Guinea fowl (*Numida meleagris*) eggs and free-range housing: a convenient alternative to laying hens' eggs in terms of food safety?

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ABSTRACT The aim of this study was to evaluate the impact of the genotype (guinea fowl, native breed Leghorn, and commercial hybrid hens), storage time (0, 14, 28 d) and storage temperature (fresh, 5, 20°C) on eggshell quality traits and microbiological contamination of eggshell, eggshell membranes, and albumen. A total of 150 hens (50 hens per genotype—divided into 2 equal groups because of the results replication) were used. There were 150 eggs (50 per genotype) used for microbial analysis and 600 eggs used for the analysis of eggshell quality. The effects of genotype, storage time, and storage temperature were observed. Moreover, interactions between these factors were calculated. The significant effect of genotype (P = 0.0001) was found in egg weight, in all observed parameters of eggshell quality (proportion, thickness, strength, surface, and index), eggshell contamination of *Escherichia coli* (**EC**) and total number of microorganisms (**TNM**), penetration of TNM into eggshell membranes (P = 0.0014), and penetration of TNM into albumen (P = 0.0019). Storage time significantly affected egg weight and all parameters of eggshell quality except the eggshell strength and index. It also significantly affected count of *Enterococcus* (**ENT**) on eggshell, TNM in eggshell membranes and TNM in albumen. Storage temperature significantly influenced egg weight (P = 0.0001) and all parameters but eggshell thickness and surface. Regarding the microbial contamination, storage temperature significantly affected a count of ENT on shell, TNM in shell membranes, and TNM in albumen. Concerning significant interactions, the interaction among genotype and storage time was found significant (P = 0.0148). Fresh and 28-day-old commercial hybrid eggs were the most contaminated, whereas guinea fowl eggs (fresh and 14 d old) and Leghorn hen eggs (fresh, 14, 28 d old) had the lowest level of contamination by EC. When looking for an alternative to laying hens, guinea fowls should be taken into consideration due to their higher resistance to diseases, ability of adaptation to different environmental conditions, and especially in terms of eggshell quality and therefore egg safety.

Key words: genotype, Guinea fowl, microbial contamination, storage temperature, storage time

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INTRODUCTION

Enriched cages came into force in 2012 in the EU and just noncages systems (free range, organic systems, barns, and aviaries) were allowed as suitable housing systems (Dikmen et al., 2016). An alternative housing of poultry, such as free-range and organic systems, is nowadays on the rise in reason of availability to the costumers (Siderer et al., 2005). Organic farming should be a tool that approaches more environmental-friendly production systems (Rigby and Cáceres, 2001). Nevertheless, the study of Williams et al. (2006) compared free-range and organic systems with results of a higher global warming potential in the organic production (counted for 20,000 eggs). Housing hens in enriched cages is nowadays under pressure of European animal welfare organization (Mench et al., 2011) because in developed countries, it is an interest in free-range housing due to reasons of welfare and consumer discomposure. In consumers' point of view, free-range eggs are healthier than these from cages (Miao et al., 2005). During manipulation (storage, transport, sale), eggs can be cracked and bacteria can be transported to yolk and albumen. Also, the aging of eggs influences the

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amount of bacteria on the shell (Pesavento et al., 2017). Eggshell quality is an essential factor of egg quality as well as its contamination in point of egg safety (De Reu et al., 2006). The amount of bacteria on eggshell limits their ability to penetrate through the shell (Shawkey et al., 2009), so cleanliness of the egg surface is really important. Ambient temperature also has a major effect on the development of bacteria (Madigan et al., 2005). The bacteria intracontamination is on the highest level in oviduct in cloacal region and its microflora is mostly represented by micrococci, enterococci, and coli-aerogenes organisms. Nevertheless, the eggshell contamination is most common after laying by interaction with superficies of litter or floor (Board, 1966). That is caused by the contact with feces, which contain bacteria, for example, *Enterococcus* spp., that influence bacterial communities on eggshells (Brandl et al., 2014). The composition of the shell is adapted to form a barrier to the entry of bacteria. It was developed as response to environmental stimuli, physiological needs of embryo and pressure of microorganisms (D'Alba et al., 2015). An amount and type of microorganism, which can be found on surface of eggs depends on housing system and its conditions (Huneau-Salaün et al., 2010). In free-range systems, one of the most favorite housing system in European countries (Leenstra et al., 2014), the occurrence of higher microbial content on the eggshell seems to be higher in comparison of cage-housing systems (Parisi et al., 2015).

