Arch Lebensmittelhyg 70,
99–110 (2019)
DOI 10.2376/0003-925X-70-99

ISSN 0003-925X

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Amino acid decarboxylase activity, biofilm formation and antibiotic resistance of gram-negative bacteria isolated from marine fish, calf meat and chicken

Aminosäuren-Dekarboxylase-Aktivität, Biofilmbildung und Antibiotikaresistenz gramnegativer Bakterien isoliert aus Meeresfischen, Kalbs- und Hähnchenfleisch

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Summary

The present study was carried out to test amino acid decarboxylase activity, biofilm formation and antibiotic resistance of 404 Gram-negative bacteria isolated from marine fish, minced veal and chicken. The following isolates were identified: *Esherichia coli, Enterobacter cloacae, Klebsiella oxytoca, Citrobacter freundii, Hafnia alvei, Serratia marcescens, Pantoea agglomerans, Serratia fanticola, Proteus vulgaris, Citrobacter amalonaticus, Rahnella aquatilis, Morganella morganii, Escherichia vulneris, Klebsiella pneumoniae, Providencia rettgeri, Aeromonas hydrophila, Aeromonas caviae, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas oryzihabitans, Acinetobacter lwoffii, Acinetobacter baumannii* and *Shewanella putrefaciens.* Two *E. coli* O157 isolates were isolated from minced veal. Decarboxylase activity was quite common for Gram-negative bacteria and over 70% of isolates could decarboxylate at least one amino acid, and lysine was the most frequently decarboxylated amino acid. According to our results, 60.3% and 62.6% of the Gram-negative bacteria produced slime and biofilm, respectively. In the antimicrobial susceptibility test, the isolates were highly resistant to ampicillin, and ß-lactamase inhibitors. Multiple antibiotic resistance indices are ranged from 0.29 to 0.64, suggesting exposure to antibiotic contamination. One hundred forty four (35.6%) out of 404 isolates were identified as extended spectrum ß-lactamase (ESBL)-producers.

Keywords: Amino acid decarboxylase, antibiotic resistance, biofilm, chicken, fish, Gram-negative bacteria, minced veal

Zusammenfassung

Die vorliegende Studie wurde durchgeführt, um die Aminosäure-Decarboxylase-Aktivität, die Biofilmbildung und die Antibiotikaresistenz von 404 gramnegativen Bakterienstämmen zu untersuchen, die aus Meeresfischen, Kalbshack- und Hähnchenfleisch isoliert wurden. Folgende Isolate wurden identifiziert: *Escherichia coli, Enterobacter cloacae, Klebsiella oxytoca, Citrobacter freundii, Hafnia alvei, Serratia marcescens, Pantoea agglomerans, Serratia fanticola, Proteus vulgaris, Citrobacter amalonaticus, Rahnella aquatilis, Eitrobacter amalonaticus, Aeromonas hydrophila, Aeromonas caviae, Pseudomonas aeruginosa, Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas oryzihabitans, Acinetobacter lwoffii, Acinetobacter baumannii* und *Shewanella putrefaciens.* Zwei *E. coli* O157-Isolate wurden aus Kalbshackfleisch isoliert. Die Decarboxylase-Aktivität war bei gramnegativen Bakterien verbreitet, über 70% der Isolate konnten mindestens eine Aminosäure decarboxylieren. Lysin war die am häufigsten decarboxylierte Aminosäure. Nach unseren Ergebnissen produzierten 60,3% und 62,6% der gramnegativen Bakterien Schleim bzw. Biofilm. Im antimikrobiellen Empfindlichkeitstest waren die Isolate gegen Ampicillin- und ß-Lactamaseinhibitoren hochresistent. Mehrere Antibiotikaresistenzindizes lagen im Bereich von 0,29 bis 0,64, was auf eine Exposition gegenüber Antibiotikakontamination hindeutete. 144 (35,6%) von 404 Isolaten wurden als Extended-Spectrum Beta-Lactamasen (ESBL) Produzenten mit erweitertem Spektrum identifiziert.

Schlüsselwörter: Aminosäuredecarboxylase, Antibiotikaresistenz, Biofilm, Fisch, gramnegative Bakterien, Kalbshackfleisch

Journal of Food Safety and Food Quality 70, Heft 4 (2019), Seiten 91–124

Introduction

The microbiological content of raw meat purchased by consumers depends mostly on the slaughter process, sanitation during processing and packaging, maintenance of adequate cold chain storage from the processing to retail and to the consumer and finally sanitation during handling at the retail end. *Enterobacteriaceae* are a large family of Gram-negative bacteria that includes a number of important foodborne pathogens such as *Salmonella*, *Yersinia enterocolitica*, pathogenic *Escherichia coli* (including *E. coli* O157:H7), *Shigella* spp. and *Cronobacter* spp. Coliform bacteria within this family namely *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia* and *Escherichia* are considered as indicator organisms to define sanitary quality of food and water (Tekiner and Özpinar, 2016). *Klebsiella* spp, *Serratia* spp. and *Citrobacter* spp. are regarded as opportunistic pathogens, especially in clinical settings. *Enterobacteriaceae* species are inhabitants of soil, water, plants and the intestinal tract of a wide range animals. That means they could enter into the food chain and contribute to disease and spoilage. *E. coli*, *Citrobacter*, *Enterobacter*, *Klebsiella* and *Serratia* are the most prevalent bacteria isolated from beef, pork and poultry (Schwaiger et al., 2012; Kilonzo-Nthenge et al., 2013;Wong et al., 2015). Bacteria in the genera *Citrobacter*, *Enterobacter*, *Klebsiella*, *Serratia*, *Shewanella*, *Pseudomonas*, *Photobacterium*, *Aeromonas*, *Acinetobacter*, *Morganella* and *Vibrio* have been found in the main spoilage flora of fresh seafood products (Chakravarty et al., 2015).

The amino acid decarboxylase activity of numerous bacteria generates high level of biogenic amines in contaminated foods. The decarboxylation of histidine, tyrosine, lysine and ornithine yields to histamine, tyramine, cadaverine and putrescine, respectively, which are the food amines. The formation of biogenic amines in food is important for health associated with food spoilage. Amino acid decarboxylases are present in many microorganisms of food concern. They have been found in genera of the family *Enterobacteriaceae*, such as *Citrobacter*, *Klebsiella*, *Escherichia*, *Proteus*, *Salmonella* and *Shigella* and *Micrococcaceae*, such as *Staphylococcus*, *Micrococcus* and *Kocuria* (Marino et al., 2000). Furthermore, species of the genera *Bacillus*, *Pseudomonas*, *Photobacterium*, as well as many lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*, *Enterococcus*, *Carnobacterium*, *Pediococcus*, *Lactococcus* and *Leuconostoc* are able to decarboxylate amino acids (Zaman et al., 2010; Tembhurne et al., 2013).

The formation of bacterial biofilm on the surface of food processing equipment increases the threat of a crossover contamination of the product. This can have an effect on the quality and safety of the final product, especially if pathogenic bacteria or spoilage organisms become dominant in the biofilm. Several types of food-contaminated-bacteria are found to be biofilm-forming, including *L. monocytogenes*, *Vibrio* spp., *Salmonella* spp., *Bacillus* spp., *Aeromonas* spp., and *Pseudomonas* spp. It was noted that the presence of *Pseudomonas* spp. would significantly enhance the colonization of *L. monocytogenes* on stainless steel (Mahapatra et al., 2015).

