Journal of Current Veterinary Research

ISSN: 2636-4026

Journal home page: http://www.jcvr.journals.ekb.eg

Food safety and public health

Prevalence and Antimicrobial Susceptibility of Ceftiofur-Resistant *Enterobacteriaceae* in Raw Cow's Milk and Kareish Cheese: Implications for Public Health

Asmaa I. El Refaey[1]; Heba Hussein[1]; Rabee A. Ombarak[1]; Nasser H. Abbas[2] and Ahmed M. Hammad[1*].

(1) Department of Food Hygiene and Control, Faculty of Veterinary Medicine, University of Sadat City, Sadat City 32897, Egypt.
(2) Department of Environmental Biotechnology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City 32897, Egypt.

Corresponding author: **hammadfood@gmail.com* *Received: 11/6/2023 Accepted: 16/6/2023*

ABSTRACT

Ceftiofur is primarily approved for the treatment of respiratory tract infections in food-producing animals. The rise of ceftiofur resistance among *Enterobacteriaceae* has allegedly been attributed to the misuse and abuse of this popular antibiotic. In this study, we aimed to investigate the prevalence and antimicrobial susceptibility profiles of ceftiofur-resistant *Enterobacteriaceae* in raw milk and kareish cheese samples collected from Menoufia governorate, Egypt. A total of 80 samples, 40 each of raw cow's milk and kareish cheese, were analyzed by enrichment in *Enterobacteriaceae* enrichment broth supplemented with ceftiofur (8 µg/ml) and plated on violet, red bile glucose agar plates with ceftiofur (8 µg/ml). The isolates were subjected to antimicrobial susceptibility testing against 10 antibiotics. The study found that 90% of raw cow's milk samples and 95% of kareish cheese samples had ceftiofur-resistant *Enterobacteriaceae*. Higher resistance rates to cefepime (80.95%), cefotaxime (95.24%), and ceftriaxone (95.24%) were found among raw milk isolates compared to ciprofloxacin (69.05%) and oxytetracycline (52.38%). Conversely, ceftazidime (14.29%), gentamicin (2.38%), meropenem (2.38%), and colistin (2.38%) showed lower resistance rates. Similarly, *Enterobacteriaceae* isolates of kareish cheese samples showed high resistance rates to cefepime (97.61%), ceftriaxone (78.57%), cefotaxime (71.43%), oxytetracycline (71.43%), and ciprofloxacin (69.05%), but low resistance rates to gentamicin (11.90%) and ceftazidime (9.52%). No resistance was observed to meropenem and colistin. Our findings suggest that raw milk and karish cheese may serve as potential reservoirs for the transmission of ceftiofur-resistant *Ente*robacteriaceae to humans. It is critical to monitor the frequency of ceftiofur-resistant bacteria in food items and modify the legislation controlling the use of ceftiofur in food animals.

Keywords: Antimicrobial resistance, Ceftiofur, Egypt, Enterobacteriaceae, Kareish cheese and Raw milk.

INTRODUCTION

Milk and dairy products are increasingly favored as sources of animal protein due to their high levels of essential nutrients, including high-quality protein, carbohydrates, fats, vitamins, and minerals (Mladenovi *et al*., 2021). However, contamination of raw milk and dairy products with bacteria during milking process or from environmental factors poses a significant risk to public health (Van Asselt *et al*., 2017). *Enterobacteriaceae* is a diverse group of Gram-negative rods that are commonly found in the intestinal microbiota of humans and animals. It comprises several genera, including *Enterobacter*, *Escherichia coli*, *Salmonella*, *Shigella*, *Klebsiella*, *Serratia*, and *Proteus*. They are widely distributed in the environment and can contaminate milk through various sources, as fecal matter, bedding, uncleaned teats, milk bad handling, and contaminated equipment (Cohen *et al*., 2017). As a result, *Enterobacteriaceae* is often used as a reliable indicator of fecal contamination and the sanitary quality of food, particularly dairy products (Martin *et al.*, 2010). Furthermore, the presence of *Enterobacteriaceae* in dairy products can cause changes in product quality, which can render them unsellable and unsuitable for human consumption.

Antimicrobial resistance (AMR) is a phenomenon that describes the ability of microorganisms to withstand the bactericidal effects of antimicrobial treatments, thereby allowing them to survive and proliferate (Matamoros-Recio *et al*., 2021). The use of antibiotics in animals can impact the food industry, as antibiotic-resistant bacteria from animals can be transmitted to food products (Rolain, 2013). Additionally, there is an indirect risk of resistance genes being transferred to harmful microbes at various points in the food chain (Capita and Alonso-Calleja, 2013 and Capita *et al*., 2020). The transfer of resistant microorganisms from animals to humans is a primary concern related to AMR in the context of livestock production, particularly in dairy systems (Sharma *et al*., 2018).

A bacterium that resists many drugs is referred to as a multi-drug resistant organism (MDR). The multi-antibiotic resistance (MAR) index, a cost-effective and valid method, is utilized to track antibiotic-resistant organisms and is calculated by dividing the number of antibiotics to which an isolate is resistant by the total antibiotics to which it is exposed. A MAR index >0.2 indicates a high contamination risk in areas with frequent antibiotic usage (Davis and Brown, 2016; Sandhu *et al*., 2016). *Enterobacteriaceae*'s resistance to multiple antibiotics has become a significant issue in modern medicine (Rock and Donnenberg, 2014). Remarkably, some Enterobacterales are capable of producing enzymes known as extended-spectrum beta-lactamases (ESBLs) that allow it to resistant the routinely used antibiotics, such as penicillins and cephalosporins including third generation cephalosporins, as they are broken down and destroyed by ESBL enzymes, rendering them useless for treating infections. Of note, the World Health Organization's (WHO) most recent list of medically necessary antimicrobials includes third generation cephalosporins as "highest priority critically important antimicrobials." Therefore, it is crucial for public health to continually monitor the emergence and dissemination of antibiotic resistance in food of animal origin (Hammad *et al*., 2022).

Ceftiofur is an appealing antimicrobial drug option for producers due to its broad-spectrum therapeutic range and its parenterally administered formulations have zero hour milk and 9 days pre-slaughter withdrawal periods (Pereira *et al*., 2019). It is commonly used for treating mastitis and

other diseases in dairy cattle (USDA, 2014; Redding *et al*., 2019). A serious public health concern is that the usage of ceftiofur and other drugs in food-producing animals is not regulated in Egypt. Furthermore, it is unclear how the use of ceftiofur in food-producing animals affects the emergence and spread of antibiotic-resistant bacteria, such as *Enterobacteriaceae*, in food products (Wittum, 2012). Therefore, the objective of our study was to investigate the incidence and prevalence of antimicrobial resistance phenotypes among a collection of ceftiofur-resistant *Enterobacteriaceae* isolates obtained from raw cow's milk and kareish cheese samples collected from local markets in Menoufia governorate, Egypt.

