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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

Isolation of *Escherichia coli* and *Klebsiella pneumoniae* from Raw Milk, Phenotypic Investigation of ESBL, AmpC and Carbapenemase Resistance, and Evaluation of the Antimicrobial Activity of Selected Antimicrobial Agents

Çiğ Sütten Escherichia coli ve Klebsiella pneumoniae İzolasyonu, ESBL, AmpC ve Karbapenemaz Direncinin Fenotipik İncelenmesi ve Seçilmiş Antimikrobiyal Ajanların Antimikrobiyal Aktivitesinin Değerlendirilmesi

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ABSTRACT

Introduction: While milk and dairy products are essential contributors to human nutritional intake, they simultaneously serve as potential vehicles for the dissemination of zoonotic agents. Members of the *Enterobacteriaceae* family, such as *Escherichia coli* and *Klebsiella pneumoniae*, present in raw milk, pose a public health threat due to the production of extended-spectrum beta-lactamases (ESBL), AmpC, and carbapenemases.

Material and Methods: A total of 200 raw milk samples collected from farms in Ankara, Türkiye and surrounding regions were microbiologically analyzed. Isolated bacteria were identified using biochemical tests, and their ESBL, AmpC, and carbapenemase activities were evaluated phenotypically. Antibiotic susceptibility was assessed using the Kirby–Bauer disk diffusion method. In addition, the antimicrobial activities of hydrogen peroxide, acetic acid, benzoic acid, nisin, natamycin, lactoferrin, sorbic acid, and propionic acid were examined using the agar well diffusion method.

Results: The overall isolation rate of *E.coli* and *K. pneumoniae* was 10%, with 60% of the isolates identified as *K. pneumoniae* and 40% as *E.coli*. Among the isolates, 40% were ESBL-positive; all ESBL-positive isolates exhibited AmpC resistance, while 50% demonstrated carbapenemase activity. Moreover, 75% of the isolates were resistant to at least one antibiotic, and 45% were multidrug-resistant. A significant association was observed between ESBL positivity and multidrug resistance. Among the antimicrobial agents tested, hydrogen peroxide was the most effective, while acetic and benzoic acids exhibited moderate activity.

Conclusion: The presence of multidrug-resistant bacteria producing ESBL, AmpC, and carbapenemases in raw milk indicates a potential risk for the dissemination of antibiotic resistance genes through the food chain. Therefore, ensuring hygiene during milk production, controlling antibiotic usage, and evaluating natural antimicrobial agents as protective additives are of critical importance.

Keywords: *E.coli, K. pneumoniae*, ESBL, carbapenemase, antimicrobial agents

ÖZ

Giriş: Süt ve süt ürünleri, insan beslenmesinde temel bir yere sahip olup, zoonotik patojenlerin bulaşması açısından da önemli bir risk kaynağıdır. Çiğ sütlerde bulunan *Escherichia coli* ve *Klebsiella pneumoniae* gibi *Enterobacteriaceae* üyeleri, genişlemiş spektrumlu beta-laktamaz (GSBL), AmpC ve karbapenemaz üretimiyle halk sağlığı açısından tehdit oluşturmaktadır.

Materyal ve Metodlar: Ankara ve çevresindeki çiftliklerden toplanan 200 çiğ süt örneği mikrobiyolojik olarak incelenmiştir. İzole edilen bakteriler biyokimyasal testlerle tanımlanmış, GSBL, AmpC ve karbapenemaz aktiviteleri fenotipik yöntemlerle değerlendirilmiştir. Antibiyotik duyarlılık testleri Kirby–Bauer disk difüzyon yöntemiyle yapılmış; ayrıca hidrojen peroksit, asetik asit, benzoik asit, nisin, natamisin, laktoferrin, sorbik ve propiyonik asitlerin antimikrobiyal etkinlikleri agar well difüzyon yöntemiyle incelenmiştir.

Bulgular: Toplam *Escherichia coli* ve *Klebsiella pneumoniae* izolasyon oranı %10 olup, bu izolatların %60'ı *K. pneumoniae*, %40'ı *E. coli*'dir. İzolatların %40'ı GSBL pozitifken, GSBL pozitif izolatların %100'ünde AmpC direnci yönünden pozitiflik, %50'sinde ise karbapenemaz aktivitesi yönünden pozitiflik tespit edilmiştir. Suşların %75'i en az bir antibiyotiğe, %45'i çoklu ilaçlara dirençlidir. Genişlemiş spektrumlu beta-laktamaz pozitifliği ile MDR arasında anlamlı ilişki saptanmıştır. Antimikrobiyal katkılar arasında hidrojen peroksit en etkili madde olarak belirlenmiş, asetik ve benzoik asit orta düzey aktivite göstermiştir.

