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Journal of the Hellenic Veterinary Medical Society

Vol 74, No 1 (2023)

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doi: 10.12681/jhvms.27918

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To cite this article:

Guran, H., Esen, S., & Alali, W. (2023). Microbial and Antibiotic Resistant E. coli Contamination in Retail Eggs Produced by Alternative and Conventional Rearing Methods. *Journal of the Hellenic Veterinary Medical Society*, *74*(1), 5155–5164. https://doi.org/10.12681/jhvms.27918 (Original work published April 11, 2023)

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J HELLENIC VET MED SOC 2023, 74 (1): 5155-5164
ΠΕΚΕ 2023, 74 (1): 5155-5164

Research article
Ερευνητικό άρθρο

Microbial and Antibiotic Resistant *E. coli* Contamination in Retail Eggs Produced by Alternative and Conventional Rearing Methods

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ABSTRACT : The aims of this study were to determine: i) the presence of total mesophilic aerobic bacteria (TMAB), total psychrophilic aerobic bacteria (TPAB) coliform, *E. coli*, *Enterobacteriaceae* and *Salmonella* in retail eggs produced in different poultry rearing systems, ii) *Enterobacteriaceae* species diversity, and iii) antibiotic resistance profile of *E. coli* isolates. A total of 350 retail eggs produced by four different rearing systems [organic (n= 100), free-range (n= 100), barn (n= 50) and conventional-cage (n=100)] were collected and characterized microbiologically. Out of the 350 eggs tested, the eggshell surface samples were contaminated, with TMAB, TPAB, coliform, *E. coli* and *Enterobacteriaceae* at positive percentages of 100%, 100%, 49.1%, 18.6% and 38%, respectively. The positive percentage of coliform, *E. coli* and *Enterobacteriaceae* were statistically significant between rearing systems ($P<0.05$). However, only mean numbers of TMAB were statistically significant between rearing systems ($P<0.05$). The contamination percent positive of crushed eggshell samples, with TMAB, TPAB, coliform, *E. coli* and *Enterobacteriaceae* were 100%, 100%, 50%, 33.7% and 42.5%, respectively. *Salmonella* was detected from only one *Enterobacteriaceae* isolate obtained from an organic eggshell pool but not from eggshell surfaces or egg yolks. A total of 35 *E. coli* isolates were recovered from *Enterobacteriaceae* isolates. Twelve (34.3%) of them exhibited resistance to at least one antibiotic tested. The dominant type of resistance was to ampicillin detected in all 12 *E. coli* isolates. This study provides valuable baseline data of the occurrence of species diversity of *Enterobacteriaceae* and antibiotic resistant *E. coli* in retail eggs produced by alternative or conventional-caged rearing methods which can be used for future risk assessments.

Keywords: Retail egg, rearing system, microbial contamination, *E. coli*, antibiotic resistance.

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Date of initial submission: 31-08-2021
Date of acceptance: 13-10-2022

INTRODUCTION

Eggs are a nutritious food item, that are rich in bioactive components, quite accessible and inexpensive worldwide. In the past couple of years, both the global production and per capita consumption of eggs have increased (Park et al., 2015; Mottet and Tempio, 2017). In 2018, it was estimated that Turkey produced 1,250,075 tons of eggs (ranked first in Europe and eighth globally) with annual per capita consumption of 224 eggs (Yum-Bir, 2018).

In 2012, the EU Member States banned the use of conventional battery caged egg production system as it does not meet animal welfare standards (European Council Directive, 1999). The EU Member States stipulated the use of two alternative systems: non-cage floor systems (organic, free-range, barn range) and enriched cage systems (enriched cage, aviary). However, while these egg production rearing systems exist in Turkey, conventional battery cages are still being used. The Turkish Food Codex (TFC) mandate that "Class/Grade A" eggs are marked with a code indicating the type of poultry rearing system which is similar to the EU regulation (European Commission, 2003; Turkish Food Codex, 2017). According to the TFC coding system, organic eggs, free-range eggs, barn eggs, and eggs from caged hens are marked with the numbers 0, 1, 2 and 3, respectively.