MATERIALS AND METHODS

Sample collection, preparation, and transportation

Sample collection was carried out over a four-month period from April to July 2022. A total of 80 samples 40 each of raw cow's milk and kareish cheese samples, were randomly purchased from various sources, including dairy shops, street vendors, and small-scale supermarkets in Menoufia governorate, Egypt. The samples were immediately transferred in aseptic packages and transported, under sterile conditions in an insulated ice box, to the Food Hygiene and Control Laboratory, Faculty of Veterinary Medicine, University of Sadat City, Egypt.

Isolation and identification of Enterobacteriaceae

Each raw milk samples (10 ml) and kareish cheese samples (10 g) were enriched in *Enterobacteriaceae* enrichment broth (EE broth) (Oxoid, UK) supplemented with ceftiofur (8 µg/ml) and incubated for 24 hours at 37°C. The enriched samples were then streaked onto violet, red-bile glucose agar (VRBG agar) (Merck, Germany) plates containing ceftiofur (8 µg/ml) and incubated for 18 to 24 hours under aerobic conditions at 37°C. All colonies showing different colors and morphologies were selected and purified on MacConkey agar plates with ceftiofur (8 µg/ml). Subsequently, Gram staining was performed on positive samples. All *Enterobacteriaceae*-like colonies were subjected to a panel of biochemical tests, which included triple sugar iron agar(TSI) test, lysine iron agar (LIA) test, kligler's iron agar (KIA) test, motility test, hydrogen sulfide test, indole test, methyl red test, voges proskauer test, citrate utilization test, urease test, ornithine decarboxylase test, phenylalanine deaminase test, beta-glucuronidase test, oxidase test, catalase test, and nitrate reduction test, as described by (Brown and Smith, 2017and Procop *et al*., 2017). A 25% glycerol stock was prepared for the bacterial culture and stored at -20°C for future use.

Antimicrobial susceptibility test

All isolates were subjected to antimicrobial susceptibility testing against ten antimicrobials (Oxoid, Hampshire, UK) using a disc diffusion technique according to the Clinical and Laboratory Standards Institute (CLSI) recommendation (CLSI, 2020). The antimicrobials tested, along with their corresponding µg concentrations, were as follows: ceftiofur (30), cefepime (30), cefotaxime (30), ceftazidime (30), ceftriaxone (30), ciprofloxacin (5), gentamicin (30), meropenem (10), colistin (10), and oxytetracycline (30). To determine the multiple antibiotic resistance (MAR) index for each strain, the procedure outlined by Osundiya *et al*. (2013) was followed. This involved dividing the number of antibiotics to which an isolated showed resistance by the total number of antibiotics tested.

Visualization of antibiotic resistance patterns and incidence of isolates

Complex Heat map (v2.6.2) R package (Gu *et al*., 2016) was used to plot a summary heatmap for the presence or absence of resistance to antibiotics. The incidence of isolates was visualized using Excel program.

RESULTS AND DISCUSSION

1. *Prevalence of ceftiofur-resistant Enterobacteriaceae in raw cow's milk and kareish cheese:*

A. Raw cow's milk

The findings of our investigation revealed a high prevalence of *Enterobacteriaceae* in raw milk samples. Out of the 40 raw milk samples analyzed, 36 (90%) were found to be positive for *Enterobacteriaceae*, indicating a potential risk of exposure to antimicrobial-resistant bacteria through the consumption of raw milk and milk products. Interestingly, *Citrobacter youngae* was the most commonly isolated ceftiofur-resistant *Enterobacteriaceae* strain in the raw milk samples (16strains, 36.11%). This may be explained by the overproduction of chromosomal AmpC β-lactamase and reduced outer membrane permeability in *Citrobacter* species (Harris *et al*., 2018). *Klebsiella pneumoniae* was the second most commonly isolated strain, with 8 strains (19.05%) identified. Other strains isolated from the raw milk samples included *Citrobacter braakii* (6 strains, 14.29%), *Citrobacter freundii* (4 strains, 9.52%), *Proteus mirabilis* (3 strains, 7.14%), as outlined in Table (1) and Figure (1-A).The identification of *Klebsiella pneumoniae* as the second most commonly isolated strain is concerning, as this species is known to produce extended-spectrum beta-lactamases (ESBLs) that confer resistance to a broad range of beta-lactam antibiotics, including cephalosporins. Furthermore, the identification of other *Enterobacteriaceae* strains in the raw milk samples, such as *Citrobacter braakii*, *Citrobacter freundii*, and *Proteus mirabilis*, also raises concerns about the potential transmission of antimicrobial-resistant bacteria through milk products.

B. Kareish cheese

The results from this study indicate that kareish cheese, which is a popular soft cheese variety in Egypt, is highly contaminated with ceftiofur-resistant *Enterobacteriaceae*. Specifically, 95% of the examined kareish cheese samples were found positive for these bacteria. The examination of Kareish cheese samples revealed the presence of eight distinct genera of ceftiofur-resistant *Enterobacteriaceae*, namely *Citrobacter*, *Klebsiella*, *Escherichia*, *Enterobacter*, *Yersinia*, *Kluyvera*, *Pantoea*, and *Hafnia*, with *Citrobacter braakii* being the most common species, accounting for 28.57% (12 strains). Different species were identified including *Citrobacter youngae* (14.29%), *Citrobacter amalonaticus* (9.52%), *Citrobacter farmeri* (4.76%), *Citrobacter koseri* (2.38%), *Klebsiella pneumoniae* (14.29%), *E. coli* (7.14%), *Enterobacter gergoviae* (2.38%), *Yersinia pseudotuberculosis* (4.76%), *Yersinia kristensenii* (4.76%), *Kluyvera* spp. (2.38%), *Pantoea agglomerans* (2.38%),and *Hafnia alvei* (2.38%) as presented in Table (1) and Figure (1-B). These findings highlight the potential risk of exposure to antimicrobial-resistant bacteria through the consumption of kareish cheese. Additionally, the identification of a diverse range of bacterial strains in the kareish cheese samples suggests that multiple sources of contamination may be contributing to the prevalence of antimicrobial-resistant bacteria in this milk product.