Sonuç: Çiğ sütlerde GSBL, AmpC ve karbapenemaz üreten çoklu dirençli bakterilerin varlığı, gıda zinciri yoluyla antibiyotik direnç genlerinin yayılma riskini göstermektedir. Bu nedenle süt üretiminde hijyenin sağlanması, antibiyotik kullanımının kontrolü ve doğal antimikrobiyal maddelerin koruyucu olarak değerlendirilmesi önem taşımaktadır.

Anahtar Sözcükler: *E. coli*, *K. pneumoniae*, GSBL, karbapenemaz, antimikrobiyal ajanlar

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Introduction

Milk and dairy products are indispensable sources of carbohydrates, proteins, vitamins, and essential fats for humans, playing a crucial role in daily nutrition (1). The transmission of zoonotic microorganisms through milk, particularly due to the consumption of unpasteurized dairy products or milk subjected to insufficient pasteurization, poses a significant risk to public health (2). Emerging evidence suggests that bacterial isolation is not restricted to mastitic udders but may also occur in milk derived from udder quarters without clinical signs of mastitis. The detected bacterial populations are hypothesized to originate from extramammary sources such as environmental reservoirs, calf oral microbiota, and the endogenous microbiota of the teat skin (3). Therefore, farm animals act as reservoirs for many pathogens of zoonotic importance (4).

Beyond causing infections, these zoonotic agents pose an additional public health concern owing to the increasing prevalence of antimicrobial resistance genes developed by bacteria exposed to antibiotics used for prophylactic or therapeutic purposes. Furthermore, these resistance genes can be transferred to other bacterial species, amplifying their dissemination (5). The emergence and spread of extended-spectrum β-lactamase (ESBL)-producing bacteria have become a major public health concern in recent years (6). ESBL-producing bacteria can be transmitted through the consumption of raw milk and dairy products, and their genetic traits may be transferred to humans (7). Bacteria exhibiting multidrug resistance (MDR) can disseminate both among humans and from animals to humans via the consumption of animal-derived foods (8).

Resistance to β-lactam antibiotics, widely used in bacterial infection treatment, has become a global issue. Extended-spectrum beta-lactamases resistance mechanisms are particularly common among members of the *Enterobacteriaceae* family, notably *Escherichia coli* and *Klebsiella pneumoniae* (9,10). These bacteria, known for their antimicrobial resistance, are capable of spreading rapidly and easily through various routes such as the food chain and cross-contamination. Strains of *E. coli* and *K. pneumoniae* exhibiting ESBL, AmpC, and carbapenemase activities pose serious challenges to both animal and public health (9).

The ESBL genes, secreted via plasmids and transposons, render bacteria resistant to penicillins, cephalosporins, and monobactams and can be transferred genetically (11). According to the Ambler classification, the AmpC gene, categorized within group C β-lactamases, encodes a chromosomal cephalosporinase. Plasmid-mediated AmpC genes have been reported to emerge alongside ESBL genes (12). Carbapenems, the broadest-spectrum β-lactam antibiotics, are used against bacteria producing ESBL and AmpC. However, recent studies have revealed the emergence of carbapenem-resistant microorganisms (13,14). Carbapenemase enzymes hydrolyze carbapenems, conferring resistance to both carbapenems and other β-lactam antibiotics. These mechanisms contribute to the development of multidrug resistance, prolong treatment duration, worsen clinical outcomes, and increase mortality rates (15).