Contamination of eggs is mostly due to contact with feces, dust and poultry house material (feeders, waterers, etc.) (Moyle et al., 2016; Neira et al., 2017). Contamination of eggs occurs mostly on the outer surface (i.e., eggshell). Irrespective of the rearing system, the content of eggs (egg yolk and albumen) laid by healthy hens is considered sterile. However, the eggshell's outer surface maybe contaminated with a variety of microorganisms, including the non-typhoidal *Salmonella* spp., *E. coli*, and coliforms (Jones et al., 2012; Parisi et al., 2015; Kalupahana et al., 2017; Cardoso et al., 2021). Microorganisms on the surface of the eggshell may pass beyond this physical barrier and settle in the eggshell pores and membranes, and can even access the egg content (De Reu et al., 2006; Chousalkar et al., 2010; Chousalkar and Roberts, 2012). In fact, some studies have demonstrated that pathogens not detected on the outer surface of the eggshell can be present in the eggshell pores and membranes (Chousalkar et al., 2010; Chousalkar and Roberts, 2012). Previous studies in Turkey have been mostly focused on microbiological assessment of retail eggs produced from conventional caged-layers (Erkan et al., 2008; Temelli et al., 2015; İncili et al., 2019; Sandikci et al., 2020). To the authors' knowledge, there are limited data available on comparative microbial analysis of the eggshell surface, eggshell (pores and membranes), and egg yolk of retail organic eggs, free-range eggs, barn eggs and conventional-cage eggs. Additionally, while reports are available on the antimicrobial resistance profile of *E. coli* strains isolated from conventional layer farms in Turkey (Gökhan and Osman 2015; Pehlivanoglu et al., 2017), the antimicrobial resistance profile of *E. coli* in retail eggs remains unknown. In this context, the objectives of this study were undertaken to: 1) compare the presence and counts of total mesophilic aerobic bacteria (TMAB), total psychrophilic aerobic bacteria (TPAB), coliform, *E. coli*, *Enterobacteriaceae* and *Salmonella*; 2) determine the distribution of *Enterobacteriaceae* isolates at species level, and 3) assess the antibiotic resistance profile of *E. coli* in retail eggs produced by different rearing systems in Turkey.

MATERIALS AND METHODS

Study design and collection of the eggs

A cross-sectional study was conducted between October 2018 and February 2019. Three hundred and fifty commercial grade A retail eggs, produced by four different rearing systems (organic [code 0], free-range [code 1], barn [code 2] and conventional [cage, code 3]) and sold at retail in accordance with the relevant national and EU legislation (European Commission, 2003; Turkish Food Codex, 2017), were purchased from different supermarkets in Diyarbakir, Turkey. The supermarkets were selected based on the egg product availability during the study period of the sample collection. Moreover, the supermarkets selected sold eggs that were representing different national companies in Turkey. During each visit to the supermarket, a sample of 20 organic eggs, 20 free-range eggs, 10 barn eggs, and 20 eggs from caged hens were collected. The brands of the sampled organic, free-range and cage eggs represented four national companies; whereas, barn eggs represented two other national companies. The sampling was repeated five times on different dates. At each repeated sampling visit, we ensured that the sampled eggs were matched by production company, production plant, and the same sell-by date as the previous visits. The samples collected were sold in their original packaging and within the limits specified in the legislation (Turkish Food Codex, 2017).

Preparation of the outer surface of the eggshell

The presence and counts of microbial contamination on the eggshell outer surface were determined with a slightly modified version of the rinse method described by Musgrove et al. (2005). For this purpose, each egg was transferred into a sampling bag under aseptic conditions, then 20 ml of buffered peptone water (BPW) was added to each bag and the egg was gently hand-rubbed for 1 min to allow the microorganisms on the outer surface of the eggshell to pass into the solution. Then, one milliliter aliquots (10^0) directly from each rinsate were 10-fold serially diluted in 0.1% BPW and used for microbiological analyses.

Eggshell crush method

The microorganisms on the eggshell with intact shell membranes were determined using the crush method as described by Musgrove et al. (2005). After the rinsing procedure, each egg was aseptically removed from the sampling bag and dipped into 70% ethanol for one minute to kill any bacteria on the outer surface of the shell, and then allowed to air-dry in a biosafety cabinet. Next, the eggs were cracked open into a sterile container. The inner surface of the eggshells were washed with sterile 0.1% peptone water to remove the adhering egg albumen. The shells and shell membranes of five eggs, belonging to the same rearing method and production company were pooled into one sample. The pooled samples were then transferred into a sterile bag and crushed gently. A total of 50 ml of BPW was added to each bag and then vigorously shaken for 1 min. Then, one milliliter aliquots (10^0) directly from each crushed shell pooled sample were 10-fold serially diluted in 0.1% BPW and used for microbiological analyses.

Egg yolk

The egg yolks from five eggs belonging to the same brand were pooled in a sterile beaker by carefully separating the yolk from the albumen. After being vigorously mixed, 3 ml of egg yolk was transferred to sterile polypropylene tubes containing 12 ml of BPW and then the suspension was homogenized by shaking the tubes (Chousalkar and Roberts, 2012). This suspension was used for determining the targeted microorganisms.