Food-related and environmental bacteria resistant to antibiotics represent a major threat to humans, because they can act as a reservoir for the maintenance and spread of antibiotic resistance genes. Multidrug-resistance, including resistance to ß-lactams, fluoroquinolones, carbapenems and aminoglycosides, is frequently observed among *Enterobacteriaceae*, *Pseudomonas* spp., *Acinetobacter* spp. and *Shewanella* spp. (Matyar et al., 2008). Most *Aeromonas* spp. strains are typically resistant to penicillin, ampicillin, carbenicillin, methicillin, erythromycin, clindamycin and vancomycin (Arslan and Kucuksarı, 2015). The presence of extended spectrum beta lactamase (ESBL) and metallo beta lactamase (MBLS) genes among bacterial communities is of great concern, as they confer resistance to beta-lactam antibiotics as well as aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole. Antibiotic resistance has turned into a global public health problem in all over the world. Excessive or incorrect use of antimicrobials in human and veterinary medicine and without proper prescription are mediated on the development of antibiotic resistance. Antibiotics are extensively used in Turkey and this situation remains uncontrolled at both the community and hospital levels. Turkey has been identified as the country with the highest antibiotic use out of 42 countries in the broader European region (Kuzucu et al. 2011; Karabay et al. 2016).

The aim of this study was to identify Gram-negative bacteria that were isolated from marine fish, raw minced veal and chicken breasts, and to determine amino acid decarboxylase activity, slime formation, biofilm formation, antibiotic resistance and extended spectrum beta lactamase (ESBL) production that may influence food safety.

Materials and Methods

Sample collection

Ninety samples of marine fish (Black sea anchovy, *Engraulis encrasicolus*), 90 samples of raw minced veal and 90 samples of chicken breasts were purchased from different fish markets and butcher shops in Ankara, Turkey, between June 2012 and December 2013. Samples were collected in sterile polyethylene packs, placed on ice, immediately transported to the laboratory, and processed within 2 h after collection.

Sampling, Isolation and Identification

Twenty-five grams of each food sample was homogenized with 225 ml of sterile buffered peptone water (Merck, Darmstadt, Germany) and homogenized for 2 min using a stomacher (Lab. Lemco 400. Of each prepared sample, 0.1 ml was evenly spread on MacConkey agar (Merck). The inoculated media was incubated aerobically at 37 °C for 24–48 h. After incubation, at least five red (lactose positive) and colorless (lactose negative) colonies were picked from the plates and restreaked on fresh MacConkey agar to purify. Pure isolates were characterised by colony and cell morphology, Gram staining, oxidase and catalase activity, OF glucose and gelatin liquefaction tests and indol reaction (Matyar et al., 2008). Isolates were then identified using the BBL® Crystal™ ENF system (Becton Dickinson and Company, Maryland, USA).

For the determination of *E. coli* O157:H7 serotype, 25 g of each sample was homogenised in tryptone soya broth (Oxoid) supplemented with novobiocin (20 mg/L) and incubated at 37 °C for 24 h. The enrichment samples were streaked onto sorbitol MacConkey agar (Merck, Darmstadt, Germany) plates supplemented with cefexime (0.5 mg/L) and potassium tellurite (2.5 mg/L), and incubated at 37 °C for 24 h. After incubation, the plates were checked for the presence of sorbitol-negative, colourless colonies

1–2 mm in diameter. Subsequently, these presumptive colonies were confirmed serologically using an *E. coli* O157 latex agglutination test (Oxoid) and H7 antisera (Denka SeikenCo., Tokyo, Japan), as described by the manufacturers (AOAC, 1998).

Amino Acid Decarboxylase Activity

Amino acid decarboxylase activity of the isolates was qualitatively assessed by observing their ability to grow on modified Niven agar (0.5 % tryptone, 0.5 % yeast extract, 0.5 % Na CI, 0.1 % $CaCO_3$, 3 % agar, and 0.006% bromcresol purple, pH 5.3) containing 1 % of each precursor amino acid; L-histidine hydrochloride, L-lysine hydochloride, L-ornithine hydochloride and L-tyrosine hydrochloride (Sigma, St. Louis, MO) (Niven et al., 1981). The inoculated plates were incubated at 37 °C for 24–72 h. A colour change from yellow to purple indicated a positive reaction, i. e. that the respective amino acid decarboxylase was present.

Slime Formation

Production of slime from all isolates was studied by cultivation of the isolates on Congo Red Agar (CRA). CRA plates (sucrose 50 g (Sigma), brain heart infusion broth 37 g (Oxoid, Basingstoke, Hampshire, UK), agar 10 g, congo red 0.8 g (Sigma, St. Louis, MO), distilled water 1000 ml) were incubated at 37 °C for 24 h. After incubation, bright black colonies were established as slime positive (Gundogan et al., 2013).

Biofilm-forming ability

Biofilm-forming ability was measured by determination of adhesion to polystyrene microtiter plates according to the protocol of Christensen et al., (1985). Briefly, isolates were inoculated in Tryptic Soy Broth (TSB; Oxoid) and incubated for 18 h at 37 °C. Afterwards a 1:40 dilution in TSB supplemented with 0.25 % glucose, 200 µl of each dilution was distributed in flat-bottom 96-well polystyrene plates (Oxyvital, Hong Kong, China). The plates were incubated for 18 h at 37 °C, washed 3x with phosphate buffer saline (PBS), pH 7.0, air-dried for 1 h at 60 °C and stained with 0.25 % crystal violet for 1 min. After washing, optical density (OD) of each well content was measured at 570 nm using an automated microplate reader (Thermo Scientific Multiskan Microplate Reader GIO de Vita E C; Rome, Italy). We defined the cut-off OD (OD_c) for the microtiter-plate test as three standard deviations above the mean OD of the negative control. The adherence ability of the tested strains was classified into four categories based on the OD: "$OD \leq OD_c$: non–adherent, $OD_c < OD \leq 2XOD_c$: weakly adherent, $2XOD_c < OD \leq 4XOD_c$: moderately adherent, $4XOD_c < OD$: strongly adherent". All tests were carried out three times and the results were averaged.

Antibiotic susceptibility testing

Susceptibility testing of the isolated organisms was done by a disk diffusion method using the Kirby-Bauer technique and following the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2006). All disks were obtained from Bioanalyse (Ankara, Turkey): amikacin (30 µg), gentamicin (30), imipenem (10 µg), ertapenem (10 µg), ampicillin (10 µg), amoxicillin-clavulanic acid (20 and 10 µg), ampicillin-sulbactam (each 10 µg), piperacillin-tazobactam (100 and 10 µg), ceftazidime (30), cefotaxime (30), cefepime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), and aztreonam (30 µg). A standard reference strain of *K. pneumoniae* (ATCC 700603) sensitive to all antimicrobial drugs being tested was used as a control. For each isolate, a standard inoculum was prepared by adjusting the bacterial suspension in Lactose Broth (LB; Merck) to a final optical density of 0.5 McFarland units.

Detection of ESBL by Double Disk Synergy Test

ESBL was detected by a double disk synergy technique in which an augmentin disk (20 µg of amoxicillin and 10 µg of clavulanic acid) was placed in the center of a plate, and cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), and aztreonam (30 µg) disks were placed 30 mm (center to center) from the augmentin disk. The enhancement of the zone of inhibition of any one of the four drug disks toward the disk containing clavulanic acid suggested the presence of ESBLs. *Escherichia coli* NCTC 10418 was used as an ESBL-negative control, and *K. pneumoniae* ATCC 700603 was used as an ESBL-positive control (Gundogan et al., 2011).

Multiple Antibiotic Resistance (MAR) Index

The MAR index was calculated as the ratio (a/b) between the number of antibiotics to which the isolate was resistant (a) and the total number of antibiotics tested (b). A MAR index value >0.2 is observed when the isolates are exposed to high risk sources of human or animal contamination, where antibiotics use is common. In contrast, a MAR index value ≤0.2 is observed when antibiotics are seldom or never used (Matyar et al., 2014).

Statistical Analysis

Chi-square (x^2) tests were used to determine statistically significant differences in the prevalence of Gram-negative bacteria in food samples. P values of less than 0.05 were considered significant.