Various preservatives are employed to control microbial spoilage in foods, commonly referred to as antimicrobial additives. These additives are used to inhibit or suppress the growth of undesired microorganisms such as pathogens, molds, and yeasts that may contaminate food products. To prevent microbiological spoilage and extend shelf life without altering the quality of food, compounds such as sorbic acid, sulfur dioxide, acetic acid, benzoic acid, propionic acid, nitrites, and nitrates are utilized (16). In the dairy industry, several antimicrobial agents primarily hydrogen peroxide, sorbic acid and its salts, nisin, natamycin, lactoferrin, lactoperoxidase, propionic acid, acetic acid, and benzoic acid are applied. Antimicrobial compounds used against Gram-negative bacteria such as *E. coli* and *K. pneumoniae* can exert bactericidal or bacteriostatic effects through various mechanisms, including disruption of cell membrane permeability, inhibition of enzymatic activity, or interference with iron binding. For instance, lactoferrin exhibits inhibitory activity against *E. coli* and *K. pneumoniae* through its iron-chelating mechanism, whereas the lactoperoxidase system via hydrogen peroxide and thiocyanate demonstrates pronounced bactericidal activity particularly against *E.coli*. Sorbic acid has been reported to completely inhibit *E.coli* growth within 48 hours at a concentration of 0,75%, while lower concentrations (0,075%) exert only bacteriostatic effects. Although nisin displays limited efficacy against Gram-negative bacteria, it may exert synergistic antimicrobial activity against *E.coli* when combined with the lactoperoxidase system. Natamycin primarily targets yeasts and molds; therefore, its activity against these pathogenic bacteria is minimal. Propionic and acetic acids inhibit *E.coli* growth by creating an acidic environment, whereas benzoic acid demonstrates more pronounced inhibitory effects against Gram-negative bacteria under pH values <4,5 (29,30).

The effective concentrations of antimicrobial agents used in milk and dairy products against *E. coli* and Klebsiella species vary depending on product matrix and pH conditions. Reported effective dose ranges include: hydrogen peroxide (50 µM when used within the lactoperoxidase system), sorbic acid (0,3–0,75%; approximately 1000 mg/kg), nisin (10–200 IU/mL; 12,5 mg/kg), natamycin (1–10 ppm; 1 mg/kg), lactoferrin (0,02–0,35 mg/mL), lactoperoxidase (10–30 µg/mL), propionic acid (1%), acetic acid, and benzoic acid (0,05–0.1 mg/kg; with a legal limit of 50 mg/kg). These concentrations are fine-tuned based on the intrinsic characteristics of the product, such as its pH, constituent composition, and existing microbial burden (29). The permissible limits of these substances are regulated nationally by the Turkish Food Codex and internationally by organizations such as the World Health Organization (WHO),

the Food and Agriculture Organization (FAO), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (16).

In light of this information, the present study aimed to isolate *E. coli* and *K. pneumoniae* strains from raw milk samples collected from local farms, determine their ESBL, AmpC, and carbapenemase resistance phenotypes, and evaluate the antimicrobial activity of selected antimicrobial agents against these isolates.

Material and Methods

Raw Milk Sample Collection

A total of 200 raw milk samples were collected under aseptic conditions from dairy farms located in Ankara and surrounding regions in Türkiye. The samples were placed in sterile tubes and transported to the laboratory under cold chain conditions as quickly as possible. Before milking, teat ends were cleaned with antiseptic water and wiped with sterile cotton; the first few streams of milk were discarded, and approximately 15–20 mL of milk was collected into sterile Falcon tubes for analysis.

Isolation and Identification of Bacterial Strains

For the purpose of bacterial isolation and characterization, milk samples were aseptically inoculated onto MacConkey agar, 5% sheep blood agar, and eosin methylene blue (EMB) agar. The inoculated plates were incubated at 37°C for 24 to 48 hours. Bacterial colonies were preliminarily evaluated based on their morphological features and Gram staining results. Isolates exhibiting phenotypic characteristics suggestive of the Enterobacteriaceae family were subsequently subjected to a series of biochemical assays to confirm the presence of *Escherichia coli* and *Klebsiella pneumoniae.* Oxidase-negative isolates were further tested using Triple Sugar Iron (TSI), citrate, urease, and indole tests, followed by incubation at 37°C for another 24–48 hours. Confirmed *E. coli* and *K. pneumoniae* isolates were transferred to stock media and stored at −20°C for subsequent testing of ESBL, AmpC, and carbapenemase activity (32,33).

Antimicrobial Susceptibility Testing

The antibiotic resistance profiles and multidrug resistance (MDR) status of the isolates were evaluated using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar, utilizing a panel of antibiotic disks that included cefotaxime, aztreonam, ceftazidime, amoxicillin/clavulanic acid, amikacin, trimethoprim/sulfamethoxazole, gentamicin, imipenem, tobramycin, ciprofloxacin, cefepime, meropenem, and cefoxitin. Results were interpreted according to the EUCAST standards for *Enterobacterales*. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC BAA-1705™ were used as positive control strains (32,33).

