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Determination of Multiple Antibiotic Resistance Profiles of *Enterococcus* Species Isolated From Fermented Meat Products Consumed in Ankara

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Abstract

The aim of this study was to determine the multiple antibiotic resistance profiles of *Enterococcus* spp. isolated from the fermented meat products consumed in Ankara, Turkey. A total of 134 *Enterococcus* spp. were isolated and identified from 80 fermented meat samples. The highest prevalence of enterococci in the fermented food samples was found in sucuk (a Turkish fermented sausage) samples (50%), followed by sausage (25.4%), pastirma (a Turkish dry-cured meat product) (18.6%), and salami, respectively. Of a total of 134 *Enterococcus* isolates, 110 (82.1%) were found to be resistant to one or more of the antibiotics tested. The highest resistance rate was seen against rifampicin (73.2%), streptomycin (36.5%) and erythromycin (20.2%), and 28 (20.9%) of Enterococcus isolates were resistant to multiple antibiotics. The presence of multiple antibiotic resistant *Enterococcus* in foods of animal origin raises alarm because of the risk of carrying these bacteria to humans via the food chain.

Keywords: *Enterococcus*, fermented meat products, multiple antibiotic resistance

1. INTRODUCTION

Enterococcus species are found in many environments such as soil, food and water and they are also found in the microbiota of the gastrointestinal system of humans and warm-blooded animals [1]. They have also shown to be present in some ripened cheeses and fermented salami as part of established starter cultures and are used in the production of some meat and dairy products because of their important contribution in the process and fermentation of flavors and their probiotic properties. Therefore by intestinal and/or environmental exposure, enterococci can colonize raw foods of animal origin (milk, meat) where they can survive and reproduce during fermentation processes. Due to their tolerance to environmental conditions (temperature, pH, salinity), they are often isolated from both fermented and heat treated meat products [2].

Enterococcus species have been shown to be among the most common nosocomial pathogens, important cause of multiple antibiotic resistance as well as urinary tract, central nervous system, intraabdominal and pelvic infections, endocarditis, bacteremia and infections [3, 4]. There is no consensus, however, on the importance of their existence in food items. In the field of veterinary medicine, antibiotics are commonly used to control and treat infections and

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feed additives to promote growth results in the selection of resistant enterococci in the animal intestinal flora. The ingestion of antibiotic-resistant bacteria through foods is a potential mechanism for the transition of antibiotic resistance determinants to human or animal adapted strains [5, 6].

The present study was designed to determine the multiple antibiotic resistance profiles of enterococci isolated from fermented meat products mainly derived from sucuk (a Turkish fermented sausage), sausage, salami, and pastirma (a Turkish dry-cured meat product) in Turkey.

2. MATERIAL AND METHODS

2.1. Sampling

Eighty samples of fermented meat (20 sucuk, 20 sausages, 20 salami, and 20 pastirma) were collected from markets in Ankara between February 2017 and December 2017. All the samples were kept at 4^0C before analysis and transported to the laboratory, immediately.

2.2. Isolation and Identification

The fermented meat samples were transferred to the laboratory under cold chain conditions, diluted 1:10 with sterile buffered peptone water (BPW) (Oxoid, CM 509, Basingstoke, UK) and homogenized for about 10 min. Isolation was done by selecting typical pink and purple colonies on Slanetz Bartley Agar (SBA; Oxoid, CM 377), selective medium for *Enterococcus*. Estimated identification of isolates was performed by Gram staining, production of catalase and oxidase, growth at 10 °C and 45 °C, growth in the presence of 6.5% NaCl, growth at pH 9.6, determination of esculin hydrolysis on bile-esculin agar (Merck, 48300).

The species identification was confirmed with Becton Dickinson (BD) BBL Crystal Identification Systems and Gram-Positive ID kit (Becton, Dickinson and Company, USA [7].

