

## Quality assessment and detection of multiple drug-resistant food-borne aerobic bacteria in frozen quail in Luxor and Aswan city

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### Abstract

Quail meat is a delicate white game meat with extremely low skin fat and low cholesterol value. It is rich in micronutrients and vitamins including vitamin B6, niacin, thiamin, pantothenic acid and riboflavin, folate and vitamin E and K. It is therefore recommended for people with high cholesterol levels. Fifty random samples were collected from different restaurants in Luxor and Aswan cities, Egypt to evaluate the quality of frozen wild quail meat. The investigation revealed that (8%), (8%), and (12 %) of the examined samples were contaminated with *E.coli*, *Staphylococcus aureus*, and *Salmonella* spp., respectively as well as the mean values of APC, Coliform, and *S. aureus* counts were  $4.4 \times 10^4 \pm 0.074$ ,  $2.2 \times 10^3 \pm 0.094$  and  $1.2 \times 10 \pm 1.1$  respectively. Serotyping revealed that the investigated *E. coli* isolates belonged to 3 different O-serogroups comprising O<sub>125</sub> (50%), O<sub>55</sub> (25%), and O<sub>86a</sub> (25%) while the examined *Salmonella* isolates including *Salmonella* Othmarschen (16.6%), *Salmonella* Livingstone (16.6%), *Salmonella* Kentucky (16.6%), *Salmonella* Tado (16.6%) and *Salmonella enterica* Subspecies *Salamae* (33.3%). Antimicrobial susceptibility testing for *E. coli* isolates revealed that they were sensitive to Colistin sulfate, Nalidixic acid, and Ceftriaxone while they were resistant to Gentamycin, Streptomycin. *S. aureus* isolates were sensitive to Ampicillin and Vancomycin while resistant to Erythromycin, Chloramphenicol, and Tetracycline. In addition, *Salmonella* isolates were sensitive to Amoxicillin and Streptomycin while resistant to Colistin sulfate. Moreover, the mean values of pH, total basic nitrogen (TVB-N mg/100gm), and Thiobarbituric acid (TBA mg/Kg) were  $5.9 \pm 0.01$ , and  $12.1 \pm 0.2$  and  $0.72 \pm 0.03$ , respectively.

### Keywords:

Aerobic bacteria, Meat quality, pH, Quail, Thiobarbituric acid, Total volatile basic Nitrogen.

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## Introduction

Quail meat is a sweet and subtle white game meat with extremely low skin fat and low cholesterol value; also it is rich in micronutrients and a wide range of vitamins including vitamin B6, niacin, thiamin, pantothenic acid and riboflavin, and vitamin E and K (MichaelImchen, 2014). Quail meat is an ideal food for all groups of ages, due to its high meat yield, low shrinkage during cooking and serving (El-Dengawy et al. 2010) and considered superior to red meat because it contains low fat, low cholesterol, and has a high amount of iron (Liu et al., 2012) so the quail meat production represents a promising source of protein in developing countries including Egypt. The wild quails were exposed to many stress factors which cause depletion of muscle glycogen and make the gut more permeable to bacteria resulting in a high bacterial population in muscle and reducing meat quality and shelf-life (Mousa et al. 2016). Despite the high value of quail meat, there is no accurate control and inspection of quail carcasses. Therefore, the possibility for transmission of some bacteria such as *E. coli*, staphylococcus, and salmonella is one of the main causes of food poisoning. Hence, quail carcass contamination during slaughtering and processing is a major risk for subsequent foodborne infections in humans (Freitas et al. 2013; Kanwal et al. 2015). Furthermore, foodborne pathogens are of major public health concern throughout the world. Raw and undercooked quail meat is a rich

source of *E. coli*, *Salmonella* and *Shigella* (Darwish et al. 2015). These pathogens can be transmitted to humans by consuming contaminated food and can lead to the risk of food-borne illness (Hara-Kudo et al. 2012). Such pathogenic strains have special adhesion fimbriae, and intestinal mucosa, damaging the absorptive surface of the intestine and leading to diarrhea (Vincent et al. 2010). Several reports took into an account the isolation and characterization of bacterial pathogens such as *E. coli*, *Staphylococci*, and *Salmonella*, from quail and quail products (Wang et al. 2010). The possible sources of quail meat contamination, the Public health importance of the isolated bacteria, and the hygienic measures which should be imposed were discussed. Therefore, the present study aimed to throw light on the bacteriological and chemical criteria of frozen quail meat and its suitability for human consumption.