Phenotypic Detection of ESBL, AmpC, and Carbapenemase Activity

Extended-spectrum beta-lactamases screening was conducted by comparing the inhibition zones of ceftazidime and ceftazidime/clavulanic acid; a ≥5 mm increase in zone diameter with clavulanic acid was interpreted as ESBL positive. Cefoxitin-resistant isolates were evaluated for AmpC production, while meropenem zone diameters were assessed for carbapenemase activity (32,33).

Evaluation of the Antimicrobial Activity of Selected Antimicrobial Agents

The antimicrobial activity of the selected agents, including hydrogen peroxide, sorbic acid, nisin, natamycin, acetic acid, lactoferrin, benzoic acid, and propionic acid, was evaluated using the agar well diffusion method. Each compound was tested at two different concentrations, and the inhibition zones were measured after incubation on Mueller-Hinton agar at 37°C for 16–18 hours. Initially, the isolated *E. coli* and *K. pneumoniaee* strains were adjusted to a 0.5 McFarland standard and uniformly inoculated onto Mueller-Hinton agar plates using sterile swabs. Subsequently, wells prepared in the agar were filled with the antimicrobial agents.

The agents were applied at both full and half concentrations, which were defined as follows: acetic acid, 4 µL/mL (full) and 2 µL/mL (half); benzoic acid, 1 µg/mL (full) and 0,5 µg/mL (half); sorbic acid, 2 mg/mL (full) and 1 mg/mL (half); hydrogen peroxide, 0,2 mg/mL (full) and 0,1 mg/mL (half); propionic acid, 5 mg/mL (full) and 2,5 mg/mL (half); nisin, 10 µg/mL (full) and 5 µg/mL (half); natamycin, 1 µg/mL (full) and 0,5 µg/mL (half); lactoferrin, 0,1 mg/mL (full) and 0,05 mg/mL (half). After incubation, the diameters of the inhibition zones were measured for each concentration, and the antimicrobial efficacy of the compounds against the bacterial isolates was determined.

Statistical Analysis

The statistical analyses of the data obtained in this study were performed using the IBM Statistical Package for Social Sciences (SPSS) program software package. The antibiotic resistance rates of *E. coli* and *K. pneumoniae* isolates were evaluated using descriptive statistics (percentages and mean ± standard deviation). Relationships between categorical variables were assessed using the Chi-square (χ^2) test, or Fisher's Exact test when expected cell frequencies were insufficient.

To compare inhibition zone diameters produced by different concentrations of antimicrobial agents, one-way analysis of variance (ANOVA) was conducted for multi-group comparisons. In cases where ANOVA revealed significant differences, Tukey's post hoc multiple comparison test was applied to determine the source of variation among groups. In instances where data distributions deviated from normality, non-parametric methods, including the Mann–Whitney U test for two-group comparisons

and the Kruskal–Wallis test for multiple-group comparisons, were utilized. A significance level of p <0,05 was considered for all statistical evaluations. The results were reported as mean inhibition zone diameters, standard deviation values, and corresponding significance levels.

Results

In this study, a total of 200 raw milk samples collected under appropriate conditions from dairy farms located in different regions of Ankara, Türkiye and its surroundings were examined. Following microbiological analysis, 6% (n=12) of the total samples were identified as *Klebsiella pneumoniae* and 4% (n=8) as *Escherichia coli*.

Based on biochemical test results, *E.coli* isolates were indole and lactose positive but citrate and urease negative, whereas *K. pneumoniae* isolates were urease, citrate, and lactose positive but indole negative. These findings are consistent with species-level identification characteristics (Table 1).

Phenotypic detection of ESBL activity among the total 20 *E.coli* and *K. pneumoniae* isolates was performed using the ceftazidime (CAZ) and ceftazidime–clavulanic acid (CAZ–CLA) disk diffusion test. According to zone diameter differences, 8 isolates (40%) were identified as ESBL-positive. Both *E.coli* and *K. pneumoniae* isolates exhibited ESBL activity; however, the rate was higher among *K. pneumoniae* strains.

Evaluation of the cefoxitin (FOX) disk diffusion results for ESBL-positive isolates revealed that all eight showed resistance to FOX, indicating AmpC production. Notably, isolates S-2, S-1, T-10, and G-2 demonstrated pronounced FOX resistance, further supporting the AmpC phenotype.

Regarding carbapenemase activity, approximately half of the ESBL-positive isolates exhibited resistance to meropenem, which was confirmed by the modified Hodge test, indicating 50% carbapenemase positivity among ESBL-producing isolates. Specifically, isolates S-1, S-2, S-3, and T-10 were positive for ESBL, AmpC, and carbapenemase activity (Table 2).