Results and discussion

Prevalence of Gram-negative Bacteria in Marine fish, Minced veal and Chicken

Raw meat can be contaminated with a variety of microorganisms, including those capable of spoiling the product during storage, and certain foodborne pathogens (Schwaiger et al., 2012; Kilonzo-Nthenge et al., 2013; Wong et al., 2015). In this study, 404 Gram-negative bacteria isolated and identified from 270 samples of marine fish, minced veal, and chicken breasts were investigated for amino acid decarboxylase activity, slime and biofilm production, ESBL production and antibiotic resistance. We found that there was a significant difference in the Gram-negative bacterial contamination levels among fish, minced meat and chicken, with the highest contamination level seen in fish ($P < 0.05$).

Enterobacteriaceae are useful marker for the identification of either fecal contamination to raw meats during the slaughter processs or secondary contamination along the processing chain (Tekiner and Özpinar, 2016). In this study, out of the 404 Gram-negative bacterial isolates, 309 (76.5%) isolates were belonging to the family *Enterobacteriaceae*. As shown in Table 1, the most predominant species present in the isolates was *Escherichia coli* (53 isolates), followed by *Enterobacter cloacae* (45 isolates), *Klebsiella oxytoca* (35 isolates), *Citrobacter freundii* (26 isolates), *Hafnia alvei* (24 isolates), *Serratia marcescens* (24 isolates), *Pantoea agglomerans* (15 isolates), *Serratia fantico-*

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TABLE 1: *Gram-negative bacteria isolated from fish, minced veal and chicken.*

Bacteria	Fish	Meat	Chicken	Number of total isolates	%
Escherichia coli	25	10	18	53	13.1
Enterobacter cloacae	13	14	18	45	11.1
Aeromonas hydrophila	43	–	–	43	10.6
Klebsiella oxytoca	7	16	12	35	8.6
Citrobacter freundii	10	6	10	26	6.4
Hafnia alvei	8	8	8	24	5.9
Serratia marcescens	4	12	8	24	5.9
Pantoea agglomerans	4	5	6	15	3.7
Serratia fanticola	4	2	6	12	2.9
Proteus vulgaris	4	6	2	12	2.9
Citrobacter amalonaticus	5	7	–	12	2.9
Rahnella aquatilis	3	4	5	12	2.9
Pseudomonas aeruginosa	11	–	–	11	2.7
Morganella morganii	9	1	1	11	2.7
Escherichia vulneris	6	2	2	10	2.4
Klebsiella pneumoniae	4	2	3	9	2.2
Providencia rettgeri	5	3	1	9	2.2
Shewanella putrefaciens	6	–	–	6	1.4
Pseudomonas putida	6	–	–	6	1.4
Pseudomonas fluorescens	6	–	–	6	1.4
Aeromonas caviae	6	–	–	6	1.4
Acinetobacter lwoffii	6	–	–	6	1.4
Acinetobacter baumannii	6	–	–	6	1.4
Pseudomonas oryzihabitans	5	–	–	5	1.2
Total	206	98	100	404	

la (12 isolates), *Proteus vulgaris* (12 isolates), *Citrobacter amalonaticus* (12 isolates), *Rahnella aquatilis* (12 isolates), *Morganella morganii* (11 isolates), *Escherichia vulneris* (10 isolates), *Klebsiella pneumoniae* (9 isolates), and *Providencia rettgeri* (9 isolates). Similar to our results, a large number of *Enterobacteriaceae* species isolated from fish, red meat and chicken meat products have been reported in Turkey (Matyar 2007; Ondes and Ozpinar, 2016; Tekiner and Ozpinar, 2016), In USA (Kilonzo-Nthenge et al., 2013), in Germany (Gwida et al., 2014), in Nepal (Shrestha et al. 2017), and in China (Ye et al., 2018).

In this study, the important foodborne pathogens such as *Salmonella*, *Yersinia enterocolitica*, *Shigella* spp. and *Cronobacter* spp. were not detected in any of the food samples. However; two organisms of concern were *E. coli* and *K. pneumoniae*, opportunistic pathogens of humans and animals responsible for a wide range of infections, such as urinary tract infections, pneumoniae, wound infections and septicemia (Gundogan et al., 2011; Gundogan and Avci, 2014). The presence of *E. coli* in foods is a matter of concern because some strains may be pathgenic (Gundogan and Avci, 2014).We isolated two (2.2%) *E. coli* O157 isolates from 90 minced veal. *E. coli* O157:H7 serotypes, identified as enterohaemorrhagic *E. coli* (EHEC) and grouped as verotoxin-producing *E. coli* (VTEC), are recognised as the primary cause of haemorrhagic colitis (HC) and the diarrhoea-associated form of haemolytic-uremic syndrome (HUS) (Gundogan and Avci, 2014). In some studies conducted in different cities of Turkey, *E. coli* O157 was detected in 7.6%–8.8% of ground beef, minced meat and ground meat (Sarimehmetoglu et al., 2009; Cetin et al., 2010; Temelli et al., 2012). These values are higher than that obtained from this study. The intestinal tract of cattle was reported as the principal reservoir of *E. coli* O157 (Gundogan and Avci, 2014). Therefore, preventing faecal material from contaminating meat is an important step in reducing the prevalence of *E. coli* O157 in raw meat and its products.

In our country, the largest proportion of fish yields were from the Black Sea where anchovy (*Engraulis encrasicolus*) was the dominant fish in caught with about 51 percent of total between 2003 and 2012 (Turkish Statistical Institute, 2013). Black sea anchovy is most commonly consumed fish species in Turkey due to its cheaper price compared to other fish species (Gucu et al., 2017). The following 95 Gram-negative isolates *Aeromonas hydrophila* (43 isolates), *Aeromonas caviae* (6 isolates), *Pseudomonas aeruginosa* (11 isolates), *Pseudomonas putida* (6 isolates), *Pseudomonas fluorescens* (6 isolates), *Pseudomonas oryzihabitans* (5 isolates), *Acinetobacter lwoffii* (6 isolates), *Acinetobacter baumannii* (6 isolates), and *Shewanella putrefaciens* (6 isolates) have been isolated from the fish only (Table 1). In the present study, *Aeromonas hydrophila* were found the most frequently comparing with species from *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* ($P < 0.05$). Similarly, Grigoryan et al. (2013) reported that although bacteria from *Enterobacter*, *Klebsiella*, *Citrobacter*, *Serratia*, *Pseudomonas*, *Alcaligenes* and *Vibrio* genera have been isolated and identified from rainbow trout, bacteria form genera *Aeromonas* have been found in prevailing quantities (96%). *Aeromonas* spp. have emerged as an important human pathogens because of diarrhea related to foodborne outbreaks. These bacteria are also predominantly pathogenic to aquatic animals, especially fish (Arslan and Küçüksari, 2015).

Compared to our results, higher contamination rates of fish with *Aeromonas* spp. were reported as 44.1%, 42.8% and 77.9%, respectively by Arslan and Kucuksari (2015), Yucel and Erdogan (2010) and Yucel et al. (2005) in Turkey. In Malaysia *A. hydrophila* isolates were isolated from 11.5% of the fish (Radu et al., 2003), while in Brazil, the percentage of these bacteria was 50% (Da Silva et al., 2010). There may be several reasons for these variations, such as differences in geographic location and season and difference in fish species studied. The fish samples were obtained from several sources and storage conditions which bring about different results. According to Mol and Saglam (2004), fish boxes are generally laid on the floor, and this is a major cause of bacterial contamination in Turkish fish markets. Furthermore, the transportation of fish from seaside cities to Ankara will take at least 5 hours. During the transportation, sprinkling of fish with contaminated water, packing it with contaminated ice, coupled with unhygienic handling may explain the high prevalence of Gram-negative bacteria in fish in the markets.