Microbiological analyses

In the microbiological analyses, the pour plate technique was used for the determination of total mesophilic aerobic bacteria (TMAB), total psychotropic aerobic bacteria (TPAB), coliforms, *E. coli*, and *Enterobacteriaceae* in the egg samples. The TMAB and TPAB counts were performed on plate count agar (PCA) after incubation at 30°C for 72 hours (ISO 4833, 2003), and on PCA after incubation at 7°C for 10 days (Alvarez-Fernandez et al., 2012), respectively. Coliforms, *E. coli* and *Enterobacteriaceae* were determined on violet red bile lactose agar (VRB) after incubation at 37°C for 24 hours (ISO 4832, 2006), on tryptone bile X-glucuronide agar (TBX) after incubation at 44°C for 24 hours (ISO 16649-2, 2001), and on violet red bile glucose agar (VRBG) after incubation at 37°C for 48 hours (Musgrove et al., 2005), respectively. The detection limit for each microorganism was considered as >10 CFU/ml.

Determination of *Enterobacteriaceae*

In order to determine the distribution of the *Enterobacteriaceae* isolates at species level, up to three suspected colonies on VRBG agar were transferred to tryptone soy agar (TSA) and incubated for 18-24 h at 37°C. After incubation, the colonies that grew on TSA agar were identified at genus and species level by using Gram-negative cards in a VITEK 2 microbial identification system following the manufacturer's instructions (Biomerieux, France).

Antibiotic susceptibility testing of *E. coli*

Antibiotic susceptibility testing was performed on the *E. coli* isolates identified at the species level from the *Enterobacteriaceae* isolates obtained from egg samples. The susceptibility tests were conducted using the BD Phoenix™ 100 Automatic Microbiology Identification System in accordance with the manufacturer's instructions. A Phoenix NMIC-400/ID Panel (BD Diagnostic Instrument Systems, Sparks, MD, USA) that contained the following 16 antibiotics (amikacin, amoxicillin-clavulanate, ampicillin, aztreonam, cefepime, ceftazidime, ceftriaxone, ciprofloxacin, ertapenem, gentamicin, imipenem, meropenem, netilmicin, piperacillin-tazobactam, tigecycline and trimethoprim-sulfamethoxazole) was used. The minimal inhibitory concentration (MIC) values were interpreted as susceptible, intermediate, or resistant according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2021).

Isolation and confirmation of *Salmonellae* in the egg samples

Salmonella contamination of the outer surface of the eggshell, shell crush and egg yolk were deter-

mined with a slightly modified version of the method described by the FDA/BAM protocol (FDA/BAM 2011). Fifteen-ml of the rinsate from the outer surface of the eggshell, 45 ml of crushed eggshell pooled samples, and 15 ml of egg yolk pooled samples were pre-enriched by incubation at 37 °C for 18-24 h. After incubation, 0.5 ml of each pre-enriched solution was transferred to 10 ml of tetrathionate (TT) broth and incubated for 24 h at 42 °C (selective enrichment). Next, one loopful of TT broth was streaked onto xylose-lysine-tergitol 4 agar (XLT4) and incubated (37 °C, 24 h). Up to five presumptive *Salmonella* colonies on the XLT4 plates were selected and streaked onto TSA and incubated (37 °C, 24 h). *Salmonella* colonies on TSA were inoculated onto triple-sugar iron agar (TSI) and lysine iron agar (LIA) slants and incubated at 37 °C for 24 h. Isolates with typical *Salmonella* reactions on TSI and LIA were then confirmed by using Vitek Gram negative cards in a VITEK 2 microbial identification system (Biomerieux, France).

Statistical analysis

Results expressed as CFU/ml were $\log_{10}$ transformed to approximate normality. Numbers of TMAB and TPAB on the outer surface of the eggshell (outcome variables) were analyzed using the general linear model (GLM) (N = 350 eggs) procedure of SPSS version 17.0 (IBM, NY, USA). The main effects of this model were the rearing system (organic, free-range, barn and conventional cage), packaging type (cardboard and polystyrene foam) and visual appearance (clean, dirty and very dirty). The percent-positive of TMAB, TPAB, coliforms, *E. coli*, and *Enterobacteriaceae* were compared by the main effect variables using a chi-square analysis in SPSS.