Antimicrobial susceptibility profiles for nine antibiotics were evaluated across all 20 isolates. High levels of resistance were observed, with 75% (n=15) of isolates resistant to at least one antibiotic. Among these, 45% (n=9) were classified as multidrug-resistant (MDR), while isolate T-10 exhibited an extensively drug-resistant (XDR) phenotype.

The highest resistance rates were recorded against fluoroquinolones, β-lactam/β-lactamase inhibitor combinations, and monobactams. Resistance rates to ciprofloxacin and amoxicillin-clavulanic acid were 40% and 50%, respectively, while aztreonam and cefepime resistance were both observed at 40%. In contrast, aminoglycosides demonstrated high efficacy, with susceptibility rates of 85%, 80%, and 82% for gentamicin, tobramycin, and amikacin, respectively.

The relationship between ESBL production and multidrug resistance was also assessed. Among the 20 isolates, 8 (40%) were ESBL-positive, while the overall MDR rate was 45% (9/20). Of the ESBL-positive isolates, 75% (6/8) exhibited an MDR phenotype, whereas only 25% (3/12) of ESBL-negative isolates were MDR. Fisher's Exact Test revealed a statistically significant association between ESBL production and MDR development (p=0,041). The odds ratio was 9,0, indicating that ESBL-positive isolates were nine times more likely to develop MDR compared to ESBL-negative ones (95% CI: 1,12–72,43) (Table 3).

Table 1. Biochemical test results of *E. coli* and *K. pneumoniae* isolates

Isolate	Oxidase	Indole	Urease	Citrate	Lactose	Identification Result
S-1	-	-	+	+	+	*K. pneumoniae*
S-2	-	-	+	+	+	*K. pneumoniae*
T-10	-	-	+	+	+	*K. pneumoniae*
K-3	-	-	+	+	+	*K. pneumoniae*
K-6	-	-	+	+	+	*K. pneumoniae*
S-12	-	-	+	+	+	*K. pneumoniae*
S-5	-	-	+	+	+	*K. pneumoniae*
B-3	-	+	-	-	+	*E.coli*
F-52	-	+	-	-	+	*E.coli*
K-2	-	-	+	+	+	*K. pneumoniae*
K-22	-	-	+	+	+	*K. pneumoniae*
K-9	-	-	+	+	+	*K. pneumoniae*
K-1	-	-	+	+	+	*K. pneumoniae*
S-3	-	-	+	+	+	*K. pneumoniae*
A-1	-	+	-	-	+	*E.coli*
F-54	-	+	-	-	+	*E.coli*
H-1	-	+	-	-	+	*E.coli*
G-2	-	+	-	-	+	*E.coli*
H-3	-	+	-	-	+	*E.coli*
G-1	-	+	-	-	+	*E.coli*

Table 2. AmpC and carbapenemase activity of ESBL-positive isolates

Isolate	CAZ (mm)	CAZ-CLA (mm)	FOX (mm)	MEM (mm)
G-1	22	30	11*	32
S-3	16	23	9*	19**
S-2	14	16	R*	20**
S-1	12	16	R*	20**
T-10	10	15	R*	16**
K-6	18	30	12*	30
G-2	21	25	R*	32
F-54	21	25	12*	30

CAZ: ceftazidime; CAZ CLA: ceftazidime/clavulanic acid; FOX: cefoxitin; MEM: meropenem; * resistance FOX; ** resistance MEM.

Table 3. Number (n) and percentages (%) of isolates regarding ESBL (Extended-spectrum β-lactamase) and MDR (Multi drug resistance) properties

ESBL-MDR property	n	%
ESBL (+) & MDR (+)	6	30%
ESBL (+) & MDR (−)	2	10%
ESBL (−) & MDR (+)	3	15%
ESBL (−) & MDR (−)	9	45%

The antibacterial activity of antimicrobial agents was examined at both full and half concentrations against *E.coli* and *K. pneumoniae* isolates. Hydrogen peroxide demonstrated the highest inhibition zones across all isolates, with mean diameters of 14,3±2,9 mm at full concentration and 11,3±2,5 mm at half concentration. Acetic acid and benzoic acid showed moderate activity, producing inhibition zones of 10,5±1,6 mm and 10,1±1,7 mm at full concentration, respectively, with significantly reduced effects at half concentration ($p < 0,05$). Sorbic acid and propionic acid exhibited limited or no activity against Gram-negative isolates, while nisin, natamycin, and lactoferrin were ineffective at both concentrations. Repeated-measures ANOVA indicated a statistically significant difference among additives ($F=28,7$, $p < 0,001$), and Tukey's post-hoc analysis identified hydrogen peroxide as the most effective agent, followed by acetic and benzoic acids ($p < 0,05$) (Table 4).