In comparison to a study conducted by Gaffer *et al*. (2019), our investigation reveals a higher prevalence of antimicrobial-resistant bacteria in kareish cheese samples, specifically ceftiofur-resistant

Enterobacteriaceae including *E. coli*, *Citrobacter* spp., *Klebsiella pneumonia*, *Enterobacter gergoviae*, *Proteus mirabilis*, *Yersinia* spp., *Kluyvera* spp, *Pantoea agglomerans*, and *Hafnia alvei*. In contrast, Gaffer *et al*. (2019) found that only 36% of their kareish cheese samples tested positive for cefotaxime resistant *Enterobacteriaceae* such as *E. coli*, *Klebsiella pneumonia*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Citrobacter diversus*, and *Serratia liquefaciens*. Notably, Sobeih *et al*. (2020) identified *Hafnia*, *E. coli*, and *Serratia marcescens* as the most frequently identified strains in kareish cheese samples, which differs from our study. Of note, *Citrobacter braakii*was the most isolated strain of ceftiofur-resistant *Enterobacteriaceae* in our samples Dutil *et al*. (2010) observed a higher prevalence of ceftiofur-resistant commensal *E. coli* in Canadian retail chicken samples compared to ceftiofur-resistant Salmonella Heidelberg. This disparity in findings might be attributed to variances in the types of samples analyzed, as well as discrepancies in sanitary practices during food manufacturing and handling.

Table (1): Incidence of ceftiofur-resistant *Enterobacteriaceae* isolated from raw milk and kareish cheese.

	Raw milk	**Kareish cheese**
***Enterobacteriaceae* species**	**Numbers of isolated strains (%) (n=42)**	**Numbers of isolated strains (%) (n=42)**
E. coli	0	3 (7.14%)
Citrobacter youngae	16 (38.09%)	6 (14.29%)
Citrobacter braakii	6 (14.29%)	12 (28.57%)
Citrobacter freundii	4 (9.52%)	0
Citrobacter amalonaticus	0	4 (9.52%)
Citrobacter farmeri	1 (2.38%)	2 (4.76%)
Citrobacter koseri	1 (2.38%)	1 (2.38%)
Klebsiella pneumoniae	8 (19.05%)	6 (14.29%)
Enterobacter gergoviae	1 (2.38%)	1 (2.38%)
Proteus mirabilis	3 (7.14%)	0
Yersinia pseudotuberculosis	1 (2.38%)	2 (4.76%)
Yersinia kristensenii	1 (2.38%)	2 (4.76%)
Kluyvera spp.	0	1 (2.38%)
Pantoea agglomerans	0	1 (2.38%)
Hafnia alvei	0	1 (2.38%)

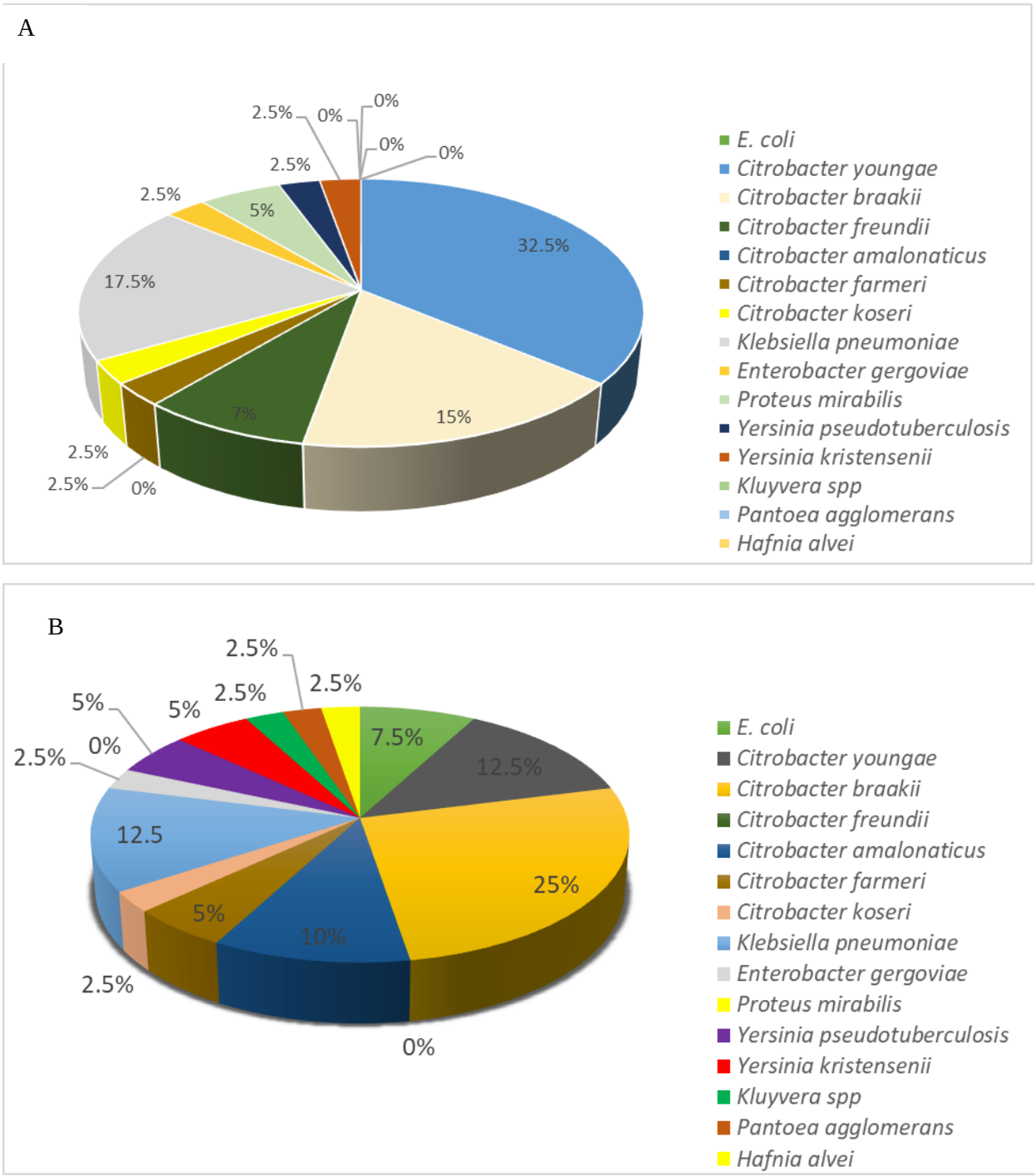

Figure (1): Incidence of ceftiofur-resistant *Enterobacteriaceae* among the examined raw milk (A) and kareish cheese (B) samples.

2- Antimicrobial resistance among Enterobacteriaceae:

A- Raw milk

Table (2) presents a comprehensive analysis of the antimicrobial resistance of ceftiofur-resistant *Enterobacteriaceae* in raw milk samples. Our investigation of raw milk samples revealed that the isolated

Enterobacteriaceae exhibited a higher degree of resistance to cefepime (97.61%), cefotaxime (95.24%), and ceftriaxone (95.24%) compared to ciprofloxacin (69.05%) and oxytetracycline (52.38%). Moreover, resistance to ceftazidime, gentamicin, meropenem, and colistin was relatively low with percentages of 14.29%, 2.38%, 2.38%, and 2.38%, respectively. The data presented in Table 2 underscore the high degree of antimicrobial resistance in *Enterobacteriaceae* isolated from raw milk samples, with resistance to modern beta-lactam antibiotics being particularly notable. This could be attributed to their frequent use in animal husbandry. The observed resistance to other antibiotics such as ciprofloxacin and oxytetracycline suggests the potential overuse and misuse of these antibiotics in animal production and/or co-selection because of using ceftiofur.