RESULTS

Eggshell surface

Out of the 350 eggshell samples, 100%, 100%, 49.1%, 18.6% and 37.7% were contaminated with TMAB, TPAB, coliforms, *E. coli* and *Enterobacteriaceae*, respectively (Table 1). The *E. coli*, coliform and *Enterobacteriaceae* contamination percentages differed significantly (P<0.05) by rearing systems but not by packaging type or visual appearance. The TMAB $\log_{10}$ mean counts differed significantly (P<0.05) by rearing systems (Table 2). However, the TPAB counts were not significantly different by any of the main effects. Furthermore, none of the eggshells were contaminated with *Salmonellae*.

Eighty-five (24.3%) of the 350 analyzed samples were visually classified as clean; whereas 35.1%, and 40.6% were dirty and very dirty, respectively. There was no significant correlation between the visual contamination categories and type of rearing system (Table 1). Overall, the *E. coli* positivity percentage was significantly (P<0.05) higher in the very dirty samples (47.7%) compared to that in the clean samples, but not significantly (P>0.05) different from that in the dirty samples. The positivity percentages of the other microorganisms were not significantly different between the visual contamination categories of the eggs. Out of the 350 eggshells, 210 (60%) were sampled from cardboard cartons and 140 (40%) were sampled from polystyrene foam packages. Out of the 210 samples from cardboard cartons, 100%, 100%, 11.9% and 76.2% were contaminated with TMAB, TPAB, *E. coli* and *Enterobacteriaceae*, respectively (Table 3). On the other hand, out of the 140 samples from polystyrene foam packages, 100%, 100%, 41.4%, and 88.5% were contaminated with TMAB, TPAB, *E. coli* and *Enterobacteriaceae*, respectively (Table 3). When we assessed the interaction effect between the main study variables (rearing system x packaging type x visual appearance) on the TMAB and TPAB counts of the eggshell surfaces, there was no significant interaction.

Shell crushed

Fourteen pooled eggshell crush samples (one sample = 5 pooled shells) from each visit and in total 70 pooled samples were tested. The percent-positive for TMAB, TPAB, coliforms, *E. coli* and *Enterobacteriaceae* were 100%, 100%, 50%, 33.7% and 42.5%, respectively. The coliform, *E. coli*, and *Enterobacteriaceae* contamination percentage determined for the different rearing systems were as follows: 40%, 25%, and 45%, respectively for organic eggs; 30%, 40%, and 45%, respectively for free-range eggs; 80%, 40%, and 50%, respectively for barn eggs; and 50%, 30%, and 30%, respectively for eggs from conventional cages. The correlation between the contamination percentage and rearing systems was not significant. Also, there was no *Salmonella* detected in the shell crush samples.

Egg yolk

Fourteen pooled egg yolk samples (one sample = 5 pooled yolks) from each visit, and in total 70 yolks were tested for *Enterobacteriaceae* and *Salmonellae*. *Enterobacteriaceae* and *Salmonellae* were not detected in any of the egg yolk samples.

Table 1. Contamination percentages of microorganism in eggshell surface by microorganism and visual appearance

Microorganism	Rearing system*	No. of total positive sample (%)	Visual appearance: Clean No. of positive sample (%)	Dirty No. of positive sample (%)	Very dirty No. of positive sample (%)
TMAB	0	100 (100)	27 (27)	36 (36)	37 (37)
	1	100 (100)	29 (29)	31 (31)	40 (40)
	2	50 (100)	14 (28)	15 (30)	21 (42)
	3	100 (100)	15 (15)	41 (41)	44 (44)[l]
Overall		350 (100)	85 (100)	123 (100)	142 (100)
TPAB	0	100 (100)	27 (27)	36 (36)	37 (37)
	1	100 (100)	29 (29)	31 (31)	40 (40)
	2	50 (100)	14 (28)	15 (30)	21 (42)
	3	100 (100)	15 (15)	41 (41)	44 (44)
Overall		350 (100)	85 (100)	123 (100)	142 (100)
Coliform	0	38 (38)[a]	14 (14)	10 (10)	14 (14)
	1	64 (64)[c]	19 (19)	22(22)	23 (23)
	2	21 (42)[a,b]	6 (12)	7 (14)	8 (16)
	3	49 (49)[b]	8 (8)	21 (21)	20 (20)
Overall		172 (49.1)	47 (27.3)	60 (34.9)	65 (37.8)
E. coli	0	9 (9)[a]	4 (4)	0 (0.0)	5 (5)
	1	31 (31)[c]	7 (7)	10 (10)	14 (14)
	2	10 (20)[b,c]	1 (2)	3 (6)	6 (12)
	3	15 (15)[b]	3 (3)	6 (6)	6 (6)
Overall		65 (18.6)	15 (23.1)[k]	19 (29.2)[k,l]	31 (47.7)[l]
Enterobacteriaceae	0	24 (24)[a]	4 (4)	6 (6)	14 (14)
	1	47 (47)[b]	14 (14)	15 (15)	18 (18)
	2	21 (42)[b]	9 (18)	6 (12)	6 (12)
	3	40 (40)[b]	5 (5)	17 (18)	18 (18)
Overall		132 (37.7)	32 (24.2)	44 (33.3)	56 (42.4)