These findings suggest that oxidative agents and acidic environments produced by organic acids can provide limited but statistically significant inhibition against Gram-negative pathogens such as *E. coli* and *K. pneumoniae*, whereas protein-based natural antimicrobials are ineffective due to the permeability barrier of the Gram-negative outer membrane.

When comparing the susceptibility of ESBL-positive and ESBL-negative isolates to antimicrobial agents, hydrogen peroxide, acetic acid, and benzoic acid produced slightly smaller inhibition zones in ESBL-positive strains; however, these differences were not statistically significant. Neither nisin, natamycin, nor lactoferrin produced inhibition zones in any of the isolates, confirming their ineffectiveness against Gram-negative bacteria ($p > 0,05$) (Table 5).

Table 4. Doses, zone diameters, and activities of antimicrobial agents

Antimicrobial agent	Full dose (mm)	Half dose (mm)	Activity reduction (mm)	Activity level
Acetic acid	10,5	8,3	↓2,2	Medium
Benzoic acid	10,1	8,6	↓1,5	Medium
Hydrogen peroxide	14,3	11,3	↓3,0	High
Sorbic acid	R	R	–	Ineffective
Propionic acid	Very low	R	Lost	Ineffective
Nisin	0	0	–	Ineffective
Natamycin	0	0	–	Ineffective
Lactoferrin	0	0	–	Ineffective

Table 5. Effects of antimicrobial agents on ESBL (+) (Extended-spectrum β-lactamase) microorganisms

Antimicrobial agent	Resistance in ESBL (+)	Resistance in ESBL (−)	Statistical significance
Acetic acid	High	Medium	$p \approx 0,12$
Benzoic acid	High	Medium	$p \approx 0,11$
H_2O_2 (Hydrogen peroxide)	Slightly more resistant	More susceptible	$p \approx 0,08$
Sorbic acid	Ineffective	Ineffective	Not significant
Propionic acid	Ineffective	Ineffective	Not significant
Nisin	Ineffective	Ineffective	Not significant
Natamycin	Ineffective	Ineffective	Not significant
Lactoferrin	Ineffective	Ineffective	Not significant

Overall, these results indicate that ESBL-positive strains may display a tendency toward higher resistance to certain natural antimicrobial agents, although this association could not be statistically confirmed within the current dataset.

Discussion

In this study, *Escherichia coli* and *Klebsiella pneumoniae* isolates obtained from 200 raw milk samples collected from farms around Ankara, Türkiye were examined phenotypically for ESBL, AmpC, and carbapenemase activities; their antibiotic resistance profiles and the antimicrobial effects of some antimicrobial agents were also evaluated. The total isolation rate of *E. coli* and *K. pneumoniae* from raw milk was determined as 10% (20/200). Among the isolates, 60% (12/20) were *K. pneumoniae* and 40% (8/20) were *E. coli.* When examining the percentages relative to the total sample number, these rates were 6% for *K. pneumoniae* and 4% for *E. coli*. Similar studies have shown highly variable *E. coli.* isolation rates; for instance, in a study conducted in Egypt, the isolation rates of *E. coli* and *K. pneumoniae* were found to be 10% and 4%, respectively, while *E. coli.* prevalence in raw milk in Romania was reported as 22,45% by Drugea et al. (17,18). In Bangladesh, 22,45% of all isolates were *E. coli.* among which 27,51% were multidrug-resistant (MDR) and ESBL-producing strains were identified (19). The comparatively lower isolation rate in this study is likely due to differences in sampling methods, hygiene standards, farm management programs, and regional variations.