Decarboxylase Activity of Gram-negative Bacteria

The detection of bacteria possessing amino acid decarboxylase activity is of main importance to assess the risk of foods to contain biogenic amine and to prevent their accumulation in food products. Formation of biogenic ami-

nes in foods is important for health and also for unfavorable effects on flavor (Marino et al., 2000; Maifreni et al., 2013). Therefore, we studied 404 Gram-negative bacteria isolates for their capability to decarboxylate tyrosine, ornithine, lysine and histidine (Table 2). Decarboxylase activity was quite common for Gram-negative bacteria and over 70% of the isolates could decarboxylate at least one amino acid. Lysine was the most frequently decarboxylated amino acid, followed by ornithine, tyrosine and histidine. This is an important food safety concern, considering the these isolates were potential cadaverine, putrescine, tyramine and histamine producers in fish, veal and chicken. Durlu-Ozkaya et al. (2001) reported that conversion of ornithine, lysine, tyrosine and histidine respectively, to putrescine, cadaverine, tyramine and histamine was found in ≤ 82% of *Enterobacteriaceae* isolates. Authors indicated that high levels of these biogenic amines in ground meat and meat products can be an indicator of the hygienic quality of meats. Marino et al. (2000) and Maifreni et al. (2013) reported that most of the *Enterobacteriaceae* species were shown to have the ability to decarboxylate mainly lysine and ornithine, which were consistent with our results.

The results obtained from this study also indicated that depends on the isolates, the ability of microorganisms to decarboxylate amino acids were highly variable. The ability to decarboxylate the two amino acids (histidine-tyrosine or lysine-ornithine or tyrosine-ornithine) was present in all isolates of *Enterobacteriaceae*, in *Shewanella putrefaciens* and some isolates of *Acinetobacter* spp. In this study, only few isolates of the *Enterobacteriaceae* species could decarboxylate histidine-ornithine. Isolates from *Aeromonas* spp. and *Pseudomonas* spp. were able to decarboxylate histidine-ornithine or lysine-tyrosine (Table 2). Histamine is considered to be the most active amine and is related to almost all food amines poisoning incidences. However, the occurrence of putrescine and cadaverine which may enhance the toxicity of histamine should not be underestimated (Zaman et al., 2010). The result observed is that, even if the microorganisms had the capability to produce more than one decarboxylase, the decarboxylating activity is highest towards lysine and ornithine with consequent production of cadaverine and putrescine, respectively which was in agreement with the results reported by Marino et al. (2000) and Maifreni et al. (2013).

Previous studies have also revealed the capability of Gram-negative bacteria to produce decarboxylase enzymes was variable. Durlu-Ozkaya et al. (2001) showed that *E. coli* and *M. morganii* possess both histidine and lysine decarboxylases, *P. mirabilis* has both histidine and ornithine decarboxylases and *Enterobacter* spp. have both lysine and ornithine decarboxylases. Ozogul and Ozogul (2005) reported that *E. coli*, *K. oxytoca, H. alvei* and *P. vulgaris* possess histidine decarboxylase activity while *Pseudomonas* spp., *K. oxytoca* and *H. alvei* could decarboxylate both histidine and lysine. In their study, all Gram-negative rods decarboxylated ornithine but none of the *Acinetobacter* spp. isolates had lysine, ornithine and

TABLE 2: *Amino acid decarboxylase activity of gram-negatvie bacteria.*

Species	his	lys	tyr	orn	his-tyr	his-orn	lys-tyr	lys-orn	tyr-orn	his-lys tyr	his-tyr orn	lys-tyr-orn	his-lys-tyr-orn	Total
E. coli (n=53)	7	12	11	7	3	2	2	1	3	1	2	–	1	52
E. cloacae (n=45)	1	18	9	5	3	–	1	1	2	1	–	–	–	41
A. hydrophila (n=43)	1	1	1	1	–	6	2	–	–	1	–	2	1	16
K. oxytoca (n=35)	2	9	4	8	2	–	1	1	1	1	2	–	–	31
C. freundii (n=26)	7	1	2	5	1	–	–	1	1	–	–	1	1	20
H. alvei (n=24)	1	2	2	8	1	1	1	2	1	–	1	1	1	22
S. marcescens (n=24)	1	7	2	2	2	1	1	1	1	–	–	–	–	18
P. agglomerans (n=15)	–	–	1	–	1	–	–	1	1	–	–	–	–	4
S. fanticola (n=12)	–	–	1	1	1	1	–	1	1	–	–	–	–	6
P. vulgaris (n=12)	1	–	1	–	1	1	–	1	1	–	–	–	–	6
C. amalonaticus (n=12)	1	1	–	1	1	1	–	1	1	–	–	–	–	7
R. aquatilis (n=12)	–	1	–	–	1	–	–	1	1	–	–	–	–	4
P. aeruginosa (n=11)	1	–	–	1	–	1	2	–	–	1	–	–	1	7
M. morganii (n=11)	2	–	1	–	1	1	–	1	1	–	–	1	1	9
E. vulneris (n=10)	–	1	–	–	1	–	–	1	1	–	–	–	–	4
K. pneumoniae (n=9)	1	1	1	1	1	1	–	1	1	–	–	–	–	8
P. rettgeri (n=9)	–	–	–	–	1	–	–	1	1	–	–	–	–	3
S. putrefaciens (n=6)	1	–	–	1	1	–	–	1	1	–	–	–	1	6
P. putida (n=6)	–	1	1	–	–	1	1	–	–	1	–	–	1	6
P. fluorescens (n=6)	1	1	–	–	–	1	1	–	–	1	–	–	1	6
A. caviae (n=6)	–	1	1	–	–	1	1	–	–	1	–	–	–	5
A. lwoffii (n=6)	–	1	–	–	–	–	–	2	3	–	–	–	–	6
A. baumannii (n=6)	–	1	–	–	–	–	–	2	2	–	–	–	–	5
P. oryzhabitans (n=5)	1	1	–	–	–	1	1	–	–	1	–	–	–	5
Total	29	60	38	41	22	20	14	21	24	9	5	5	9	297

his: histidine; lys: lysine; orn: ornithine; tyr: tyrosin

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histidine decarboxylase activity. However, in this study, *Acinetobacter* spp. isolates have the capacity to decarboxylate these amino acids. Tembhurne et al. (2013) reported that 63 out of 202 *Enterobacteriaceae* isolates from Indian Mackerel gave positive results in the Niven medium, indicating histidine decarboxylase activity. In the same study, *Shewanella putrefaciens* was found as non-*Enterobacteri*al histamine-producing bacteria, which was similar to our result. Other study showed histidine decarboxylase activity in 84% of 152 Gram-negative bacteria from fish using modified Niven method whereas *E. coli* isolates were detected as non-histamine producers (Bjornsdottir et al., 2009). This result is not in agreement with the result of our study. We observed that few isolates from *Klebsiella* spp., *Pseudomonas* spp., *Aeromonas* spp., *E. coli*, *C. freundii, H. alvei, M. morganii,* and *S. putrefaciens* also decarboxylated three or four amino acids. Our results provide new information regarding the decarboxylase activity of *S. fanticola, R. aquatilis, E. vulneris, K. pneumoniae, P. rettgeri* and *A. baumannii* (Table 2). Differences between the reults may be due to the difference methods that were used for the detection of amino acid decarboxylase-producing bacteria.

Nevertheless, in this study, it is observed that a high proportion of *Enterobacteriaceae*, *Aeromonas* spp., and *Pseudomonas* spp. isolates had amino acid decarboxylase activity. The presence of microbial populations with decarboxylase activity and availability of free amino acids are considered the most important factors affecting the production of biogenic amines in raw and processed foods (Ozogul and Ozogul 2005). The results obtained regarding isolation of these organisms from raw meats highlight the need to improve hygienic practices to prevent further proliferation of decarboxylase positive microflora on fish, veal and chicken.

Slime and biofilm formation of Gram-negative bacteria

Microorganisms in food are able to form biofilms on the food and food processing equipment surfaces. Biofilms can also be transferred onto food, such as fish, meat and poultry, when these foods come in contact with contaminated surfaces (Silagyi et al., 2009). The present study showed that slime and biofilm-forming Gram-negative bacteria contaminate fish, meat and chicken (Table 3). According to our results, 244 (60.4%) and 253 (62.6%) out of the 404 Gram-negative bacteria had slime and biofim formation, respectively. All of the *Pseudomonas* spp., *Aeromonas* spp., *Acinetobacter* spp., and *S. putrefaciens* isolates produced slime.