2.3. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of the isolates was determined using the disc diffusion method following Clinical and Laboratory Standard Institute guidelines, 2012 [8] on Muller Hinton Agar (MHA) plates (Becton Dickinson Microbiology Systems, Cockeysville, USA). All strains were incubated at 37^0C for 48 h. The antimicrobials and their concentrations (BBL Microbiology Systems, Cockeysville, USA) were as follows: ampicillin chloramphenicol (C, 30 μg), ciprofloxacin (CIP, 5 μg), erythromycin (E, 15 μg), gentamicin (CN, 120 μg), nitrofurantoin (F, 300 μg), penicillin (P, 10 μg), rifampicin (RF, 5 μg), streptomycin (S, 300 μg), teicoplanin (TEC, 30 μg), tetracycline (TE, 30 μg), vancomycin (VA, 30 μg). *Enterococcus faecalis* ATCC 29211 was used as a control strain. The MAR (multiple antibiotic resistance) indexes were calculated as described by Krumperman [9].

3. RESULTS

3.1. Isolation and Identification

Overall, one hundred thirty-four *Enterococcus* spp. were isolated from the eighty fermented meat samples (twenty sucuk, twenty sausages, twenty salami, twenty pastirma). In this study, the highest prevalence of enterococci in the fermented food samples was found in sucuk samples (50%), followed by sausage (25.4%), pastirma (18.6%) and salami (6%), respectively (Table 1).

Table 1 Distribution in the 134 *Enterococcus* strains isolated from the fermented meat samples

Types of fermented meat	*Enterococcus* isolated from fermented meat						Total
	E. faecium	*E. faecalis*	*E. gallinarum*	*E. durans*	*E. avium*	*E. hirae*	
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
sucuk	48 (71.6%)	6 (8.9%)	9 (13.4%)	3 (4.5%)	1 (1.5%)	-	67 (50%)
sausage	11 (32.3%)	20 (58.8%)	3 (8.8%)	-	-	-	34 (25.4%)
salami	6 (75%)	1 (12.5%)	1 (12.5%)	-	-	-	8 (6%)
pastirma	14 (56%)	8 (32%)	-	2 (8%)	-	1 (4%)	25 (18.6)

n: Number of Enterococcus isolates

E. faecium (59%) and *E faecalis* (26.1%) strains were predominantly isolated from all the fermented meat classes in this study (Table 2).

Table 2 Distribution of the *Enterococcus* strains isolated from the fermented meat samples

Enterococcus species	Fermented meat samples	
	Number of	%
E.faecium	79	59
E.faecalis	35	26.1
E.gallinarum	13	9.7
E.durans	5	3.7
E.avium	1	0.7
E.hirae	1	0.7
Total	134	100

3.2. Antimicrobial Susceptibility Testing

The prevalence of antibiotic resistance determined in Enterococcus species isolated from fermented meat samples in our study is shown in Table 3.

Table 3 Antimicrobial resistance in *Enterococcus* isolates

Antibiotics	Resistant isolates (%)						
	E. faecium n=79	*E. faecalis* n=35	*E. gallinarum* n=13	*E. durans* n=5	*E. avium* n=1	*E. hirae* n=1	Total n=134
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Ampicillin	1 (%1.3)	-	-	-	-	-	1 (%0.8)
Chloramphenicol	-	2 (%5.7)	-	-	-	-	2 (%1.5)
Ciprofloxacin	4 (%5)	4 (%11.4)	-	-	-	-	8 (%6)
Erythromycin	19 (%24)	6 (%17.1)	1 (%7.7)	1 (%20)	-	-	27 (%20.2)
Gentamicin	1 (%1.3)	1 (%2.8)	1 (%7.7)	-	-	-	3 (%2.3)
Nitrofurantoin	12 (%15.1)	3 (%8.5)	4 (%30.7)	1 (%20)	-	-	20 (%15)

Penicillin	1 (%1.3)	-	-	-	-	-	1 (%0.8)
Rifampicin	59 (%74.6)	27 (%77.1)	8 (%61.5)	2 (%40)	1 (%100)	1 (%100)	98 (%73.2)
Streptomycin	32 (%40.5)	12 (%34.2)	4 (%30.8)	-	-	1 (%100)	49 (%36.5)
Teicoplanin	2 (%2.5)	-	1 (%7.7)	-	-	-	3 (%2.3)
Tetracycline	5 (%6.3)	3 (%8.5)	-	-	-	-	8 (%6)
Vancomycin	-	-	-	-	-	-	-

Of a total of 134 *Enterococcus* isolates, 110 (82.1%) were found to be resistant to one or more of the antibiotics tested. Multiple antibiotic resistance status of *Enterococcus* isolates are given in Table 4.