In the study, 40% (8/20) of the *E. coli.* and *K. pneumoniae* isolates were ESBL positive, with a higher rate of ESBL positivity detected in *K. pneumoniae*. Of this 40% positive rate, 62,5% were *K. pneumoniae* and 37,5% were *E. coli.* isolates. These findings differ from international studies. Tyasningsih et al. reported an ESBL prevalence over 38% in 176 *E. coli.* isolates from 250 raw milk samples in Indonesia (20). A recent study in Egypt found that 43,3% of *E. coli.* isolates and 33,3% of *K. pneumoniae* isolates produced ESBL (17). Mim et al. reported ESBL prevalence in *E. coli* and *K. pneumoniae* isolates from milk in Bangladesh as 47,5% and 42,5%, respectively (22). A similar study in Pakistan found ESBL production in 54% of *K. pneumoniae* strains and 46% of *E. coli.* strains (23). A 2022 study in Italy reported that 87,5% of *K. pneumoniae* isolates from raw cow milk exhibited ESBL activity (24). In Türkiye, ESBL positivity in *E. coli.* isolates was determined as 17,03% (21). The ESBL positivity rate in this study aligns reasonably with international data and reflects the current state of the industry. When compared with similar studies, it is thought that the prevalence of ESBL-producing strains in the Enterobacteriaceae family varies depending on hygienic production systems, antibiotic use practices, biosecurity measures on farms, and regional epidemiological developments.

In evaluating ESBL-positive isolates, isolates resistant to cefotaxime disk were assessed for the AmpC phenotype. Among the eight ESBL-positive isolates studied, 100% resistance to cefoxitin (FOX) was observed. Resistance was especially prominent in strains S-2, S-1, T-10, and G-2, which also demonstrated production of ESBL, AmpC, and carbapenemase. Regarding carbapenemase, activity was detected in 50% of ESBL-positive isolates, all of which were identified as *K. pneumoniae*. The ESBL/AmpC/carbapenemase positivity found in this study was mostly observed in strains S-1, S-2, S-3, and T-10, and these isolates approached the pandrug-resistant phenotype severely limiting treatment options. Bonardi et al. reported in Italy that 75% of *K. pneumoniae* isolates from raw cow milk exhibited AmpC enzyme activity and 75% were OXA-48 carbapenemase positive (24). In China, 45% of *K. pneumoniae* isolates from raw cow milk were ESBL and AmpC positive, while 30% tested positive for carbapenemase in 2020 (25). Another study in Egypt found ESBL prevalence of 68% in Enterobacteriaceae isolates from cow milk, with 45% AmpC and 35% carbapenemase positivity (26). These results highlight a serious threat to the food chain and show a critical limitation of treatment options.

The resistance profiles against nine antibiotics were evaluated for the 20 isolates, and 75% (n=15) were found to be resistant to at least one antibiotic. Sensitivity rates to the aminoglycosides gentamicin, tobramycin, and amikacin were 85%, 80%, and 82%, respectively, indicating high efficacy of these antibiotics. Conversely, resistance rates for fluoroquinolone ciprofloxacin, beta-lactam/beta-lactamase inhibitor amoxicillin-clavulanic acid, and monobactam aztreonam were 40%, 50%, and 40%, respectively. The MDR rate was determined as 45% (9/20). In a 2025 study in Bangladesh, *E.coli* and *K. pneumoniae* isolates exhibited 95% MDR and 85% ciprofloxacin resistance (23). Mim et al. found ciprofloxacin, gentamicin, and amikacin resistance rates in *E. coli* isolates as 40%, 45%, and 50%, respectively (22). In Colombia, ampicillin, tetracycline, and trimethoprim/sulfamethoxazole resistance rates were between 85–95% in milk isolates of *E. coli* and *K. pneumoniae* (27). These results suggest that the aminoglycoside group is not the primary choice on farms, explaining the high sensitivity, while resistance is high against beta-lactams and fluoroquinolones, which are more commonly used and affected by ESBL production.

Among ESBL-positive isolates, 75% (6/8) exhibited MDR phenotype, whereas only 25% (3/12) of ESBL-negative isolates showed MDR. Fisher's Exact Test revealed a significant association between ESBL production and MDR development (p=0,041), with an odds ratio of 9.0, indicating that ESBL-positive isolates are nine times more likely to develop MDR than ESBL-negative isolates. This suggests that plasmids encoding ESBL commonly carry other antimicrobial resistance genes. Similarly, a study from Egypt showed higher resistance levels to non-beta-lactam antibiotics among ESBL-producing isolates compared to non-ESBL producers (26). In Bangladesh, the association between MDR (95%) and ESBL (50%) presence in *E. coli* and *K. pneumoniae* isolates was significant (23). Mim et al. reported that ESBL-producing strains were significantly more resistant to gentamicin, ciprofloxacin, and carbapenems than ESBL-negative strains (22). Penati et al. in Italy found ESBL and AmpC-producing *E. coli* isolates had significantly higher resistance to all tested antibiotics, with MDR prevalence reaching 88% (28). In China, ESBL-positive *K. pneumoniae* isolates demonstrated significant resistance to cephalosporins, fluoroquinolones, aminoglycosides, and carbapenems (25). Collectively, findings from this study also indicate that ESBL-positive isolates possess MDR phenotypes.