A recent study showed that slime-producing *Pseudomonas* spp. isolates were the most abundant bacteria on slaughterhouse surfaces after cleaning and sanitizing treatments (Bakhtiary et al. 2016). The supportive activity of *Pseudomonas* isolates for the attachment and biofilm formation of *S. aureus* and *L. monocytogenes* has also been reported (Bakhtiary et al. 2016). On the other hand, Arslan et al. (2011) showed that *Pseudomonas* spp. isolates did not produce slime. Furthermore, Orozova et al. (2009) reported that *Aeromonas* isolates were negative for slime production. The results of our study do not confirm these findings. Also, our results only partially agree with Arslan and Kucuksari (2015) who found that slime activity in 45.2% of *A. caviae* isolates but none of the A. *hydrophila* isolates produced slime while the A. *hydrophila* isolates in this study had slime activity. Some of the previous studies have shown that the slime/biofilm formation is largely dependent on the origin of the isolates as well as temperature and time, and associated with nutrient content of the growth medium (Orozova et al., 2009; Reynisson et al. 2009). Meanwhile we found that none of the *P. agglomerans, S. fanticola, C. amalonaticus, R. aquatilis, E. vulneris* and *P. rettgeri* isolates had slime activity. However, *E. coli* (66%), *E. cloacae* (62.2%), *K. oxytoca* (54.3%), *C. freundii* (53.8%), *H. alvei* (66.7%), *S. marcescens* (54.2%), *P. vulgaris* (66.7%) *M. morganii* (81.8%) and *K. pneumoniae* (77.8%) isolates had a great tendency to produce slime. Furthermore, except *S. marcescens*, *S. fanticola*, *R. aquatilis*, *M. morganii* and *P. rettgeri* isolates, remeaning isolates were characterised by moderate to strong biofilm-forming ability (Table 3). The species in the biofilm originated from the all samples studied and therefore be expected to play a role in biofilm formation in food contact surfaces. This is not suprising because similar results have already been reported by several authors. Bagge et al. (2001) found that *S. putrefaciens*, a fish spoilage bacterium, is able to attach and form biofilms on food processing surfaces. Møretrø et al. (2013) showed that *Enterobacter* spp., *Pseudomonas* spp., *Citrobacter* spp., *Acinetobacter* spp., *Serratia* spp. and *Listeria monocytogenes* isolates from meat abattoir process surfaces were strong biofilm producers. Also Liu et al. (2015) observed

TABLE 3: *Slime formation and biofilm-forming ability of gram-negative bacteria.*

Bacteria	Slime formation	Biofilm-forming ability			
		Absent	Weak	Moderate	Strong
E. coli	53/35	26	–	15	12
E. cloacae	45/28	19	20	3	3
A. hydrophila	43/43	21	–	13	9
K. oxytoca	35/19	1	17	9	8
C. freundii	26/14	12	3	10	1
H. alvei	24/16	5	–	12	7
S. marcescens	24/13	6	8	10	–
P. agglomerans	15/-	3	–	9	3
S. fanticola	12/-	–	12	–	–
P. vulgaris	12/8	7	–	4	1
C. amalonaticus	12/-	4	–	8	–
R. aquatilis	12/-	10	–	–	2
P. aeruginosa	11/11	3	3	4	1
M. morganii	11/9	–	11	–	–
E. vulneris	10/-	5	3	1	1
K. pneumoniae	9/7	4	3	1	1
P. rettgeri	9/-	9	–	–	–
S. putrefaciens	6/6	3	1	1	1
P. putida	6/6	3	1	1	1
P. fluorescens	6/6	3	–	2	1
A. caviae	6/6	–	3	2	1
A. lwoffii	6/6	4	–	1	1
A. baumannii	6/6	1	1	3	1
P. oryzhabitans	5/5	2	–	2	1
Total	404/244	151	86	111	56

that *P. lundensis* isolated from spoiled Chinese pork had a high capacity to produce biofilms and was able to adhere to the contact surfaces. According to Bitrian et al.(2012), Yaron and Römling (2014) and Bakhtiary et al. (2016), in meat-processing environments, all surfaces and materials are likely to be colonized by microorganisms if sanitation procedures are inadequate and/or insufficient. Moreover the attachment properties and the biofilm formation of bacteria on surfaces facilitate cross-contamination. If pathogens are present, consumption of the contaminated foods may pose a health risk (Reynisson et al., 2009). In addition, biofilm formation creates major problems in the food industry, because biofilms represent an important source of contamination, increased food spoilage and can support microbial growth. Therefore, for quality and safety of foods, preventive and control strategies like hygienic plant lay-out and design of equipment, choice of materials, correct use and selection of detergents and disinfectants coupled with physical methods can be suitably applied for controlling biofilm formation on food-contact surfaces (Reynisson et al., 2009; Mahapatra et al., 2015).

Antimicrobial Resistance

During the past decade, multidrug resistance in Gram-negative bacteria is increasing throughout the world (Woodford et al., 2014). This increase is mainly the result of an increased prevalence of ESBL-producing *Enterobacteriaceae* and non-lactose fermenting bacteria such as *Pseudomonas* and *Acinetobacter* species (Tekiner and Ozpinar, 2016).

The results for 404 isolates that were tested against 14 antimicrobial agents are presented in Table 4.

Aminoglycosides are broad-spectrum antibiotics of high potency that have been used for the treatment of serious Gram-negative infections (Arslan et al., 2011; Gundogan et al., 2011). According to the results reported here, all the isolates of *Enterobacteriaceae*, *Aeromonas* spp., *Pseudomanas* spp., *Acinetobacter* spp., and *S. putrefaciens* were susceptible to aminoglycosides (amikacin and gentamicin), which is in agreement with previously published data for *Enterobacteriaceae*, *Aeromonas* spp., *Pseudomonas* spp., and *A. baumanii* isolates isolated from food (Arslan et al., 2011; Schwaiger et al., 2012; Kilonzo-Nthenge et al., 2013). Resistance to amikacin and gentamicin was most common in clinical isolates of *Aeromonas* spp., *Acinetobacter* spp., *Pseudomonas* spp. and *Enterobacteriaceae* (Kucukates, 2005). However, Chakravarty et al. (2015) reported that prevalence of gentamicin resistance of coliform bacteria in Indian foods was 50%. Furthermore, Gundogan and Avci (2014) observed that 53.7% of gentamicin resistance amongst *E. coli* isolates. Arslan and Küçüksari (2015) reported low levels of resistance to gentamicin (4.1%) and amikacin (4.1%) in *Aeromonas* spp. isolates while high level of gentamicin resistance was reported in *Aeromonas* spp. isolates (54%) by Yucel et al. (2005).