Notably, *Citrobacter youngae* isolates from raw milk exhibited high resistance rates to seven antibiotics, with the highest resistance rates observed for cefotaxime, ceftriaxone, and cefepime (100% each), followed by oxytetracycline and ciprofloxacin (68.75% each), and gentamycin (6.25%). Similarly, *Citrobacter braakii* isolates from raw milk were highly resistant to all seven antibiotics, except for ciprofloxacin, which showed resistance rate of 50%. *Citrobacter freundii* isolates from raw milk also showed high resistance rates to cephalosporins (100%) and ciprofloxacin (75%), with lower resistance rates to ceftazidime, oxytetracycline, and meropenem (25% each). Moreover, *Citrobacter farmeri* and *Citrobacter koseri* isolates from raw milk demonstrated high resistance rates to ceftiofur, cefepime, ceftriaxone, cefotaxime, and ciprofloxacin (100% each) in addition to oxytetracycline (100 %) for *Citrobacter koseri*. Adegun *et al*. (2019) also reported resistance to ceftriaxone, cefotaxime, and gentamycin in *Citrobacter* species isolated from fruits and vegetables.

Klebsiella pneumoniae is a pathogen of significant concern as it is known to cause both hospital-acquired infections and foodborne illnesses including septicemia, liver abscesses, and diarrhea, which have been increasingly reported in recent years. Despite this, there remains a limited understanding of the characteristics of *K. pneumoniae* isolated from food sources (Zhang *et al*., 2018). Our study reveals that isolated *K. pneumoniae* strains (n=8) obtained from raw milk samples displayed high levels of resistance to several antibiotics, including cefotaxime, ceftriaxone, and cefepime (100% each), followed by ciprofloxacin, oxytetracycline (87.5% each), ceftazidime, and colistin (12.5% each). Enferad and Mahdavi (2020) similarly found resistance to multiple antibiotics in *K. pneumoniae* isolated from raw milk samples in Iran. Furthermore, our study identified high levels of resistance to various antibiotics such as cefotaxime, ceftriaxone, cefepime, ciprofloxacin, and oxytetracycline (100 % each) in *Enterobacter gergoviae* however, were sensitive to ceftazidime, colistin, meropenem, and gentamycin. *Proteus mirabilis*, known to cause urinary tract infections in humans, displayed high levels of resistance to ceftiofur, ceftriaxone, cefotaxime, and cefepime (100% each) in our study, while exhibiting sensitivity to gentamycin, ceftazidime, ciprofloxacin, and meropenem. Ronanki *et al*. (2022) similarly reported sensitivity to gentamycin, ceftazidime, and ceftriaxone in *P. mirabilis* isolates from milk samples. *Yersinia*, a foodborne pathogen with multiple species, was also found to be present in raw milk samples, with *Yersinia pseudotuberculosis* showed notable resistance levels to ciprofloxacin, ceftiofur, and oxytetracycline (100% each), while *Yersinia kristensenii*

showed significant resistance to ciprofloxacin and cefepime (100% each). Jamali *et al*. (2015) similarly reported resistance to tetracycline and ciprofloxacin in *Yersinia* isolates from food samples.

Interestingly, several strains showed multidrug resistance phenotype. Overall, out of 84 isolates, 44.05% (37/84) exhibited resistance to one or two categories of antimicrobials, with MAR index values ranging from 0.1 to 0.6, while 55.95% (47/84) showed resistance to three or four categories and were classified as MDR. Notably, 45.45% (20/44) of the MDR isolates in raw milk displayed resistance to three categories of antimicrobials with MAR index values ranging from 0.6 to 0.7. This finding contrasts with the results of previous studies that reported lower rates of MDR *Enterobacteriaceae* in milk samples (Amador*et al*., 2011 and Sadi *et al*., 2014).

Of note, as illustrated in Figure 2, a total of 9 resistance patterns were observed in raw milk, with the most common being the resistance pattern "CFM (cefepime) + CRO (ceftriaxone) + CTX (cefotaxime) + EFT (ceftiofur) + CIP (ciprofloxacin) + OT (oxytetracycline)." This resistance pattern was exhibited by *Citrobacter youngae* (n=7), *Citrobacter freundii* (n=1), *Citrobacter koseri* (n=1), *Klebsiella pneumonia* (n=6), and *Enterobacter gergoviae* (n=1) (Figure 2). The emergence of resistance to carbapenems in *Enterobacteriaceae* is concerning, including an isolate of *Citrobacter freundii* from raw milk that exhibited resistance to meropenem and other antibiotics. This isolate displayed a considerable multidrug resistance pattern of "CAZ (ceftazidime) + CFM (cefepime) + CRO (ceftriaxone) + CTX (cefotaxime) + EFT (ceftiofur) + MEM (meropenem) + CIP (ciprofloxacin)".

Table (2): Antimicrobial resistant *Enterobacteriaceae* isolated from raw milk samples:

	Number and percentages of antimicrobial-resistant isolates										
***Enterobacteriaceae* species**	**Number of isolates**	**EFT***	**CTX**	**CRO**	**CFM**	**CAZ**	**CT**	**CIP**	**OT**	**CN**	**MEM**
C. youngae	16	16(100%)	16(100%)	16(100%)	16(100%)	4(25%)	0	11(68.75%)	11(68.75%)	1(6.25%)	0
C. braakii	6	6(100%)	6(100%)	6(100%)	6(100%)	0	0	3	0	0	0
C. freundii	4	4(100%)	4(100%)	4(100%)	4(100%)	1(25%)	0	3	1(25%)	0	1(25%)
C. farmeri	1	1(100%)	1(100%)	1(100%)	1(100%)	0	0	1(100%)	0	0	0
C. koseri	1	1(100%)	1(100%)	1(100%)	1(100%)	0	0	1(100%)	1(100%)	0	0
K. pneumoniae	8	8(100%)	8(100%)	8(100%)	8(100%)	1(12.5%)	1(12.5%)	7(87.5%)	7(87.5%)	0	0
E. gergoviae	1	1(100%)	1(100%)	1(100%)	1(100%)	0	0	1(100%)	1(100%)	0	0
P. mirabilis	3	3(100%)	3(100%)	3(100%)	3(100%)	0	0	0	0	0	0
Y. pseudotuberculosis	1	1(100%)	0	0	0	0	0	1(100%)	1(100%)	0	0
Y. kristensenii	1	1(100%)	0	0	1(100%)	0	0	1(100%)	0	0	0
Total number	42	42	40	40	41	6	1	29	22	1	1
Percentage %		100 %	95.24%	95.24%	97.61%	14.29%	2.38%	69.05%	52.38%	2.38%	2.38%