The antimicrobial efficacy of antimicrobial agents tested in the study was assessed by agar well diffusion. Hydrogen peroxide produced the largest inhibition zones among all isolates, with an average of 14,3±2,9 mm at 0,2 mg/ml and 11,3±2,5 mm at half concentration. Acetic acid and benzoic acid, at full concentrations of 4 and 1 µg/ml respectively, showed moderate activity of 10,5±1,6 mm and 10,1±1,7 mm, with significant reduction in inhibition at half doses. Sorbic and propionic acids showed no activity against gram-negative isolates; nisin, natamycin, and lactoferrin exhibited no effect at either concentration on any isolates.

The strong antimicrobial activity of hydrogen peroxide is supported by several studies. A recent review by Teshome et al. noted hydrogen peroxide as the most effective agent against gram-negative pathogens like *E. coli* and *K. pneumoniae*. Peroxide damages lipids, proteins, and DNA, rendering bactericidal effects against gram-negative and gram-positive bacteria (29). In Türkiye, Alan and Öksüztepe reported high hydrogen peroxide activity against enterobacteria in milk, recommending its frequent use in pre-pasteurization processing (30). Another Turkish study confirmed its bacteriostatic and bactericidal effects against *E. coli* and *K. pneumoniae* isolates (31).

Regarding organic acids, acetic and benzoic acids showed moderate activity consistent with international literature. Wei et al. reported limited antimicrobial properties of benzoic acid against *Enterobacteriaceae* (32). Alan and Öksüztepe observed that full concentrations of acetic and benzoic acids inhibited *E. coli* but partial concentrations reduced effectiveness (30). Sorbic and propionic acids' inactivity against gram-negative isolates aligns with reports by Yilmaz and Tosun, who indicated that sorbic acid is effective against gram-positive bacteria but not Enterobacteriaceae (31). Alan and Öksüztepe also confirmed propionic acid's strong activity against molds and yeasts but minimal effects on *E. coli* and *K. pneumoniae* (30). The inactivity of nisin, natamycin, and lactoferrin against *E. coli* and *K. pneumoniae* is related to gram-negative bacterial membrane structure. Field et al. detailed that nisin acts strongly on the peptidoglycan cell wall of gram-positive bacteria, but

the lipopolysaccharide (LPS) layer in gram-negatives impedes peptide entry, rendering nisin ineffective.

The relationship between ESBL-positive isolates and antimicrobial food additive efficacy was also investigated. Although hydrogen peroxide, acetic acid, and benzoic acid showed lower inhibition zones against ESBL-positive strains, differences were not statistically significant. Neither nisin, natamycin, nor lactoferrin exhibited inhibition zones on ESBL-positive or ESBL-negative isolates. These findings suggest that ESBL-positive *K. pneumoniae* and *E. coli* isolates may show resistance tendencies to specific antimicrobial agents under industrial food conditions, depending on concentration and microbial structure.

Conclusion

This study aimed to phenotypically investigate ESBL, AmpC, and carbapenemase production in *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from raw milk samples collected from dairy farms around Ankara, Türkiye, and to evaluate their antibiotic resistance profiles as well as the effectiveness of some antimicrobial agents. The findings revealed that a significant portion of raw milk samples contained members of the Enterobacteriaceae family, with widespread ESBL production and multidrug resistance (MDR) among the isolates. Furthermore, high levels of AmpC and carbapenemase activity were detected in ESBL-positive strains, indicating resistance profiles that could severely limit treatment options.

Among the antimicrobial agents tested, hydrogen peroxide demonstrated the strongest inhibitory effect, while acetic and benzoic acids showed moderate activity. Sorbic acid, propionic acid, nisin, natamycin, and lactoferrin were found ineffective against gram-negative bacteria. These results indicate that hydrogen peroxide and some organic acids possess limited but notable antimicrobial potential against pathogenic bacteria in raw milk.

In summary, the presence of resistant bacteria producing ESBL, AmpC, and carbapenemase in raw milk highlights the potential dissemination of antibiotic resistance genes through the food chain. These findings emphasize the necessity to strengthen hygiene and biosecurity in milk production, regulate antibiotic use, and consider natural antimicrobial agents as adjunct protective measures in milk hygiene. Future research should focus on molecular characterization and environmental distribution of resistance genes, which would provide valuable insights for public health.

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