## Materials and methods

Fifty (n=50) frozen quail carcass were collected randomly for 3 months during the year 2019-2020 from different supermarkets in Luxor and Aswan City. All samples were handled aseptically to prevent cross-contamination using sterile sampling materials (Middleton *et al.* 2005). The samples were wrapped, identified, and transported in an icebox container to RLQP, Animal Health Institute, Luxor branch, for bacteriological examination.

**1. Samples preparation:**

Sample preparation was done according to (APHA, 2001).

**2. Bacteriological examination:**

**A. Determination of Aerobic Plate Count** (ISO, 4833-1/2013), **Coliform count** (ISO, 4832/2006), and *S. aureus* count (Quinn *et al.* 2002).

**B. Isolation of some foodborne pathogens:**

It was done according to Quinn *et al.* (2002).

**C. Identification of some foodborne pathogens:**

It was performed as described by Quinn *et al.* "2002". Briefly, samples were inoculated into the nutrient broth at 37° for 24 hours, and a loopful was streaked into Baird Parker Agar for isolation of *S. aureus*, MacConkey agar for isolation of Gram-negative bacteria. Identification of bacteria was performed according to their colony characters, Gram's staining, and various biochemical reactions.

**D. Serotyping of food-borne isolates:**

Serological identification of *Salmonella* was performed according to (Popof and Le Minor, 2001) for determination of somatic antigen (*O*) and Flagler antigen (*H*) and *E. coli* according to (Lee *et al.* 2009).

**3. Antimicrobial susceptibility testing:**

The antimicrobial sensitivity test was performed according to the reference standard

by the Clinical and Laboratory Standard Institute (CLSI, 2018) using the disc diffusion method. Different antimicrobials were used such as Tetracycline, Erythromycin, Ampicillin, Amoxicillin, Sulphamethazone, Nalidixic acid, Streptomycin, Gentamycin, Ciprofloxacin, and Norfloxacin.

**4. Chemical evaluation:****A. Determination of pH value:**

It was carried out according to AOAC, (2012).

**B. Determination of Total Volatile Basic Nitrogen (TVBN) mg%:**

It was done according to (E.O.S 63/10, 2006).

**C. Determination of Thio-barbituric Acid Number (TBA) mg/Kg:**

It was carried out according to (E.O.S 63/9, 2006).

**Statistical analysis:**

Data statistical analysis was performed by SPSS 16.0 statistical software, (2001). Differences among means were determined using t-test.

**Result:**

The occurrence of bacterial contamination in frozen quail meat revealed contamination with *E. coli*, *S. aureus*, and *Salmonella* (Table 1).

The bacterial count (aerobic, coliform and *S. aureus*) and acceptability of the examined frozen quail meat samples were presented in Table 2.

**Table 1. Occurrence of bacterial contamination in frozen quail meat samples (n=50):**

Examined bacteria	examined samples	Isolated bacteria	
	No	No	%
<i>E. coli</i>	50	4	8
<i>S. aureus</i>	50	4	8
<i>Salmonella</i>	50	6	12

**Table 2. Prevalence of subclinical mastitis in the examined cows according to the affected quarters:**

Counts	Min.	Max.	Mean $\pm$ SE	EOS* (1090/2019)	Non-accepted samples	
					No	%
<i>Aerobic plate</i>	$1.0 \times 10^3$	$9.9 \times 10^4$	$4.4 \times 10^4 \pm 0.074$	$< 10^5$ Cuf/g	0	0
<i>Coliform</i>	$1.0 \times 10^2$	$9.0 \times 10^3$	$2.2 \times 10^3 \pm 0.094$	$< 10^2$ Cuf/g	6	12
<i>S. aureus</i>	$1.0 \times 10$	$2.0 \times 10$	$1.2 \times 10 \pm 1.1$	$< 10^2$ Cuf/g	0	0

\*EOS: Egyptian organization for standardization.

Table 3 showed the Serological identification of isolated *E. coli* in frozen quail meat samples.

The incidence of coagulase-positive *S. aureus* in examined samples was shown in Table 4. While Table 5 illustrated the serological identification of isolated *Salmonella* of examined samples.