**EFT, ceftiofur; CAZ, ceftazidime; CFM, cefepime; CIP, ciprofloxacin; CN, gentamycin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CT, colistin; CIP, ciprofloxacin; MEM, meropenem; OT, oxytetracycline.*

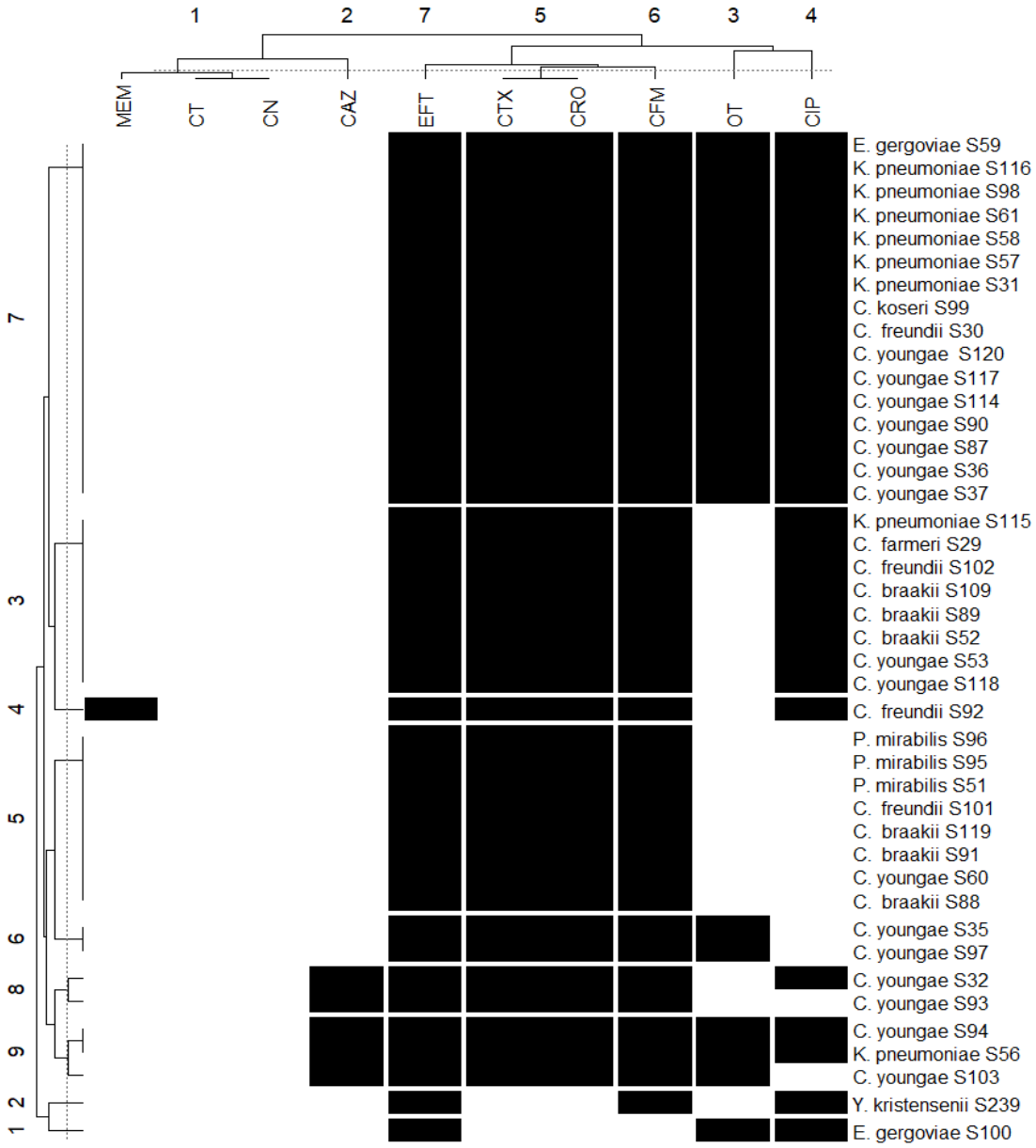

Fig (2): Heat map showing antimicrobial resistant patterns of *Enterobacteriaceae* strains isolated from raw milk. Black color indicates resistance to antibiotic and white color indicates susceptibility to antibiotic. EFT, ceftiofur; CAZ, ceftazidime; CFM, cefepime; CIP, ciprofloxacin; CN, gentamycin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CT, colistin; CIP, ciprofloxacin; MEM, meropenem; OT, oxytetracycline.

B. *Kareish cheese*

The results for the susceptibility testing of ceftiofur-resistant *Enterobacteriaceae* in kareish cheese samples are presented in Table (3). The *Enterobacteriaceae* strains obtained from kareish cheese samples exhibited high levels of resistance to cefepime (80.95%), ceftriaxone (78.57%), cefotaxime (71.43%), oxytetracycline (71.43%), and ciprofloxacin (69.05%). On the other hand, a lower level of resistance was observed for gentamicin (11.90%) and ceftazidime (9.52%). Notably, none of the *Enterobacteriaceae* strains were resistant to

meropenem and colistin. The results of our study showed that *E. coli* isolates from kareish cheese exhibited high rates of resistance to multiple antimicrobials, particularly to third generation cephalosporins (100% each) (ceftiofur, ceftriaxone, and cefotaxime), fourth-generation cephalosporin (cefepime100%), oxytetracycline, and ciprofloxacin (33.33% each). The high frequencies of resistance to these antibiotics in kareish cheese *E. coli* isolates are comparable with recent research in Egypt and Romania (Ombarak *et al*., 2018 and Imre *et al*., 2022).

Similarly, *Klebsiella pneumoniae* isolates from kareish cheese showed high resistance rates to multiple antibiotics including ceftriaxone, ciprofloxacin (100 %) followed by cefotaxime (83.33%), cefepime (66.67 %), ceftazidime (33.33 %) and gentamycin (16.67 %). These findings are in line with previous studies from Egypt (Abdel Hameed, 2017and Gaffer *et al.*, 2019). *Enterobacter gergoviae*, *Pantoea agglomerans*, *Hafnia alvei*, *Kluyvera* spp., and *Citrobacter* isolates also exhibited high rates of resistance to multiple antibiotics, including resistance to cefotaxime, ceftriaxone, cefepime, ciprofloxacin, and oxytetracycline. *Pantoea agglomerans* and *Hafnia alvei* isolates from kareish cheese, on the other hand, showed substantial resistance to ceftriaxone, ceftriaxone, cefotaxime, cefepime, oxytetracycline, and ciprofloxacin, with a resistance rate of 100% for each drug. Similarly, *Kluyvera* spp. showed resistance to the same antibiotics, except for ciprofloxacin, to which it was found to be susceptible. The observed high levels of resistance to multiple antibiotics in *Enterobacter gergoviae*, *Pantoea agglomerans*, *Hafnia alvei*, *Kluyvera* spp., and *Citrobacter* isolates from kareish cheese are consistent with previous studies (van Hoek *et al*., 2015 and Amreen *et al*., 2022).