Table 4 Antimicrobial resistance pattern and multiple resistance of *Enterococcus* isolates from the fermented meat

Antibiotics	Number of resistant *Enterococcus* isolates (%)	MAR Index
AM	1 (0.7%)	0.08
CIP	1 (0.7%)	0.08
E	1 (0.7%)	0.08
F	3 (2.2%)	0.08
RF	34 (25.3%)	0.08
S	2 (1.5%)	0.08
S-RF	19 (14.2%)	0.17
CIP-RF	4 (3%)	0.17
RF-E	9 (6.7%)	0.17
S-F	2 (1.5%)	0.17
TE-RF	4 (3%)	0.17
CIP-F	1 (0.7%)	0.17
S-E	1 (0.7%)	0.17
S-RF-F	9 (6.7%)	0.25
S-RF-TEC	1 (0.7%)	0.25
S-RF-E	7 (5.2%)	0.25
S-CIP-RF	1 (0.7%)	0.25
TE-RF-E	1 (0.7%)	0.25
RF-F-E	1 (0.7%)	0.25
S-E-TEC	1 (0.7%)	0.33
S-RF-F-E	2 (1.5%)	0.33
C-CIP-TE-E	1 (0.7%)	0.33
S-RF-CN-E	1 (0.7%)	0.33
S-TE-RF-CN	1 (0.7%)	0.33
S-RF-F-P-E	1 (0.7%)	0.42
S-RF-F-CN-TEC	1 (0.7%)	0.42

AM (Ampicillin), C (Chloramphenicol), CIP (Ciprofloxacin), CN (Gentamicin), E (Erythromycin), F (Nitrofurantoin), P (Penicillin), RF (Rifampicin), S (Streptomycin), TE (Tetracycline), TEC (Teicoplanin).

4. DISCUSSION AND CONCLUSION

In Turkey, *E. feacalis* and *E. faecium* were isolated as the dominant species in meat and meat products [10]. Likewise, *E. faecalis* and *E. faecium* were dominant species isolated from naturally fermented Turkish foods [11]. In other studies conducted on fermented meat products from Turkey [12], Canada [13] and North of Portugal [14] the researchers reported predominant isolation of *E. faecalis* and *E. faecium*. However, other species such as *E. gallinarum, E. durans, E. avium, E. hirae* are less frequently identified in our study (Table 2). Nevertheless, other studies have found *E. faecalis* as the predominant species [15, 16, 17], and the low occurrence of other *Enterococcus* species in fermented food samples resembled other research that had previously been published [10, 14, 16]. A higher prevalence of enterococci in fermented foods during the removal of the internal organs can be considered as an indicator of fecal contamination in the slaughterhouse. Due to their heat sensitivity, *Enterococcus* species can be found in many different fermented foods during fermentation without starter or in meat products processed after baking [18]. Cross-contamination can also occur at the final stages of processing, such as cutting and packaging of produce [19].

It is known that *Enterococcus* spp. is naturally resistant to many antibiotics used in treatment. In addition, they have the ability to develop genetic resistance and transfer it to commensal bacteria by genes carried by plasmids or transposons [20]. In the present study, the prevalence of antibiotic resistance was higher for *E. faecium* when compared to *E. faecalis* (Table 3). *E. faecium* showed resistance to rifampicin (74.6 %), streptomycin (40.5%), erythromycin (24%), and nitrofurantoin (15.1%). A high percentage of rifampicin resistance (73.2%) was detected among our enterococcal isolates. About 77.1% of *E. faecalis*, 74.6 % of *E. faecium* and 60% of other *Enterococcus* spp (*E. gallinarum, E. durans, E. avium and E. hirae)* presented resistance to rifampicin. Rifampicin is used nearly solely for the treatment of tuberculosis. Very significant research with enterococci was conducted in Northern Portugal using enterococcal strains isolated from traditional fermented meat products [21]. Rifampicin resistance was shown by a high proportion of strains (60%) from 182 enterococcal isolates.