The results of the sensitivity tests for *E. coli*, *S. aureus*, and *Salmonella* in the examined samples were presented in Table 6, Table 7, and Table 8, respectively.

The results of chemical parameters (pH, TVN, and TBA) and acceptability of frozen quail meat samples were presented in Table 9.

**Table 3. Serological identification of isolated *E. coli* in frozen quail meat samples:**

NO. of isolated bacteria	Serotype	No.	%
4	O125 :H2	2	50
	O55: H2	1	25
	O86a:H1	1	25

**Table 4. Incidence of coagulase-positive *S. aureus* in examined samples:**

NO. of isolated bacteria	Positive coagulase	
	No.	%
4	4	100

**Table 5. Serological identification of isolated *Salmonella* of examined samples:**

NO. of isolated bacteria	Identified strains	Group	Antigenic structure	
			O	H
6	<i>S. Othmarschen</i>	O	6,7,14	g, m,(t)
	<i>S. Livingstone</i>	O	6,7,14	d: L, W
	<i>S. Kentucky</i>	O	8,20	i: Z <sub>6</sub>
	<i>S. Tado</i>	O	8,20	C: Z <sub>6</sub>
	<i>S. enterica Subspecies Salamae</i>	O	1.9.12	H,g,m,(S),t:(1,5.7)
	<i>Salmonella enterica Subspecies Salamae</i>	O	6.7	g,m,(S),t:e,n,x

Table 6. Sensitivity test for *E. coli* in samples:

Antibiotic	<i>E. coli</i> isolates								
	O125 : H2			O86a : H1			O55 : H2		
	S	I	R	S	I	R	S	I	R
Gentamycin(10ug)			R			R			R
Neomycin(30ug)		I				R			R
Streptomycin(10ug)			R			R			R
Amoxicillin(25ug)			R			R		I	
Sulphamethoxazole(25ug)			R	S					R
Colistin sulphate(10ug)	S			S			S		
Ciprofloxacin(5ug)	S					R			R
Nalidixic acid(10ug)			R	S			S		
Ceftriaxone			R	S			S		

Table 7. Sensitivity test for *S. aureus* in samples:

Antibiotic	Sample 1			Sample 2			Sample 3			Sample 4		
	S	I	R	S	I	R	S	I	R	S	I	R
Erythromycin (15ug)			R			R		I				R
Vancomycin (15ug)	S			S			S			S		
Tetracycline (30ug)			R			R			R			R
Ampicillin (10ug)	S			S			S					R
Chlroamphenicol (30ug)			R			R			R		I	

Table 8. Sensitivity test for *Salmonella* in samples:

Antibiotic disc	<i>Salmonella</i> Isolates														
	<i>S. othmarsachen</i>			<i>S. Livingstone</i>			<i>S. Kentucky</i>			<i>S. Tado</i>			<i>S. Enterica Subsp Salamae</i>		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Gentamycin (10ug)	S			S					R				S		
Neomycin (30ug)	S				I			I			I			I	
Streptomycin (10ug)	S			S			S			S			S		
Amoxicillin (25ug)	S			S			S			S			S		
Sulphamethoxazole (25ug)	S			S					R			R	S		
Colistin sulfate (10ug)			R			R						R			R
Ciprofloxacin (5ug)	S			S			S					R	S		
Nalidixic acid (10ug)															
Ceftriaxone (10ug)	S			S					R			R	S		

**Table 9. Statistical analytical results of chemical parameters and acceptability of frozen quail meat samples (n=50):**

Chemical parameters	Min.	Max.	Mean± SE	EOS* (1090/2019)	Non-accepted samples	
					NO	%
pH	5.5	6.3	5.9±0.01	5.5-6.5	6	12
TVN mg/100gm	6.3	23.5	12.2±0.2	< 20 mg/100g	4	8
TBA mg/kg	0.12	0.91	0.52±0.03	< 0.9 mg/kg	3	6

EOS: Egyptian organization for standardization.