[*] **Rearing system:** 0 (organic, n:100), 1 (free range, n:100), 2 (barn, n:50), 3 (conventional-caged, n:100)
[a,b,c] Values in the same column that are not followed by the same lowercase letter are significantly different (P<0.05).
[k,l] Values in the same row that are not followed by the same lowercase letter are significantly different (P<0.05).

Table 2. Mean counts of TMAB and TPAB in retail egg surfaces (log_{10} CFU/ml)

	TMAB[ɪ]	TPAB[ɪ]
Rearing system		
Organic	4.27±0.95[b]	3.76±0.98
Free range	4.89±0.22[c]	3.98±0.23
Barn	4.31±0.19[b]	3.52±0.20
Conventional-caged	3.65±0.10[a]	3.49±0.10
Visual appearance		
Clean	4.42±0.11	3.52±0.12
Dirty	4.39±0.14	3.65±0.15
Very dirty	4.31±0.18	3.98±0.18
Packaging		
Cardboard	4.18±0.12	3.66±0.12
Polystyrene foam	4.65±0.12	3.83±0.13

[a,b,c] Values in the same column that are followed by the different lowercase letter are significantly different (P < 0.05).
[ɪ]: The value is expressed as the mean ± standard error

Table 3. Contamination percentages of microorganism in eggshell surface sampled from cardboard and foam package

	No. (%) positive sample	
Microorganism	**Cardboard carton (n: 210)**	**Foam package (n: 140)**
TMAB	210 (100)	140 (100)
TPAB	210 (100)	140 (100)
E. coli	25 (11.9)	58 (41.4)
Enterobacteriaceae	160 (76.2)	124 (88.5)

***Enterobacteriaceae* species**

Out of the 164 suspected *Enterobacteriaceae* isolated from 132 eggshell surfaces, 153 (93.3%) were confirmed as belonging to the *Enterobacteriaceae* family, whilst the remaining 11 (6.7%) isolates were identified as belonging to the species *Pseudomonas putida* and the *Acinetobacter baumannii complex* (Table 4). In total, nine different species of *Enterobacteriaceae* were identified from all the analyzed eggshell samples. The most common species were: *S. marcescens* (21.5%, n=33/153), *E. coli* (19.6%, n=30/153), *E. aerogenes* (16.3%, n=25/153) and *E. cloacae complex* (15.6%, n=24/153). The highest percentage of *Enterobacteriaceae* was identified from free-range eggs (32.6%, 50/153), followed by eggs from conventional cages (30%, 46/153), organic eggs (18.9%, 29/153) and barn eggs (18.3%, 28/153).

For the pooled crushed eggshell samples (n=14), out of the 19 (90.4%) of the 21 isolates recovered from these samples, there were 4 (90.4%) different bacterial species in the *Enterobacteriaceae* family, and 2 (9.6%) were of the *P. putida* species that do not belong to *Enterobacteriaceae* (Table 4). Contrary to the case with the eggshell surface, the most frequently identified species in crushed eggshell were *E. coli* (21.5%, n=5/19) and *E. hermanii* (21%, n=4/19). Furthermore, the highest percentage of *Enterobacteriaceae* species were isolated from organic eggs (50%, n=10/20), followed by free-range eggs (20%, n=5/20). One *Enterobacteriaceae* isolate recovered from organic crushed eggshell was identified as *Salmonella enterica subspecies diarizonae* with a score of 99% using a Vitek 2 automated system (bioMérieux, France).

Antibiotic resistance in *E. coli*

Out of a total of 35 *E. coli* isolates recovered, 12 (34.3%) exhibited resistance to at least one antibiotic tested (Table 5). Ampicillin resistance was dominant and was detected in 12 (34.3%) isolates. Resistance to amoxicillin-clavulanate was detected in 8 (22.8%), trimethoprim-sulfamethoxazole in 5 (14.3%), ceftriaxone in 3 (8.6%), and ciprofloxacin in 3 (8.6%) isolates. None of the isolates were resistant to amikacin, aztreonam, ceftazidime, gentamicin, imipenem, meropenem, ertapenem, netilmicin, piperacillin-tazo-

