate (V17) showed intermediate susceptibility to this antibiotics.

Around half of the studied isolates exhibited susceptibility to Levofloxacin. Resistance to this antibiotic compound was detected at a level of 42.30%. From the staphylococci, one isolate (S4), four of *Vibrio* isolates (V2, V8, V17, V15), one *Pseudomonas* isolate (P5), two *Salmonella* isolates (Sa7, OSXLD), and three from *E. coli* isolates (E.C3, EC12, EC16) showed resistance. None of the coliforms were resistant to Levofloxacin.

19.23% of the tested bacterial isolates showed resistance to Ceftriaxone. These isolates were: one *Vibrio* isolate (V2), one *Salmonella* isolate (OSXLD), two *E. coli* isolates (EC12, EC16), and one coliform isolate (MOKO1). None of the *Staphylococcus* and *Pseudomonas* isolates showed resistance. The lowest intermediate susceptibility has been detected in this case. Six bacterial isolates showed intermediate susceptibility.

Antibiotics with different mechanisms inhibit bacterial growth. These compounds prevent the synthesis of proteins involved in metabolism, DNA or RNA synthesis or have a negative impact on membrane permeability. Some bacteria with diverse strategies can avoid these effects. The dissemination of antimicrobial resistance in *Salmonella* species (and also in others) is due to horizontal gene transfer or the clonal spread of isolates. A high level of antibiotic resistance to fluoroquinolones (like Oxofloxacin, Levofloxacin) was detected in these bacterial species (*Ricke & Calo*, 2015). Similarly, the two *Salmonella* isolates exhibited resistance to these types of antibiotics. Resistance to fluoroquinolones can be resulted from target gene mutation or active efflux mechanisms, while the resistance to beta-lactams is caused by the secretion of beta-lactamases. In some reports, multidrug resistance in *Salmonella* species is determined on chromosome or plasmids (*Ricke & Calo*, 2015). It has been shown that serovars of this bacterium with multidrug resistance represent a public health concern. They appear not only in clinical samples, but they have been isolated from different foods (*Ricke & Calo*, 2015).

Vibrios appear in aquatic environments and can cause different infections. Species of this bacterium, such as *V. vulnificus*, have been isolated from different seafoods, for example, shrimp or oysters. *Vibrio casei* has been isolated and described from soft smear cheese (*Baker-Austin*, 2015; *Bleicher et al.*, 2010). It has been shown that tested *V. vulnificus* isolates exhibited different resistance to Amikacin, Ampicillin, and others; however, most of the isolates were susceptible (*Baker-Austin*, 2015). In contrast, in our results, most isolates exhibited resistance to the studied antibiotics. Our results are in accordance with researchers who detected multidrug resistance in these species (*Baker-Austin*, 2015; *Bleicher et al.*, 2010). Also, *V. parahaemolyticus* originating from cockles and seafood products carries varying antibiotic resistance (*Baker-Austin*, 2015). Antibiotic resistance of *V. cholerae* is encoded on plasmids. This strain with antibiotic resistance isolated from milk originated from the lack of good raw-milk-processing practices (*Sharma & Malik*, 2012).

The *Pseudomonas* group is involved in the spoilage of different dairy products. The species of this group usually exhibit resistance to β-lactam antibiotics (*Arslan et al.*, 2011). Our results are in accordance with this in the case of Ampicillin. In the case of Ceftriaxone, the isolates showed susceptibility (*Arslan et al.*, 2011). The *Pseudomonas* isolates showed intermediate susceptibility or strong susceptibility to Amikacin. That was like the results presented by *Arslan et al.* (2011) involving *Pseudomonas* isolates originating from homemade cheese. The mechanisms of antibiotic resistance of *P. aeruginosa* consist of outer membrane permeability, several efflux pumps, and

enzymes that inactivate the antibiotic. Acquired resistance is due to horizontal gene transfer (*Meletis & Bagkeri*, 2013). It has been shown that resistance to fluoroquinolones (Ofloxacin, Levofloxacin) is due to target-site alteration and potential efflux pump (*Meletis &Bagkeri*, 2013).

Food-related coliforms are hygienic and sanitary quality indicators. Most coliform isolates showed susceptibility to the tested antibiotics, what is in contrast with the results of *Zanella et al.* (2010), where raw milk harboured antibiotic-resistant coliforms.

Staphylococcus aureus is a commensal bacterium of the skin and nasal microbiota. It has been isolated from different types of food. It appears due to poor personal hygiene (*Castro et al.*, 2018). Several researchers from all over the world have reported the presence of multiresistant *S. aureus* in raw milk and dairy products (*Normanno et al.*, 2007; *Sasidharan et al.*, 2011; *Jamali et al.*, 2015; *Al-Ashmawy et al.*, 2016). Different mechanisms contribute to the resistance to β-lactams in *S. aureus* such as the production of penicillin-binding proteins. One of these proteins is coded by the *mecA* gene in Methicillin-Resistant Strains (MRS). This gene is spread on a mobile genetic element, designated as *SCCmec*. Diverse types of this genetic element are known in *S. aureus*. Some strains also produce β-lactamase. Other genes that are involved in methicillin and other β-lactam resistance in staphylococci have been described such as *mecB* and *mecC*. Nevertheless, penicillin susceptibility has also been observed in MRS (*Castro et al.*, 2018).