Gentamicin and streptomycin are the most effective antibiotics used in the treatment of enterococcal infections in high-level aminoglycoside resistance situations. In our study among 134 *Enterococcus* isolates, 49 (36.5%) were resistant to streptomycin, 3 (2.3%) were resistant to gentamicin. For the erythromycin, a high percentage of (20.2%) fermented food isolates was reported as resistant. Similarly, a high frequency of erythromycin resistance has been shown among enterococcal isolates from different sources [22, 23]. Resistance to erythromycin, an important representative of macrolides, is a concern because erythromycin-resistant plasmids and transposons are often found in enterococci [24]. In this study, we found low percentages of ß-lactams resistant enterococcal strains (ampicillin resistance 0.8% and penicillin resistance 0.8%), which is not in line with the generalization that enterococci are intrinsically resistant to ß-lactams [25]. Similar results were obtained by other authors [26, 27].

Vancomycin, one of the few substitutes in the treatment of enterococcal infections, has been recognized as increasingly significant in human medicine, and in the last decade, vancomycin-resistant enterococci have arisen as a common source of nosocomial infections. [28, 29]. Strong data suggest that the decreased number of VREs has been isolated since 1995 when avoparcin was first forbidden for use in livestock [30]. In this study, none of the studied isolates was resistant to vancomycin and only 2.3% of *Enterococcus* species was found resistant to teicoplanin (Table 3). Similar results have been obtained for food isolates in many studies [22, 31].

In cases where there is uncontrolled use of antibiotics in livestock, it has been determined that high and multiple resistance is seen in food and human isolates as a result of cross-resistance. The potential of fermented foods as a source for human transmission of multiple antibiotic-resistant strains of enterococci or as the cause of horizontal transfer of resistance genes between strains is particularly noteworthy [32]. The MAR index is a value determined as a result of a calculation to determine the risk of multiple antibiotic resistance of an isolate in the sample. MAR index values greater than 0.2 indicate that the samples are contaminated from sources where antibiotics are frequently used. MAR index values equal to or less than 0.2 indicate a strain originating from animals for which antibiotics are rarely or never used [33]. Because of its infamous ability to obtain and transfer resistance genes, multiple antibiotic resistance has frequently been reported for enterococci [34, 35]. The multiple antibiotic resistance index calculated for all resistant *Enterococcus* isolates and the ratio ranged from 0.08 to 0.42 values; 25.4% of the strains had a MAR index higher than 0.2 (Table 4), indicating a high risk of contamination for the consumer. This MAR index level is lower than that of the index's found in in other studies from 0.25 to 0.87 [36] and from 0.2 to 0.6 [37]. The results of this study indicated that resistance to multiple antibiotics was spread among *Enterococcus* isolates.

Enterococcus is commonly present in the digestive tract of humans and animals, with very high numbers of various species of *Streptococcus* and *Listeria monocytogenes* harboring conjugal plasmids and transposons. This information supports the notion that the intestinal tract is the most favorable ecosystem for the direct sharing of genetic information between these bacterial genera [38]. The inclusion of antibiotics in animal feed and inadequate control of prescription medications lead to the proliferation of antibiotic resistance, like enterococci, in the natural flora of healthy humans and livestock. Although antibiotic-resistant enterococci as nosocomial pathogens from clinical environments, especially *E. faecium* [39, 40] and raw food samples from livestock have been extensively studied, little is known about the antibiotic resistance of enterococci strains isolated from fermented food samples.

In conclusion, our findings suggest that the hygienic nature of raw meat may be important as a source of enterococci in the fermentation of meat. It may also provide the correct physical and biochemical conditions for the growth of enterococci during fermentation. This study, therefore, suggests the need for continuous surveillance of enterococci in meat products, considering their importance as vectors for the spread of microorganisms from animals to humans via the food chain. In addition, enterococci obtained from animal foods should be handled carefully for antimicrobial resistance. Fermented meat products are enterococcal reservoirs of antibiotic resistance. This provides useful risk assessment information indicating that foods containing antibiotic-resistant enterococci are likely to pose a potential public health risk to consumers.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

The authors contributed equally to the study. MBK: Literature research, conducting experiments, writing the article, SÇ: Literature research, writing the article, ZŞ: Literature research, conducting experiments

The Declaration of Ethics Committee Approval

This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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