However, it is important to note that the resistance patterns observed in our study and previous studies may vary depending on the geographic location, the source of the cheese, and the production process. In our study, 24 out of 44 isolates from kareish cheese (54.55%) exhibited resistance to three categories of antimicrobials and were therefore classified as MDR. Additionally, three isolates (3/84, 3.57%) from kareish cheese showed resistance to four categories of antimicrobials, with MAR index values ranging from 0.6 to 0.7. We identified 20 resistance patterns in kareish cheese, with the most common being "CFM (cefepime) + CRO (ceftriaxone) + CTX (cefotaxime) + EFT (ceftiofur) + CIP (ciprofloxacin) + OT (oxytetracycline)." This resistance pattern was exhibited by *E. coli* (n=1), *Citrobacter youngae* (n=1), *Citrobacter braakii* (n=5), *Citrobacter amalonaticus* (n=1), *Klebsiella pneumonia* (n=4), *Enterobacter gergoviae* (n=1), *Yersinia kristensenii* (n=1), *Hafnia alvei* (n=1), and *Pantoea agglomerans* (n=1) (Figure 3).The high prevalence of antibiotic-resistant bacteria found in kareish cheese isolates is a major cause for concern as this type of cheese is typically consumed without any additional treatment. This poses a risk of severe infections in humans, especially those with weakened immune systems. The causes of such resistance can be attributed to several factors, including the use of antibiotics in animal agriculture, the improper use of antibiotics in human medicine, and the presence of antibiotic-resistant bacteria in the environment. Fortunately, based on our results, meropenem and colistin remains the most effective antibiotics against ceftiofur-resistant *Enterobacteriaceae*.

Table (3): Antimicrobial resistant *Enterobacteriaceae* isolated from kareish cheese samples:

	Number and percentages of antimicrobial-resistant isolates										
***Enterobacteriaceae* species**	**Number of isolates**	**EFT**	**CTX**	**CRO**	**CFM**	**CAZ**	**CT**	**CIP**	**OT**	**CN**	**MEM**
E. coli	3	3(100%)	3(100%)	3(100%)	3(100%)	0	0	1(33.33%)	3(100%)	0	0
C. youngae	6	6(100%)	3(50%)	4(66.67%)	5(83.33%)	0	0	3(50%)	3(50%)	1(16.67%)	0
C. braakii	12	12(100%)	9(75%)	10(83.33%)	12(100%)	1(8.33%)	0	9(75%)	10(83.33%)	1 (18.33%)	0
C. amalonaticus	4	4(100%)	2(50%)	3(75%)	2(50%)	0	0	2(50%)	2(50%)	2(50%)	0
C. farmeri	2	2(100%)	2(100%)	1(50%)	2(100%)	0	0	1(50%)	1(50%)	0	0
C. koseri	1	1(100%)	0	0	0	0	0	0	0	0	0
K. pneumoniae	6	6(100%)	5(83.33%)	6(100%)	4(66.67%)	2(33.33%)	0	6(100%)	6(100%)	1(16.67%)	0
E. gergoviae	1	1(100%)	1(100%)	1(100%)	1(100%)	0	0	1(100%)	1(100%)	0	0
Y. pseudotuberculosis	2	2(100%)	1(50%)	1(50%)	1(50%)	0	0	2(100%)	1(50%)	0	0
Y. kristensenii	2	2(100%)	1(50%)	1(50%)	1(50%)	0	0	1(50%)	1(50%)	0	0
Kluyvera spp	1	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	0	1(100%)	0	0	0
P. agglomerans	1	1(100%)	1(100%)	1(100%)	1(100%)	0	0	1(100%)	1(100%)	0	0
H. alvei	1	1(100%)	1(100%)	1(100%)	1(100%)	0	0	1(100%)	1(100%)	0	0
Total number	42	42	30	33	34	4	0	29	30	5	0
Percentage %		100%	71.43%	78.57%	80.95%	9.52%	0%	69.05%	71.43%	11.90%	0%

EFT, ceftiofur; CAZ, ceftazidime; CFM, cefepime; CIP, ciprofloxacin; CN, gentamycin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CT, colistin; CIP, ciprofloxacin; MEM, meropenem; OT, oxytetracyclin.