Table 4. Distribution of Enterobacteriaceae at species level in retail eggs

	Egg Shell surface					Egg Shell crush				
Bacteria species	**No. of isolates with (%)***	**Organic** (No. of isolates)	**Free range** (No. of isolates)	**Barn** (No. of isolates)	**Conventional (caged)** (No. of isolates)	**No. of isolates with (%)**	**Organic** (No. of isolates)	**Free range** (No. of isolates)	**Barn** (No. of isolates)	**Conventional (caged)** (No. of isolates)
S. marcescens	33 (21.5)	12	8	-	13	2 (10.5)	1	-	-	1
E. coli	30 (19.6)	8	9	2	11	5 (26.3)	2	2	1	-
E. aerogenes	25 (16.3)	5	8	6	6	3 (15.7)	1	1	-	1
E. cloacae complex	24 (15.6)	-	11	9	4	3 (15.7)	2	1	-	-
Pantoea spp.	15 (9.8)	-	-	7	8	-	-	-	-	-
R. ornithinolytica	10 (6.5)	-	6	-	4	-	-	-	-	-
E. hermannii	8 (5.2)	-	8	-	-	4 (21)	2	1	1	-
S. fonticola	4 (2.6)	4	-	-	-	1 (5.2)	1	-	-	-
S. odorifera	4 (2.6)	-	-	4	-	-	-	-	-	-
Salmonella enterica subspecies diarizonae	-	-	-	-	-	1 (5.2)	1	-	-	-
Overall	**153**	**29**	**50**	**28**	**46**	**19**	**10**	**5**	**2**	**2**

**None of the yolk samples analysed was contaminated with Enterobacteriaceae*

Escherichia coli: *E. coli*; *Escherichia hermannii*: *E. hermannii*; *Serratia marcescens*: *S. marcescens*; *Serratia fonticola*: *S. fonticola*; *Serratia odorifera*: *S. odorifera*; *Enterobacter aerogenes*: *E. aerogenes*; *Enterobacter cloacae complex*: *E. cloacae complex*; *Raoultella ornithinolytica*: *R. ornithinolytica*.

Table 5. Percentages of antibiotic resistant *E. coli* isolates obtained from retail eggs by rearing system

	No. (%) antibiotic resistant *E. coli* isolates*				
Antibiotic	Organic (n = 10)	Free range (n = 11)	Barn (n = 3)	Conventional (n = 11)	Total (n = 35)
Amoxicillin-Clavulanate	0 (0%)	5 (45.4%)	1 (33.3%)	2 (18.2%)	8 (22.8%)
Ampicillin	1 (10%)	5 (45.4%)	3 (100%)	3 (27.2%)	12 (34.3%)
Ciprofloxacin	0 (0%)	2 (18.2%)	1 (33.3%)	0 (0%)	3 (8.6%)
Trimethoprim-Sulfamethoxazole	1 (10%)	2 (18.2%)	0 (0%)	2 (18.2%)	5 (14.3%)
Cefepime	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	1 (2.8%)
Cefuroxime	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	1 (2.8%)
Ceftriaxone	0 (0%)	1 (9.1%)	1 (33.3%)	1 (9.1%)	3 (8.6%)
Piperacillin	0 (0%)	1 (9.1%)	0 (0%)	0 (0%)	1 (2.8%)

*All isolates were pan susceptible/sensitive to amikacin, aztreonam, ceftazidime, gentamicin, imipenem, meropenem, ertapenem, netilmicin, piperacillin-tazobactam, tigecycline.

Table 6. Antibiotic resistance phenotypes among *E. coli* isolates obtained from retail eggs by rearing system and tested egg part

Rearing system	Egg part	Antibiotic resistance profile*
Organic	Egg surface	AMP, TMP-SUL
Free range	Egg surface	AMC, AMP, CEF, CFTX, CEFU
Free range	Egg surface	AMC, AMP, CIP, TMP-SUL, PIP
Free range	Egg surface	AMC, AMP, TMP-SUL
Barn	Egg surface	AMP, CIP
Barn	Egg surface	AMP, CFTX
Conventional (caged)	Egg surface	AMP, AMC, TMP-SUL
Conventional (caged)	Egg surface	AMP, CFTX
Free range	Egg crushed	AMC, AMP, CIP
Free range	Egg crushed	AMC, AMP
Barn	Egg crushed	AMC, AMP
Conventional (caged)	Egg crushed	AMP, AMC, TMP-SUL

* AMC: Amoxicillin-Clavulanate; AMP: Ampicillin; CEF: Cefepime; CFTX: Ceftriaxone; CEFU: Cefuroxime; CIP: Ciprofloxacin; PIP: Piperacillin; TMP-SUL: Trimethoprim-Sulfamethoxazole.

bactam or tigecycline. Overall, the highest prevalence of *E. coli* resistant isolates (to at least one antibiotic) was detected in free-range eggs (45.4%, 5/11), while the lowest prevalence was detected in organic eggs (10%, 1/10) (Table 6). In addition, 17.1 percent of the *E. coli* isolates were multidrug-resistant (i.e. resistant to three or more antibiotics from different drug classes).