### Discussion

Foodborne diseases are a global issue, and a unified and joint approach by all countries and the relevant international organizations is a prerequisite for the identification and control of all emerging foodborne problems that threaten human health and international trade (Van de Venter, 2000). In the present study, as clarified in Table (1), *E. coli* was isolated from the examined with a prevalence of (8%), these results were lower than El-Dengawy and Nassar (2010) were isolated *E. coli* in (30%) of examined samples while these results were higher than Edris et al. (2011) were recorded (4%). Isolation of *E. coli* in this study emphasizes that they could be a potential source of human infection and gives an indication of the fecal contamination during handling and processing. The presence of *E. coli* may affect the quality of meat which causes economic losses (ICMSF, 1996). As well *S. aureus* was isolated from frozen quail meat samples with a prevalence of 8%, Furthermore, in contrast to these results, very higher incidence of *S. aureus* (40%) was recorded by Edris et al. (2011) while these results higher than Mousa et al.

(2016) detected *S. aureus* in (4%) of examined samples. The higher contamination rate of *S. aureus* might be attributed to excessive handling of meat by workers during processing, contaminated water used, bad personal hygiene, and cross-contamination (Waldroup 1996). In addition, *Salmonella* was isolated from frozen quail meat with a prevalence of (12%), and these results were higher than that reported by Mousa et al. (2016) who failed to detect *Salmonella* while lower than the result obtained by Amna et al. (2015) who detected *salmonella* with an incidence of (66.6%). In these studies, *Salmonella enterica* incidence in the examined samples agreed with the results of Bacci et al. (2012) who detected *Salmonella Kentucky* with an incidence of (16.6%) and higher than Lamiaa et al. (2019) who detected *Salmonella Kentucky* with an incidence of (3.3%). In this study as illustrated in Table (2), it was found that the mean value of APC was ( $4.4 \times 10^4$  cfu/g) these results nearly similar to that recorded by Mousa et al. (2016) who recorded APC ( $5.1 \times 10^6$  cfu/g) while disagreed with those of Edris et al. (2011) who documented that APC was ( $9 \times 10^4$  cfu/g). The APC of frozen

quail may be attributed to unsatisfactory sanitation and contamination of materials during handling, processing, and distribution as well as insufficient chilling and freezing that may increase the existing organisms (Thatcher and Clark, 1973). Also, the results showed that the mean value of coliform was ( $2.2 \times 10^3$  cfu/g), the results agreed with that of El-Dengawy and Nassar (2010) who reported ( $3 \times 10^3$  cfu/g) while opposing that of Mousa *et al.* (2016) who noted coliform count was ( $8.6 \times 10^3$  cfu/g) as well Edris *et al.* (2011) who reported ( $5.7 \times 10^3 \pm 1.45 \times 10^3$  cfu/g). The occurrence of coliforms may be credited to direct or indirect fecal contamination from either human or animal sources resulting in inferior meat quality. The mean value of *S. aureus* count was ( $1.2 \times 10$  cfu/g) as reported in this study and this was nearly reported by Naeem *et al.* (2018) who detected *S. aureus* count of ( $2.21 \times 10$  cfu/g). The presence of *S. aureus* in food indicated contamination of handlers and inadequately cleaned equipment. The decrease in the count may be due to the effect of freezing application ( $-18 \pm 2^\circ\text{C}$ ) on bacteria that led to the destruction of the cell membrane and DNA denaturation of bacterial cells causing the death of the bacteria during freezing (Pavlov, 2007; Sonale *et al.* 2014;) as well *S. aureus* count of quail meat was also found to decline gradually during frozen storage of 90 days. Serotyping is a common way to characterize Shiga toxin-

producing *E. coli* strains and is based on the somatic antigen (O) and flagellar antigen (H) (Gyles *et al.* 2007). As illustrated in Table (3), serotyping of (4) *E. coli* isolates revealed that they belonged to (3) different O-serogroups including O<sub>125</sub> (50%), O<sub>55</sub> (25%), and O<sub>86a</sub> (25%), and the results agreed with findings of Varnam and Evans (1991) who recorded that O<sub>55</sub> are the most predominant serotypes of *E. coli* among examined samples. As well these results were nearly similar to the results of Kudakwashe *et al.* (2013), Zende *et al.* (2013), and Hassanin *et al.* (2014). Furthermore, the serotyping of *Salmonella* spp. isolates (Table 5) showed a major variety of serotypes which included *S. Othmarschen*, *S. Livingstone*, *S. Kentucky*, *S. Tado*, *S. enterica* Subspecies *Salamae*, *S. enterica* Subspecies *Salamae*. Harsha *et al.*, (2011) and Bacci *et al.* (2012) recorded the most frequently isolated serotypes in the quail samples *S. Enteritidis* (17.1%). Similarly, Freitas *et al.*, (2013) and Udhayavel *et al.*, (2016) isolated different types of *Salmonella* spp. identified *S. enterica* subspecies *Enterica*; *S. Corvalis*, *S. Give*, *S. Lexington*, *S. Minnesota*, *S. Schwarzengrund*, *S. Rissen*, and *S. Typhimurium*.