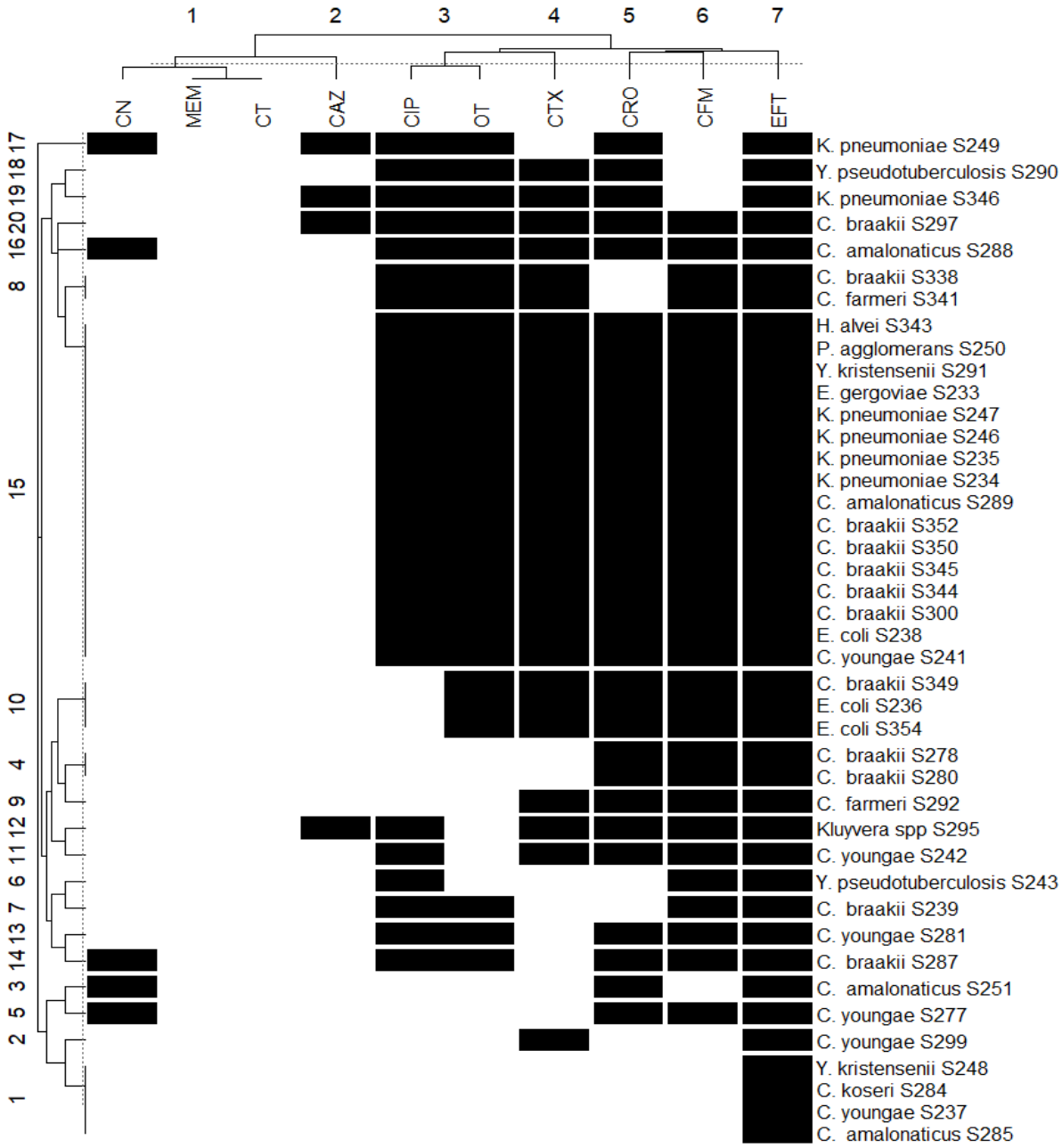

Fig (3): Heat map showing antimicrobial resistant patterns of *Enterobacteriaceae* strains isolated from Kareish cheese. Black color indicates resistance to antibiotic and white color indicates susceptibility to antibiotic. EFT, ceftiofur; CAZ, ceftazidime; CFM, cefepime; CIP, ciprofloxacin; CN, gentamycin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; CT, colistin; CIP, ciprofloxacin; MEM, meropenem; OT, oxytetracycline.

CONCLUSION

Our findings suggest that raw milk and karish cheese may serve as potential reservoirs for the transmission of ceftiofur-resistant *Enterobacteriaceae* to humans. It is critical to monitor the frequency of

ceftiofur-resistant bacteria in food items and modify the legislation controlling the use of ceftiofur in food animals. It is worth noting that there are many policies exist in Egypt which restrict the spread of multi-antibiotic-resistant bacteria in the food chain but, are either not enforced or are ignored. On the other hand, considering that *Enterobacteriaceae* is an enteric indicator of the general hygiene status of milk products (Vincenti *et al*., 2018), their high incidence detected in this study highlights the importance of the application of rigorous hygiene measures throughout the milk production process. The Egyptian authorities should establish legislation prohibiting the sale and consumption of retail raw milk and raw milk cheese.

REFERENCES

Abdel Hameed, K. G. (2017): Widespread acquisition of antimicrobial resistance of *Klebsiella pneumoniae* isolated from raw milk and the effect of cinnamon oil on such isolates. International food Food Research Journal, 24(2): 876-880.

Adegun, B. R.; Oluduro, A. O. andAregbesola, O. A. (2019): Isolation and molecular characterization of *Citrobacter* species in fruits and vegetables sold for consumption in ILE-IFE, Nigeria. Scientific African, 6, e00173.

Amador, P.; Fernandes, R.;Brito, L., andPrudencio, C. (2011): Antibiotic resistance in *Enterobacteriaceae* isolated from Portuguese deli meats. Journal of Food Safety, 31(1):1-20.

Amreen, F.; Puttananjaiaha, M. H., and Viswanath, P. (2022): Quarantine and antibiotic susceptibility of *Enterobacteriaceae* strains and other gram-negative bacteria in dairy sweetmeat milk (doodh) peda. Research square, 1974174/v2. https://doi.org/10.21203/rs.3.rs-1974174/v2.

Brown A. E. and Smith H. (2017): Benson's microbiological applications laboratory manual in general microbiology (14th ed. Concise version). McGraw-Hill Education.

Capita, R., and Alonso-Calleja, C. (2013): Antibiotic-resistant bacteria: a challenge for the food industry. Critical Reviews in Food Science and Nutrition, 53(1):11-48.

Capita, R.; Castaño-Arriba, A.; Rodríguez-Melcón, C.; Igrejas, G.; Poeta, P. and Alonso-Calleja, C. (2020): Diversity, antibiotic resistance, and biofilm-forming ability of enterobacteria isolated from red meat and poultry preparations. Microorganisms, 8(8), 1226.

CLSI. (2020): Performance Standards for Antimicrobial Susceptibility Testing, 30th ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2020.

Cohen, I.; Powderly, W.G. and Opal, S.M. (2017): Infectious diseases, 4 th Ed. El Sevier Ltd.

Davis, R., & Brown, P. D. (2016): Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica. Journal of Medical Microbiology, 65(4): 261-271.

Dutil, L.; Irwin, R. J.; Finley, R.; Ng, L. K.; Avery, B. P.; Boerlin, P and Pillai, D. R. (2010): Ceftiofur resistance in Salmonella enterica serovar heidelberg from chicken meat and humans, Canada Emerging Infectious Diseases, 16(1):48-54. https://doi.org/10.3201/eid1601.090729.

Enferad, E., and Mahdavi, S. (2020): Antibiotic resistance pattern and

frequency of some beta lactamase genes in *Klebsiella pneumoniae* isolated from raw milk samples in Iran. Journal of the Hellenic Veterinary Medical Society, 71(4):2455-2462.

Gaffer, W.; Gwida, M.; Abo Samra, R., and Al- Ashmawy, M. (2019): Occurrence and molecular characterization of extended spectrum beta-lactamase producing *Enterobacteriaceae* in milk and some dairy products. Slovenian Veterinary Research, 56(22-Suppl). https://doi.org/10.26873/SVR-785-2019.

Gu, Z.; Eils, R., and Schlesner, M. (2016): Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics (Oxford, England), 32(18):2847–2849. https://doi.org/10.1093/bioinformatics/btw313.

Hammad, A. M.; Eltahan, A.; Hassan, H. A.; Abbas, N. H.; Hussien, H., and Shimamoto, T. (2022): Loads of coliforms and fecal coliforms and characterization of thermotolerant *Escherichia coli* in fresh raw milk cheese. Foods, 11(3):332.

Harris P. N. (2015): Clinical management of infections caused by *Enterobacteriaceae* that express extended-spectrum β-lactamase and AmpC enzymes. Seminars in Respiratory and Critical Care Medicine, 36(1), 56–73. https://doi.org/10.1055/s-0034-1398387.