Carbapenem antibiotics are the last treatment option for severe, life-threatening infections caused by multiple-drug resistant pathogens. Carbapenem-resistant *Enterobacteriaceae* strains and non-fermentative gram-negative bacilli isolated from human infections have been

TABLE 4: *Antibiotic resistance of gram-negative bacteria.*

Species	AMK	GEN	IMP	ETP	AMP	AMC	SAM	PIT	CAZ	CTX	CEP	CRO	CIP	AZT
E. coli	–	–	–	3.8*	100	71.7	56.6	52.8	–	–	7.5	13.2	17	20.8
E. cloacae	–	–	–	8.9	84.4	77.8	68.9	60	13.3	–	11.1	–	–	–
A. hydrophila	–	–	–	–	100	72	72	51.1	74.4	55.8	67.4	55.8	–	–
K. oxytoca	–	–	–	8.6	100	74.3	54.3	51.4	14.3	14.3	8.6	14.3	17.1	–
C. freundii	–	–	–	–	100	73	53.8	57.7	15.3	11.5	11.5	15.3	–	–
H. alvei	–	–	–	8.3	100	70.8	58.3	58.3	12.5	–	–	–	–	–
S. marcescens	–	–	–	8.3	70.8	70.8	50	70.8	–	16.7	16.7	–	–	–
P. agglomerans	–	–	–	–	33.3	26.7	20	20	6.7	–	–	–	–	–
S. fanticola	–	–	–	–	16.7	16.7	25	16.7	16.7	–	–	8.3	–	–
P. vulgaris	–	–	–	–	75	25	16.7	16.7	–	–	–	–	–	–
C. amalonaticus	–	–	–	–	100	8.3	16.7	16.7	16.7	16.7	–	–	–	–
R. aquatilis	–	–	–	–	50	8.3	8.3	16.7	–	–	–	–	–	–
P. aeruginosa	–	–	–	–	100	72.7	54.5	81.8	63.6	63.6	72.7	54.5	–	–
M. morganii	–	–	–	–	72.7	72.7	54.5	72.7	–	–	–	–	–	–
E. vulneris	–	–	–	–	100	20	10	20	–	–	–	–	–	–
K. pneumoniae	–	–	–	11.1	66.7	77.8	55.6	77.8	11.1	–	–	–	11.1	22.2
P. rettgeri	–	–	–	–	66.7	11.1	11.1	11.1	–	–	–	–	–	–
S. putrefaciens	–	–	–	–	100	83.3	50	50	66.7	83.3	66.7	66.7	–	–
P. putida	–	–	–	–	50	16.7	16.7	16.7	66.7	66.7	66.7	83.3	–	–
P. fluorescens	–	–	–	–	50	83.3	66.7	66.7	83.3	83.3	66.7	66.7	–	–
A. caviae	–	–	–	–	100	83.3	50	50	66.7	66.7	83.3	83.3	–	–
A. lwoffii	–	–	–	–	83.3	16.7	16.7	16.7	66.7	83.3	66.7	66.7	–	–
A. baumannii	–	–	–	–	100	83.3	66.7	83.3	83.3	83.3	83.3	66.7	–	–
P. oryzhabitans	–	–	–	–	20	20	20	20	80	80	80	80	–	–

*Percentage of resistant isolates; AMK: amikacin; GEN: gentamicin; IMP: imipenem; ETP: ertapenem; AMP: ampicillin; AMC: amoxycillin/clavulanic acid; SAM: ampicillin /sulbactam; PIT: piperacillin/tazobactam; CAZ: ceftazidime; CTX: cefotaxime; CEP: cefepime; CRO: ceftriaxone; CIP: ciprofloxacin; AZT: aztroenam

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reported in many parts of the world (Woodford et al., 2014; Webb et al., 2016), including Turkey (Kuzucu et al. 2011; Karabay et al. 2016). In the present study, all isolates were susceptible to imipenem, whereas, 3.8% of *E. coli*, 8.9% of *E. cloacae*, 8.6% of *K. oxytoca*, 8.3% of *H. alvei*, 8.3% of *S. marcescens*, and 11.1% of *K. pneumoniae* isolates were resistant to ertapenem. However, in our previous studies, carbapenems were the most effective antibiotics for *Klebsiella* spp. and *E. coli* isolates, in which 100% of the isolates were susceptible to meropenem and imipenem (Gundogan et al., 2011; Gundogan and Avci 2014). A high incidence of imipenem resistance (95%) was also documented both in coliform bacteria (*E. coli*, *C. diversus*, *E. cloacae*, *K. oxytoca*, *S. fonticola*, *K. pneumoniae*, *E. aerogenes*) and in fish pathogens (A. *hydrophila*, *A. caviae, P. oryzihabitans*) isolated from Turkish trout farms (Capkin et al., 2015). Resistance to carbapenems, such as ertapenem (40.82%), meropenem (31.36%), and imipenem (10.65%) in *E. coli* isolates have also been reported by Chakravarty et al. (2015) in India. Some studies have reported the low prevalence of carbapenem resistance in *A. lwoffii* (Wang et al., 2012), *A. baumannii* (Zhang et al., 2013) *Aeromonas* spp. (Arslan and Kücüksari, 2015), *Pseudomonas* spp. (Wong et al., 2015), and *Enterobacteriaceae* (Ye et al., 2018).

Gram-negative rods are frequently associated with resistance to ß-lactam antibiotics due to a constitutively expressed ß-lactamase. We detected resistance to ampicillin in all of the *E. coli*, A. *hydrophila*, *K. oxytoca*, *C. freundii*, *H. alvei*, *C. amalonaticus, P. aeruginosa, E. vulneris, S. putrefaciens*, *A. caviae* and *A. baumanii* isolates. Resistance rates of other isolates to ampicillin varied between 16.7% and 84.4%. This is not surprising because ß-lactams are commonly used antibiotics for the treatment of Gram-negative bacterial infections in humans and animals (Ondes and Ozpinar, 2016). Similar findings have been reported in a recent study for *Enterobacteriaceae* isolates in China, where 97.9% of all isolates were resistant to ampicillin (Ye et al., 2018). Frequent occurrence of ampicillin resistance in members of *Enterbacteriaceae*, *Aeromonas* spp. and *Pseudomonas* spp. obtained from various foods have also been described previously (Kilonzo-Nthengeet al., 2013; Gundogan and Avci, 2014; Capkin et al.,2015).

Clavulanic acid, sulbactam or tazobactam, which are ß-lactamase inhibitors, regarded as good choice for inhibit ESBL-producing Gram-negative bacteria. However, high rates of resistance to amoxycillin/clavulanic acid (>70%), ampicillin/sulbactam (≥50%), and piperacillin/tazobactam (≥50%) were observed among *E. coli*, *E. cloacae*, A. *hydrophila*, *K. oxytoca*, *C. freundii*, *H. alvei*, *S. marcescens*, *P. aeruginosa*, *M. morganii*, *K. pneumoniae*, *S. putrefaciens*, *P. fluorescens*, *A. caviae* and *A. baumanii* isolates. In the present study, low incidences of resistance to ß-lactamase inhibitors was found in other isolates (<30 %). Singh et al. (2017), in India, investigated fresh seafoods for the occurrence and antimicrobial resistance patterns of ESBL-producing *Enterobacteriaceae*. These researchers reported that resistance to amoxicillin-clavulanic acid and piperacillin/tazobactam was seen in 38.46% and 40.82% of the *Enterobacteriaceae* isolates, respectively which are comparable with results of our study. Compared to our results, lower prevalences of resistance to amoxycillin/clavulanic acid and piperacillin/tazobactam has been found in isolates of *E. coli*, *C. freundii*, *P. agglomerans*, *Aeromonas* spp. and *Klebsiella* spp. (Gundogan et al., 2011; Schwaiger et al., 2012; Gundogan and Avci, 2014; Arslan and Küçüksari, 2015; Wong et al., 2015).

Ciprofloxacin is a broad spectrum fluoroquinolone antibacterial agent. The observed resistance of *E. coli*, *K. oxytoca* and *K. pneumoniae* to ciprofloxacin was 17%, 17.1% and 11.1 %, respectively. No resistance to ciptofloxacin was observed in other isolates. Gundogan et al., 2011, 2013, 2014) also reported low resistance rates of *Klebsiella* spp. (16%) and *E. coli* (29.4%-31.1%) to ciprofloxacin. Compared to our results, higher rates of ciprofloxacin resistance have been reported by Benameur et al. (2018), who observed that 90.47% of *K. pneumoniae* and 85.10% of *E. coli* isolates isolated from poultry were resistant to ciprofloxacin. Chakravarty et al. (2015) showed high prevalence of ciprofloxacin-resistant *E. coli* in foods. In other study, more than 63% of *Enterobacteriaceae* isolates showed resistance to ciprofloxacin (Ye et al., 2013). Ciprofloxacin resistance in *Klebsiella* and *E. coli* is predominantly due to a chromosomal mutation in the gyrA gene, which codes for the target of quinolone activity (Pehlivanlar-Onen et al., 2015). As resistance to ciprofloxacin emerged, resistance to ß-lactam antibiotics became prominent. This resistance was largely a result of ESBLs, which mediate resistance to newer ß-lactam agents, such as ceftazidime, ceftriaxone, cefotaxime, and aztreonam, that have an oxyamino group (Pehlivanlar-Onen et al., 2015).