The indiscriminate use of antibiotics has been paralleled by a significant increase in the number of reports of resistant bacteria isolated (Tendencia and de la Pena, 2002). In the present study (Table 6), antimicrobial susceptibility testing for *E. coli*

isolates revealed that they were resistant to Gentamycin, Amoxicillin, Sulfa methoxazole, Streptomycin, Nalidixic acid, and Cefirioxacin. These results were nearly similar to those reported by Roy et al. (2006) and Farghaly et al. (2017) on other hand Apata (2009) reported that 10% of the strains were found resistant to ciprofloxacin. Table (7) displayed the resistance of *S. aureus* to tetracycline and chloramphenicol. These results were nearly similar to those reported by Farghaly et al. (2017) while they showed the highest resistance to erythromycin were nearly similar to those reported by Yusra et al. (2019). Furthermore, increased numbers of strains resistant to erythromycin (39%), and tetracycline (14%) was observed by Apata (2009). In addition, Suleiman (2013) reported that all the 54 *S. aureus* isolates recovered were resistant to ampicillin and erythromycin but susceptible to ciprofloxacin, and gentamycin. Similar patterns of antimicrobial susceptibility have been reported by Otalú et al. (2011), Pesavento et al. (2007) Waters et al. (2011), and Leonard and Markey (2008) where the occurrence of multidrug-resistant *S. aureus* in poultry is rather frequent. However, Geidam et al. (2012b) reported a lower resistance of 53% and 85% for ampicillin and erythromycin respectively. Tables (8), show the antimicrobial susceptibility testing for *Salmonella* isolates revealed that resisted to less extent some strains of Gentamycin, Sulfa

methoxazole, clostin, ciprofloxacin, and Cefirioxacin. Moreover, these results disagreed with Haritha (2019) and Apata (2009) showed that 80% and 59% of the isolated strains were resistant to tetracycline and erythromycin, respectively. As well the result of Jahan et al. (2018) and Hyeon et al. (2011) indicated that the isolated *Salmonella* showed resistant tetracycline, Erythromycin, and Colistine sulfate. Parvej et al. (2016) found that 50% of isolates were resistant to Colistin sulfate and 80% were sensitive to Neomycin. This indicated that Colistin sulfate is becoming resistant due to indiscriminate and unwise use. All the isolates showed 100% sensitivity towards Ciprofloxacin, as reported by Ramya et al. (2013), who found 100% susceptibility of *Salmonella spp.* to Ciprofloxacin followed by Amoxicillin (82%). The antibiotic-resistant genes of these isolates may transfer to *Salmonella* which may infect both humans and animals hindering their health (Wakawa et al., 2015). In this respect, gastro-intestinal commensal bacteria constitute a reservoir of resistance genes for pathogenic bacteria. Their level of resistance is considered to be a good indicator of selection pressure for antibiotic use and for resistance problems to be expected in pathogens. Therefore, a concerted effort should be made to maintain sanitary conditions in processing, preparation and handling, packaging, transportation, and storage of

quail carcass, periodical sanitation of utensils, chilling rooms, and cold stores, and periodical examination of workers and hand washing facilities should be present. These differences in antimicrobial susceptibility may be attributed to the use of different antibiotics in different settings and purposes as well as by humans and animals in addition to the different applied hygienic measures (Saqr et al., 2016).