When a microorganism is resistant to more than two antibiotics, we can speak about Multiple Antibiotic Resistance (MAR). It can occur in cases when different antibiotic resistance determinants are spread on the same mobile genetic elements or due to overexpression of genes related to multidrug efflux pumps (*Blanco et al.*, 2016; *Nikaido*, 2009). *Figure 1* shows the Multiple Antibiotic Resistance (MAR) index of the studied bacterial isolates originating from dairy products. The calculation of Multiple Antibiotic Resistance index is a guide for the assessment of health risk (*Davis & Brown*, 2016). The *Staphylococcus* isolates exhibited two antibiotic resistance patterns (A, TStrOL) with MAR indices 0.5 and 0.125. The *Vibrio* isolates MAR index ranged between 0.25 and 0.875. In this case, five antibiotic resistance patterns have been detected (AmTStrCefOLC, AmTStrL, AmStr, AmTStr, AmTTStrCefOL). In the case of the two *Pseudomonas* isolates, the MAR index reached 0.25 and 0.5; the antibiotic resistance pattern of these isolates was AStrOL and AT. The MAR index of *Salmonella* isolates resulted in 0.125–0.5. These isolates exhibited three resistance patterns (AOL, AmCefOLC, A). Bacterial isolates obtained on selective agar medium for *E. coli* exhibited four antibiotic resis-

tance patterns (AAmTOL, AAmTStrCefOLC, T, AmTStrCefOLC) with the MAR index ranging between 0.125 and 1. The coliform isolates' MAR index ranged between 0 and 0.375. These isolates exhibited two antibiotic resistance patterns (TStr, ATC).
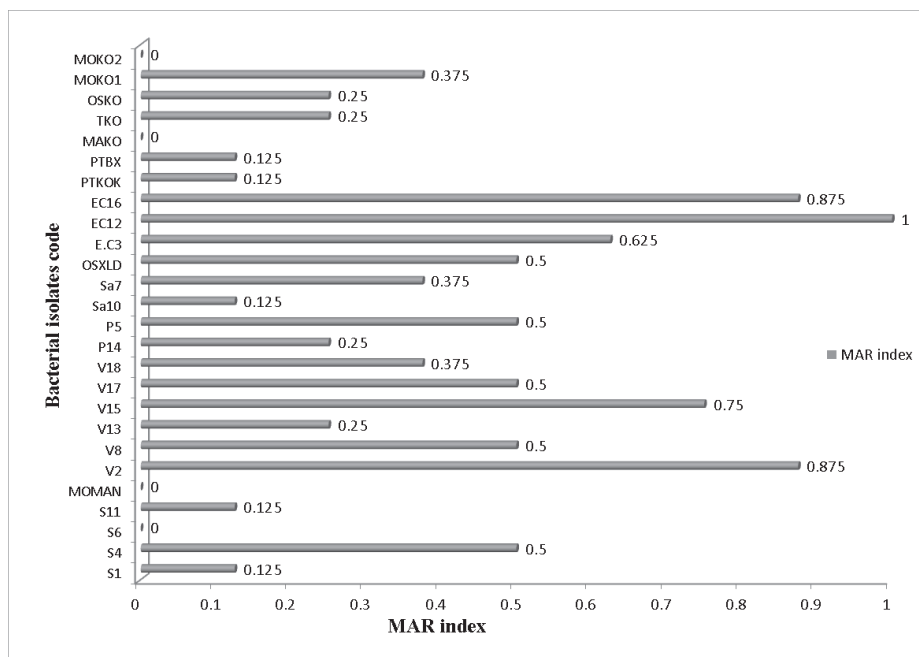

Figure 1: Multiple Antibiotic Resistance (MAR) index values of bacterial isolates

If the value of the MAR index is equal to or higher than 0.2, contamination with high risk is indicated. These values also reveal the frequent use of antibiotics in agriculture, livestock farming. Seven isolates possess values in the interval 0.2–0.4. If the value of the MAR index is equal to or higher than 0.4, faecal sources are indicated (*Adenaike et al.*, 2016). In dairy farming, antibiotics were used for prohylaxis or growth promotion (*Sharma et al.*, 2017). Ten of the studied isolates can be categorized into this group. Bacteria with a value below 0.2 can originate from an environment with less antibiotic usage. Five of the studied isolates belonged to this category. One of the *E. coli* isolates reached the value 1, which means a multidrug-resistant strain. This isolate is originated from an environment where antibiotics were often used.

4 Conclusions

According to the results of the present study, the tested bacterial isolates exhibited different resistance to the tested antibiotics. It can be summarized that the majority of the strains appeared to be susceptible to the tested agents. Also based on the Multiple Antibiotic Resistance index, there are bacterial isolates that are related to a conspicuous antibiotic use for human or animal medicine. This is an important food safety issue that calls for surveillance. Potentially, these strains can be carriers and transmission tools of multidrug resistance.

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