Imre, K.; Ban-Cucerzan, A.; Herman, V.; Sallam, K. I.; Cristina, R. T.; Abd-Elghany, S. M.; Morar, D.; Popa, S. A.; Imre, M., and Morar, A. (2022): Occurrence, pathogenic potential and antimicrobial resistance of *Escherichia coli* isolated from raw milk cheese commercialized in Banat region,
Romania. Antibiotics, 11(6):721.

Jamali, H.; Paydar, M.; Radmehr, B., and Ismail, S. (2015): Prevalence, characterization, and antimicrobial resistance of *Yersinia* species and *Yersinia enterocolitica* isolated from raw milk in farm bulk tanks. Journal of Dairy Science, 98(2):798-803.

Martín, M. C.; Martínez, N.; Del Rio, B.; Ladero, V.; Fernández, M., and Alvarez, M. A. (2010): A novel real-time polymerase chain reaction-based method for the detection and quantification of lactose-fermenting *Enterobacteriaceae* in the dairy and other food industries. Journal of Dairy Science, 93(3):860-867.

Matamoros-Recio, A.; Franco-Gonzalez, J. F.; Forgione, R. E.; Torres-Mozas, A.; Silipo, A., and Martín-Santamaría, S. (2021): Understanding the antibacterial resistance: Computational explorations in bacterial membranes. ACS Omega, 6(9):6041-6054.

Mladenović, K. G.; Grujović, M.; Kiš, M.; Furmeg, S.; Tkalec, V. J.; Stefanović, O. D., and Kocić-Tanackov, S. D. (2021): *Enterobacteriaceae* in food safety with an emphasis on raw milk and meat. Applied Microbiology and Biotechnology, 105(23): 8615–8627. https://doi.org/10.1007/s00253-021-11655-7.

Ombarak, R. A.; Hinenoya, A.; Elbagory, A. R. M., and Yamasaki, S. (2018): Prevalence and molecular characterization of antimicrobial resistance in *Escherichia coli* isolated from raw milk and raw milk cheese in Egypt. Journal of Food Protection, 81(2): 226-232.

Osundiya, O. O.; Oladele, R. O., and Oduyebo, O. O. (2013): Multiple antibiotic resistance (MAR) indices of *Pseudomonas* and *Klebsiella* species isolates in Lagos University Teaching Hospital. African Journal of Clinical and Experimental Microbiology, 14(3):164-168.

Pereira, R.; Williams, D. R.; Rossitto, P.; Adaska, J.; Okello, E.; Champagne, J.; Lehenbauer, T. W.; Li, X.; Chase, J.; Nguyen, T.; Pires, A. F. A; Atwill, E. R., and Aly, S. S. (2019): Association between herd management practices and antimicrobial resistance in *Salmonella* spp. from cull dairy cattle in Central California. PeerJ, 7, e6546. https://doi.org/10.7717/peerj.6546.

Procop, G. W.; Church, D. L.; Hall, G. S.; Janda, W. M.; Koneman, E. W.; Schreckenberger, P. C., and Woods, G. L. (2017): The nonfermentative gram-negative bacilli. Koneman's color atlas and textbook of diagnostic microbiology. 7th ed. Philadelphia: Lippincott Williams & Wilkins, 317-431.

Redding, L. E.; Bender, J., and Baker, L. (2019): Quantification of antibiotic use on dairy farms in Pennsylvania. Journal of Dairy Science, 102(2):1494-1507.

Rock, C., and Donnenberg, M. S. (2014): Human pathogenic *Enterobacteriaceae*. Reference module in biomedical sciences. https://doi.org/10.1016/B978-0-12-801238-3.00136-7.

Rolain, J. M. (2013): Food and human gut as reservoirs of transferable antibiotic resistance encoding genes. Frontiers in Microbiology, 4, 173.

Ronanki, S. P.; Ramya, P.; Babu, A. J., and Sreedevi, B. (2022): Multidrug resistant-*Proteus mirabilis* and *Proteus vulgaris* isolated from milk and meat samples in Tirupati, Andhra Pradesh: An emerging public health threat. The Pharma Innovation Journal. 2022; 11(11S): 1427-1434.

Saidi, R.; Khelef, D., and Kaidi, R. (2014): Antibiotic susceptibility of *Enterobacteriaceae* species isolated from mastitic milk in Algeria. Asian Pacific Journal of Reproduction, 3(4):311-316.

Sandhu, R.; Dahiya, S., and Sayal, P. (2016): Evaluation of multiple antibiotic resistance (MAR) index and Doxycycline susceptibility of *Acinetobacter* species among inpatients. Indian J. Microbiol. Research, 3(3):299.

Sharma, C.; Rokana, N.; Chandra, M.; Singh, B. P.; Gulhane, R. D.; Gill, J. P. S.; Ray, P.; Puniya A.K., and Panwar, H. (2018): Antimicrobial resistance: its surveillance, impact, and alternative management strategies in dairy animals. Frontiers in Veterinary Science, 4, 237.

Sobeih, A. M.; AL-Hawary, I.; Khalifa, E., and Ebied, N. (2020): Prevalence of *Enterobacteriaceae* in raw milk and some dairy products. Kafrelsheikh Veterinary Medical Journal, 18(2):9-13.

USDA. Diary 14 Part III. Health and Management Practices on U.S. Dairy Operations, 2014; USDA-APHIS-VS-CEAHNAHMS, Fort Collins, CO, USA; USDA: Washington, DC, USA, 2018.

Van Asselt, E. D.; Van der Fels-Klerx, H. J.; Marvin, H. J. P.; Van Bokhorst-Van de Veen, H., and Groot, M. N. (2017): Overview of food safety hazards in the European dairy supply Chain. Comprehensive Reviews in Food Science and Food

Safety, 16(1):59–75. https://doi.org/10.1111/1541-4337.12245.

Van Hoek, A. H.; Schouls, L.; Van Santen, M. G.; Florijn, A.; De Greeff, S. C., and Van Duijkeren, E. (2015): Molecular characteristics of extended-spectrum cephalosporin-resistant *Enterobacteriaceae* from humans in the community. PloS One, 10(6):e0129085. https://doi.org/10.1371/journal.pone.0129085.

Vincenti, S.; Raponi, M.; Sezzatini, R.; Giubbini, G., and Laurenti, P. (2018): *Enterobacteriaceae* antibiotic resistance in ready-to-eat foods collected from hospital and community canteens: analysis of prevalence. Journal of Food Protection, 81(3):424-429.

Wittum, T. E. (2012): The challenge of regulating agricultural ceftiofur use to slow the emergence of resistance to extended spectrum cephalosporins. Applied and Environmental Microbiology, 78(22), 7819-7821.

Zhang, S.; Yang, G.; Ye, Q.; Wu, Q.; Zhang, J., and Huang, Y. (2018): Phenotypic and genotypic characterization of *Klebsiella pneumoniae* isolated from retail foods in China. Frontiers in Microbiology, 9, 289.