DISCUSSION

In the present study, the TMAB counts being higher in the free-range, barn and organic eggs, compared to the eggs from caged hens, was likely attributed to the ability to effectively clean conventional battery cages. This might be explained by the air circulating in conventional cage systems containing less dust; hence, the eggshell surface of eggs have a lower number of TMAB, when compared to eggs produced in alternative systems (organic, free-range, and barn) as described elsewhere (Protais et al., 2003;, Huneau-Salaün et al., 2010). In agreement with our findings, De Reu et al. (2005) reported higher total bacteria counts for free-range and barn eggs compared to eggs from hens housed in conventional battery cages. Moreover, Belkot and Gondek (2014) reported psychrophilic bacteria counts of 3.66 log CFU/cm^2 and 4.02 log CFU/cm^2 for eggs laid by hens housed in conventional battery cages and eggs laid by hens housed in deep litter systems, respectively. Alvarez-Fernández et al. (2012) reported psychrotrophic bacteria counts of 2.19 ±0.5 log CFU/cm^2 for free-range eggs, 1.54 ±1.2 log CFU/cm^2 for cage eggs, and 1.41 ±0.5 log CFU/cm^2 for organic eggs. Similarly, in the present study, the TPAB count was highest in free-range eggs and lowest in eggs from caged hens. However, the differences between the rearing systems were not found to be statistically significant.

Many studies have shown that, compared to eggs from caged hens, those produced in alternative rearing systems (free-range, barn, organic etc.) are contaminated with a higher number of coliforms and *E. coli*, due to the hens being able to move freely, and thus, being in close contact with the environment and soil, as well as to the difficulty of cleaning the floor in these alternative systems (De Reu et al. 2008; Singh et al., 2009; Jones et al., 2012; Alvarez-Fernandez et al., 2012). In the present study, the highest levels of contamination with coliform bacteria and *E. coli* were detected in free-range eggs. This is in agreement with previous reports (Jones et al., 2012; Alvarez-Fernandez et al., 2012; Moyle et al., 2016). On the other hand, the lowest contamination levels with coliform bacteria and *E. coli* was detected in organic eggs, rather than in eggs from caged hens, which differed from the findings of previous studies. Pathogenic bacteria, such as *Salmonellae*, being classified under *Enterobacteriaceae* makes this family important in terms of the food safety of eggs (Moyle et al., 2016). In the present study, we ascertained that the level of contamination with *Enterobacteriaceae* ranged between 41-47% in free-range, barn and cage eggs, and was lowest in organic eggs (24%). While De Reu et al. (2009) reported that *Enterobacteriaceae* contamination levels significantly differed between eggs from caged hens (12%) and eggs produced in non-cage systems (6%), Roberts and Chousalkar (2014) suggested that the difference between eggs produced in conventional cage and free-range systems for *Enterobacteriaceae* contamination levels was not statistically significant.

In the present study, it was determined that the difference between the visual contamination levels and TMAB, TPAB, coliform, *E. coli* and *Enterobacteriaceae* contamination levels of eggs was not statistically significant. In agreement with our findings, previous research has shown a weak correlation between the visual contamination levels and microbial counts of eggs (Parisi et al., 2010). In this context, the prediction of microbial contamination levels of eggs based on visual contamination levels might not produce reliable results.

During transport and storage, eggs may crack or break and may cause the nutrient-rich egg content to spill onto the packaging material and create a favorable environment for microbial growth, eventually turning the package into a source of contamination. We reported that the number of microorganisms on the packaging material and eggshell surface did not show any significant relationship (P>0.05). However, since the packaging materials of the tested eggs were not sampled and analyzed for microbiological parameters, it would be speculative to suggest that the source of the microbial load of the eggshell surface could be the egg packaging material. Indeed, there are several reports, which suggest the possibility of the microbial load of eggs being related to the packaging material (Figueiredo et al. 2014; Al-Shadeedi, 2018).