Results recorded in table (9) revealed that pH values of examined frozen quail samples ranged from 5.5 to 6.3 with a mean value of  $5.9 \pm 0.01$ , these results nearly agree with Mousa et al. (2016), El-Shehry, (2012), Youssef, (2013) and Edris et al. (2014) with a mean value of 5.8, 5.91, 5.64, 5.90 and 5.85 respectively. Higher results were achieved by Shedeed, (1999), Afifi, (2000), Abd ElAll (2001), Genchev et al. (2008), and de la Torre et al. (2012) with the mean value of (6.10), (6.15), (6.6), (6.4) and (6.5) respectively. Compared to the safe permissible limits of pH recommended by EOS 1090/2019 (5.5- 6.5), 12% of the examined samples were not within the accepted level, as shown in table (8). The decrease in pH value may be attributed to the breakdown of glycogen with the formation of lactic acid and the increase in pH may be due to the partial proteolysis leading to the increase of free alkaline groups depending on the condition of such changes (Pearson and Gillette, 1996). It is evident from the results recorded in table (8)

that obtained results from TVB/N ranged from 6.3 to 23.5 with a mean value of  $12.2 \pm 0.2$ . The obtained values from the examined samples were nearly similar to those recorded by Abd El-All (2001) and Hassan (2013) with mean values of 10.94 and 11.25. This result comes in contrast to the results obtained by Youssef (2013), Edris et al., (2014), and Mousa et al., (2016) with a mean value of,  $9.11 \pm 0.33$ ,  $6.08 \pm 0.3$  and  $7.1 \pm 0.32$  but lower than the results of Afifi (2000) with a mean value of  $13.87 \pm 0.18$  (mg%). According to the safe permissible limits stipulated by EOS 1090/2019; TVB/N lower than 20mg/100gm, 8% of the examined samples were higher than the safe standard limit. TVB/N in poultry meat may be increased as the days of storage increased (Reddy et al., 1970), the increase in TVB/N value in meat during storage might be attributed to the breakdown of protein as a result of the activity of microbial strains and proteolytic enzymes (Yassien, 2003; Alina and Ovidiu, 2007). The increase to critical values indicates incipient spoilage of chicken meat product samples after different periods of storage (Hassanin and Hassan, 2003) due to ammonia is one of the most spoilage end products in spoiled meat and meat products which is directly responsible for spoilage odors and flavors, it is considered as an indicator for amino acid degradation by bacteria and it can be measured as total volatile basic nitrogen Gill, (1983). Accordingly, TVN can be

considered a reliable indicative measure for the quality of various food articles especially poultry and its products. Furthermore, from the results recorded in a table (8), it is evident that TBA (mg%) in the examined samples of quail meats varied from 0.12 to 0.91 with an average of  $0.52 \pm 0.03$ . Lower results were obtained by de la Torre *et al.* (2012), Mousa *et al.* (2016), Afifi, (2000), Youssef, (2013), and Edris *et al.* (2014) with an average of 0.18, 0.23, 0.119, 0.09 and 0.218 respectively. A furthermore higher result was reported by Abd El-All (2001) with an average of (1.1). The oxidative rancidity in poultry meat was evaluated by measuring malonaldehyde in fat meat with an improved Thiobarbituric acid (TBA) assay with antioxidant protection (Abd El-Kader, 1996). Compared to the safe permissible limits approved by EOS 1090/2019, TBA lower than 0.9mg/kg, 6% of the examined samples were higher than the safe standard value. The quality of poultry meat products during the chilling or freezing depends greatly on TBA value as recommended by (Hassan and Shaltout, 2004). The variation of TBA values of examined samples could be attributed to the variation of the fat content of different samples under examination and storage life and development of off-flavors known as rancidity is due to lipid oxidation (Owens, 2001), and so the Thiobarbituric acid value is routinely used as an index of lipid oxidation in stored meat products (Abd El-Kader,

1996). The hygienic quality precautions of foodstuff should be monitored including antemortem and postmortem examination, every so often cleaning and disinfecting the instruments used in slaughtering, processing, and sanitation of quail slaughter halls, equipment, utensils, chilling rooms, and cold stores (Tsola *et al.*, 2008).

### Conclusion

In the present study, it could be concluded that the examined frozen quail meat was contaminated with various microorganisms including *Salmonella* spp., *S. aureus*, and *E. coli* reflecting unhygienic measures and unsuitable environmental conditions during handling, transporting, processing, and storage. As well there were misused antibiotics resulting in multiple antibiotic-resistant strains of obtained bacteria. Furthermore, this study showed that differentiation in pH, TVBN, and TBA values occur between examined samples.

### Conflict of interest statement

The authors declare that they have no conflict of interest.

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