Cephalosporins are an important class of antibacterial agents in use for both humans and animals. According to the results reported here, resistance to ceftazidime, cefotaxime, cefepime and ceftriaxone was observed for >54 % of the *Aeromonas* spp., *Pseudomonas* spp., *Acinetobacter* spp., and *S. putrefaciens* isolates. *Enterobacteriaceae* isolates showed resistance to ceftazidime, cefotaxime, cefepime and ceftriaxone in the range of 0–16.7%. In a previous studies conducted by Tekiner and Ozpinar (2016) and Ye et al. (2018) on the resistance of *Enterobacteriaceae* from various foods, a high percentage (≥60%) of isolates were resistant to cephalosporins. Resistance to these group antibacterial agents for Gram-negative bacteria in aquatic environments reported to be >90% (Matyar et al., 2004; 2008; 2014; Schwaiger et al. 2012; Wasiński et al., 2014; Devarajan et al., 2017; Singh et al., (2017). The simultaneous resistance of isolates to ß-lactams, may be due to the dissemination of antibiotic resistance plasmids in the marine environment, as reported by Matyar et al. (2004).

Aztreonam is a synthetic monocyclic ß-lactam in the family of monobactams and is exclusively active (like aminoglycosides) against the aerobic gram-negative bacilli. We observed that only 22.2 % and 20.8 % of *K. pneumoniae* and *E. coli* isolates, respectively, were resistant to aztroenam. According to Gundogan et al. (2011, 2013, 2014), aztroenam had moderate activity against *Klebsiella* spp. (24%–42.9%) and *E. coli* (29.9%). Our results were not in agreement with the findings of Capkin et al. (2015) who found that a high incidence of aztreonam resistance (95%) in *Aeromonas* spp., and *Pseudomonas* spp. Environmental and food isolates of *E. coli* and *Klebsiella* spp. showed resistance to aztreonam in the range of 76.9–100% (Afifi, 2013; Ibrahimagićet al., 2016).

Excessive ampicillin usage in Turkey for treatment of infections in humans and animals can be regarded as one of the major causes of resistance to this antimicrobial among Gram-negative bacteria. Also, there are great tendency towards decreased susceptibility observed for ß-lactams, carbapenems and cephalosporins. Therefore, there remains a need for continued surveillance and judicious use of these antibiotics.

Multiple Antibiotic Resistance (MAR) Index

Increasing levels of MAR among bacteria are resulted from widespread use of antibiotics in human and veterinary medicine and as growth promoters for intensive livestock production (Arslan and Kucuksari, 2015). In addition, food handlers may cross contaminate foods during preparation and if they are carriers of MAR bacteria, they may contaminate foods themselves (Carvalheira et al., 2017). Consumable animal products have been suggested as a possible source of both resistant bacteria and resistant genes that can be transferred to humans directly (Shrestha et al., 2017).

If the bacterial isolates were resistant to four or more antibiotics, they were regarded as multi antibiotic resistant (MAR) (Matyar et al., 2014). In the present study, MAR index values in Gram-negative bacterial isolates ranged from 0.29 to 0.64, showing a resistance to 4–9 antibiotics. MAR index values >0.2 indicate that the isolates must have originated from an environment where antibiotics are often used. Our finding is in agreement with previous reports showing the prevalence of multiple-resistant bacteria in various foods in Turkey and different parts of the world.

Among the multi-resistant isolates, a significant proprtion of *Acinetobacter* spp. isolates were resistant to 4 (100%), 5 (83.3%), 6 (83.3%), 7 (66.7%) 8 (50%) and 9 (50%) antibiotics. The MAR index ranged from 0.29–0.57 (Figure 1). In Portugal, 51.2% of the *Acinetobacter* strains were considered as multidrug-resistant (Carvalheira et al., 2017). In China, isolates from aquaculture products, including *Acinetobacter* spp. were resistant to two (22%), three (36%), and four (29%) antibiotics (Ye et al., 2013). High prevalence of multi-resistance in *A. baumannii* isolates from chicken, veal, beef, pork and turkey has also been reported in Switzerland by Lupo et al. (2014).

Our results revealed that the MAR index ranging from 0.29 to 0.64 for *Enterobacteriaceae* isolates. They were resistant to 4 (55%), 5 (46.4%), 6 (43%), 7 (35.9%), 8 (35.6%) and 9 (28.8%) antibiotics. (Figure 1). Higher percentages reported by Matyar et al. (2008) who showed that 43.3% of Gram-negative bacteria isolated from different sources, including seawater, shrimp and sediment in Turkey, were resistant to 6 antibiotcs while 56.8 % of them were resistant to 7 or more antibiotcs. In USA, Kilonzo-Nthenge et al. (2013) observed that 84.9% of *Enterobacteriaceae* isolates isolated from chicken and beef displayed MAR to 3 or more antimicrobials. In their study, 19.2% of isolates showed MAR to 5 or more antimicrobials. Capkin et al. (2015) showed MAR index values ranging between 0.19–0.83 and 0.42–0.83, respectively for coliform bacteria and other Gram-negative bacteria in Turkish trout. These values are higher than that obtained from our study.

As it shown in Figure 1, isolates from *Aeromonas* spp. showed resistance to 4, 5, 6, 7 and 8 antibiotics with a frequency of 91.8%, 81.6%, 65.3% ,44.9% and 32.6%, respectively. The MAR index ranged from 0.29-0.57. *Pseudomonas* spp. isolates were resistant to 4 (89.3%) and 5 antibiotics (60.8%). The MAR index varied from 0.29 to 0.36. Our results were in agreement with Matyar et al. (2010) who reported that *Aeromonas* isolates resistant to 6 or more antibiotics and MAR index values ranged from 0.2 to 0.60. These authors have also detected multiple resistance in *Pseudomonas* isolates but reported higher MAR index values (0.2–0.73). Matyar (2007) showed that 29.4% and 8% of the *Pseudomonas* spp. isolates from Turkish sea bass (*Dicentrarchus labrax*) resistant to 7 and 8 antibiotics, respectively. In his study, higher MAR index values (ranging from 0.3–0.8) has been reported. Arslan and Kucuksari et al. (2015) reported that prevalence of multiple resistance in *Aeromonas* spp. isolates to 3 or more antimicrobial agents was 57.1%, indicating fish and ground beef were exposed to significant antibiotic contamination.

In this study, *S. putrefaciens* showed resistance to 4 (50%), 5 (50%) and 6 (16.7%) antibiotics and MAR index ranging from 0.29 to 0.43 (Figure 1). In Korea and Italy, respectively Kang et al. (2013) and Smaldone et al. (2014) detected multiple resistance of *S. putrefaciens* isolated from marine fish and shellfish involving resistance to vancomycin, oxacillin, tetracycline, penicillin and ampicillin. Authors suggested that marine environments are important reservoirs for resistance genes, and may be play an important role in transfer of drug-resistant genes between bacteria.

It was observed in our study and above-mentioned studies that there are no geographic borders for the spread of antibiotic-resistant bacteria, and that their emergence in the food chain threatens the world. MDR bacteria can continue to spread globally via the food chain as well.

ESBL Production of Gram-negative Bacteria

ESBLs are plasmidborne enzymes that can hydrolyze cephalosporins and monobactams. They are mainly producing in Gram-negative bacilli, particularly several members of *Enterobacteriaceae*, including *Escherichia*, *Klebsiella*, *Proteus*, *Citrobacter*, *Serratia*, *Salmonella* and *Shigella* (Ondes and Ozpinar, 2016).