Via the cuticle, microorganisms may spread from the outer surface of the eggshell into its pores, and via the eggshell membranes into the egg yolk and albumen (Kalupahana et al., 2017). The findings of the present study are in agreement with those of previous research suggesting, on the basis of the egg crush method, that the eggshell membranes and pores may host microorganisms (Musgrove et al., 2004; Chousalkar et al., 2010; Roberts and Chousalkar, 2014). Gole et al. (2013) determined the genus distribution of *Enterobacteriaceae* isolates recovered from the surface and pores of shells belonging to eggs from caged hens, as follows: *Cedecea*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Pantoea*, *Salmonella*, *Serratia* and *Yersinia*. Musgrove et al. (2004) reported the presence of the bacterial genera *Escherichia*, *Salmonella*, *Enterobacter*, *Serratia*, *Yersinia, Klebsiella*, *Pantoea*, *Kluyvera* and *Citrobacter* on the outer surface of eggshells. Contrary to the report of these researchers, in the present study, some of the bacteria genera were not isolated from eggshells. *E. coli* and *S. marcescens* were the predominant *Enterobacteriaceae* isolated in the current study. Similar to our study, Jones et al. (2012) reported the greatest amount of *E. coli* was from eggshells.

The egg content can be contaminated with *Salmonellae* both vertically and horizontally (De Reu et al., 2006; Gantois et al., 2009). Similar to the findings of the present study, previous studies have reported that these pathogens are mostly absent or found at very low levels in the egg content and/or on the eggshell (Daughtry et al., 2005; Erkan et al., 2008; Chousalkar et al., 2010; , Chousalkar and Roberts, 2012; Gole et al., 2013). In a recent study, *Salmonella* contamination was reported in 3.3% of table eggs purchased from different regions of Turkey. In the same study, 75% and 25% of contaminated eggs carried only *S.* Enteritidis and *S.* enterica subsp. salamae, respectively (Diker et al, 2020). In another study aimed at determining the presence of *Salmonellae* in the egg-

shell membranes and pores, Musgrove et al. (2005) reported *S*. Enteritidis and *S*. enterica subsp. Salamae in 5.3% of the eggshell crush samples. In the current study, *Salmonellae* were not isolated from eggshell crush. However, one of the *Enterobacteriaceae* isolates recovered from the shell crush pool of organic eggs was identified as *Salmonella enterica subspecies diarizonae*. *Salmonella enterica subspecies diarizonae*, which is typically associated with coldblooded animals including reptilians, an infrequent human pathogen, is reported to have been isolated from environmental samples taken from the area surrounding poultry houses (Lamas et al., 2016).

The results of the present study demonstrated a high prevalence of antibiotic resistance to ampicillin, amoxicillin-clavulanate and trimethoprim-sulfamethoxazole in *E. coli* isolates recovered from eggs, but a low overall prevalence to other antibiotics. These results agree with data from several previous studies revealing that resistance to aminopenicillins and sulphonamides is common among *E. coli* strains isolated from poultry and commercial eggs (Musgrove et al., 2005; Persoons et al., 2010; Seo and Lee, 2018). The presence of a small percentage of ciprofloxacin-resistant *E. coli* isolates is noteworthy, but not entirely surprising considering the frequent detection of quinolone resistance in *E. coli* isolated from poultry (Warren et al., 2008; Vanni et al., 2014; Sodagari et al., 2021).

CONCLUSION

In conclusion, the overall coliform, *E. coli* and *Enterobacteriaceae* contamination in eggs at retail level in Turkey was highest in free range compared to conventional-caged, barn and organic system. Moreover, in this study, our findings indicate that different bacterial species could exist not only on the eggshell surface but also in shell egg membrane (crushed egg samples) regardless of the laying hen rearing systems. None of the samples were found to be contaminated with *Salmonella* even though an *Enterobacteriaceae* isolate obtained from a shell crushed organic egg pool was identified as *Salmonella enterica subspecies diarizonae*. The overall *E. coli* prevalence was low in retail eggs, but the multidrug-resistant *E. coli* to some antibiotics, particularly to ampicillin, ciprofloxacin, amoxicillin-clavulanate (drugs of choice for treatment of *E. coli* in human) is a major public health concern. Although alternative rearing systems such as organic, free-range and barn has been considered by countries and consumers as a safer option than conventional-caged for the environment friendly production and the animal welfare issues, studies including this research, have shown that the food safety risk to public health could exist due to the presence of some bacteria of enteric origin and the multidrug-resistant *E. coli*.

ACKNOWLEDGEMENT

Data relating to determination of the microbiological profile in this study belongs to Sukran Esen's M.Sc. thesis. Sukran Esen's M.Sc. thesis was financially supported by Dicle University Scientific Research Projects Coordination Unit (Project number: DUBAP/Veteriner.18.004).

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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