In the present study, 144 (35.6%) out of 404 isolates were identified as ESBL producers. In a previous studies conducted in Turkey, the frequency of ESBL-producing *Enterobacteriaceae* ranged between 3.7%–52.7% in ground beef, chicken meat, raw milk and cheese (Ondes

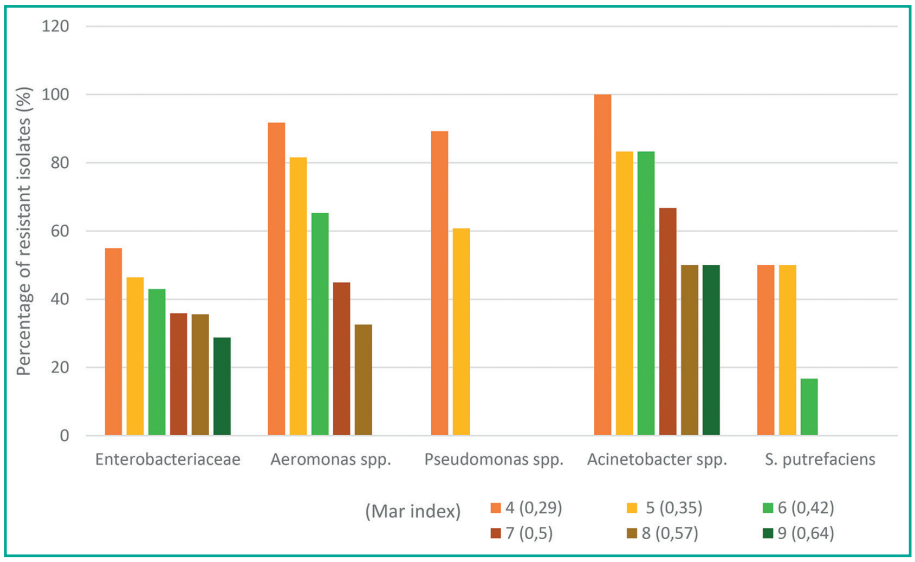

FIGURE 1: *Multiple antibiotic resistance (MAR) index of Gram-negative bacteria.*

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and Ozpinar, 2016; Tekiner and Ozpinar, 2016). Outside of Turkey, various rates of ESBL-producing *Enterobacteriaceae* were reported as 8.3%–42.5% in China (Ye et al., 2018), 13.5% in Bosnia and Herzegovina (Ibrahimagić et al., 2016), 36.9% in Nepal (Shrestha et al., 2017), and 78.6% in India (Singh et al., 2017) examined raw meat products, seafoods, ready-to-eat (RTE) foods and frozen foods. On the other hand, ESBL-producing bacteria were not detected in the Swedish meat or in the Swedish chicken (Tham et al., 2012).

The results in the work reported here showed that the majority of the ESBL producers were *K. oxytoca* (74.3%), followed by *C. freundii* (61.5%), *E. coli* (56.6%), A. *hydrophila* (51.1%), *P. aeruginosa* (45.4%), *C. amalonaticus* (41.7%), *E. cloacae* (33.3%), *S. marcescens* (33.3%), *P. vulgaris* (33.3%), *K. pneumoniae* (33.3%), *S. putrefaciens* (33.3%), *P. putida* (33.3%) and *A. baumannii* (33.3%) and *P. agglomerans* (26.7%) isolates (data not shown). Percentages of ESBL producing *E. coli* and *P. aeruginosa*, respectively were 45% in Egyptian camel meat (Elhariri et al., 2017), and 55% in Dutch chicken meat (Blaak et al., 2015) which are comparable with the results of our study. However, our results were appeared to be higher than those in several studies. For instance, Singh et al. (2017) detected ESBL-positive *K. oxytoxa* in 27% of seafood. Duan et al. (2006) reported 3.1% prevalence of ESBL producers among *E. coli* isolates from cattle in China while Petternel et al. (2014) reported an incidence of 24% from minced meat in Austria. Studies from Turkey reported 21%, 5.5% and 9.1%, respectively of ESBL producing *K. oxytoca*, *Citrobacter* spp., and *E. cloacae* isolates in red meat and chicken meat (Gundogan et al., 2014; Tekiner and Ozpinar, 2016). ESBL detection rates in *Citrobacter* spp. and *Proteus* spp. isolated from chiken meat were 26.1% and 26.3%, respectively (Shrestha et al., 2017). The frequency of ESBL-producing *E. coli*, *Klebsiella* spp., *Citrobacter* spp., *Proteus* spp., *E. cloacae*, *P. aeruginosa*, *M. morganii*, *P. rettgeri*, isolated from water, food and environmental samples were 13.6% in Bosnia and Herzegovina (Ibrahimagić et al., 2016).

Compared to our results, higher contamination rates of different types of foods with ESBL-producing *E. coli* and *Pseudomonas* spp. were reported as 77%, 84% and 100% by Nahar et al. (2018), Stuart et al. (2012) and Shrestha et al. (2017), respectively. Foods of animal origin sold in Turkey have been found as potential reservoirs for ESBL-producing Gram-negative bacteria. The National Surveillance Network by the Ministry of Health in Turkey (www.uhes.saglik.gov.tr) has reported an increasing prevalence of ESBL-producing *E. coli* (33.2% in 2008 and 48.83% in 2013) and ESBL-producing *K. pneumoniae* (40% in 2008 and 49.69% in 2013). In the current study, fish, veal and chicken meat were found to be highly contaminated with ESBL-producing *K. oxytoca* (74.3%), and *E. coli* (56.6%). Results obtained in the present study were not suprising when compared with our previous study in which ESBL production was detected in 26% of *K. oxytoca* and 44.4% of *E. coli* isolates islated from foods of animal origin (Gundogan and Avci 2013). There is a clear tendency towards increased prevalence of ESBL-producing Gram-negative bacteria in Turkish animal-derived foods. This might lead to a risk for infection and colonisation of the human intestinal flora with ESBL-producing bacteria. Transfer of ESBL-producing *Enterobacteriaceae* to humans via the food chain has been reported previously (Tekiner and Ozpinar 2016).

Although *H. alvei*, *S. fanticola*, *R. aquatilis*, *M. morganii*, *E. vulneris, P. rettgeri*, *P. fluorescens*, *A. caviae*, *A. lwoffii* and *P. oryzhabitans* isolates did not produce ESBL in the present study, it should not be underestimated that ESBL encoding genetic elements are transferable between the same and different bacterial species (Tekiner and Ozpinar, 2016). Thus, monitoring of ESBL-producing bacteria should be continued at various level (animals, human, and environment), while investigating the factors that contribute to their selection and dissemination.

Conclusion

The results obtained in this study in Turkey showed a high incidence of Gram-negative bacteria in marine fish, veal and chicken samples. Our results suggest that some of these bacteria such as *E. coli*, *E. coli* O157, and *Aeromonas* spp. represent a potential health risk. This is because some strains of these organisms are capable of producing toxins. Furthermore, bacterial characteristics that influencing the food safety such as biogenic amine production, slime and biofilm formation are quite common within the Gram-negative bacteria. In particular, *E. coli*, *E. cloacae*, *C. freundii*, *K. oxytoca*, *K. pneumoniae, P. aeruginosa*, *P. fluorescens*, A. *hydrophila*, *A. caviae*, *A. baumanni* and *S. putrefaciens* isolates were found resistant to clinically important antibiotics and most of them have multiple antibiotic resistance (MAR) patterns and produced ESBL, thus posing a health risk for the Turkish consumers. The presence of these bacteria in fish, veal and chicken seemed to be related to the unhygienic production processes and storage conditions. Thus, all potential sources of MAR and ESBL-producing bacteria should be considered and strategies devised to reduce their presence in foods. Furthermore, Turkish regulatory agencies should require food processing plants to adopt quality guarantee systems such as Hazard Analysis and Critical Control Points (HACCP) system and a better control system to prevent the presence of these products on the market.

Acknowledgements

This work was financially supported by the Gazi University Research Fund (Project No:05/2012-67). The authors wish to thank Mec. Eng. M.Sc. Tuncer Yakut for critical reading of the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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