Guinea fowls, mostly reared in free-range systems (Dahouda et al., 2007), as an alternative poultry species, occurs in Asia, Latin America, and also in Europe because of their high quality of meat and eggs (Moreki and Radikara, 2013). Also, they are more resistant to common poultry diseases and their production costs are reduced because of the ability of looking for food (Yamak et al., 2018). Guinea fowl breeding is popular not only in Africa, but their production is increasing in France, Belgium, and in Scandinavia (Baeza et al., 2001).

Compared with domestic laying hens, their eggshells are stronger and thicker (Petersen and Tyler, 1966), which could mean that penetration of microorganism through the shell will not be as easy as in hens. The risk of contaminating the interior off eggs due to lower incidence of cracks will be also lower (Pesavento et al., 2017). Reduction of bacteria growth rate depends on the temperature as the factor, which highly affects bacterial growing (Huang et al., 2011) and on the storage time (Vlčková et al., 2018).

This is the pilot study of investigation guinea fowls being a suitable alternative to hens housed in free-range system. Also, this is the first study of its kind of microbiological analyses of guinea fowl eggs. The aim of this study was to evaluate the impact of the genotype (guinea fowl, native breed Leghorn, and commercial hybrid), storage time (0, 14, 28 d), and storage temperature (fresh, 5, 20°C) on eggshell quality traits and microbiological contamination of eggshell, eggshell membranes, and albumen.

MATERIAL AND METHODS

The Ethics Committee of the Central Commission for Animal Welfare at the Ministry of Agriculture of the Czech Republic and the Ethics Committee of the Czech University of Life Sciences Prague approved this study with animals.

Animals and Management

This study included 3 genotypes (guinea fowl, native breed Leghorn, and commercial brown laying hybrid ISA Brown). Specifically, each group consisted of 50 hens. Furthermore, the hens of all 3 groups were subsequently divided into 2 equal groups and kept separately because of the results replication. All animals were kept under the same conditions. Litter housing system with the possibility of free range was used. The requirements on floor area, which are set by European Commission Directive 1999/74/EC, were met. The temperature inside was kept at 18°C to 20°C and humidity at 50 to 60%, whereas outside conditions were natural (the term of experiment was in July). Lighting regime was also natural. Feed mixture contained 16.4% of crude protein and 11.42 MJ of metabolizable energy and the access was ad libitum. Access to water was unlimited as well.

Samples

A total of 150 eggs (50 from guinea fowl, 50 from Leghorn, and 50 from commercial hybrid) were collected and used for the analysis of microbiological contamination, whereas 600 eggs (200 from each of 3 above mentioned groups) for the analysis of eggshell quality. The collection of eggs was carried out when the hens were 44 wk old. Eggs were collected for 5 consecutive days in the morning between 9:00 and 10:00 am (for microbiological analysis per treatment). The rest of eggs for eggshell quality analysis was collected after the collection for microbial testing for the whole week to reach required number of eggs. Egg collection was performed by using clean sterile plastic gloves. Eggs were then placed into sterile plastic boxes to avoid unwanted contamination. Eggs were equally divided into 5 groups concerning the storage time and conditions after the collection. The first group was a control group (day 0 group) and was analyzed immediately. The second (temperature 5°C) group and third (temperature 20°C) group were both stored for 14 d. The fourth and the fifth group were stored for 28 d (during the same temperatures as the groups 2 and 3). The eggs were stored in the fridge with internal conditions (5°C and relative humidity at 50 to 60%) in a room with thermostat (20°C and relative humidity at 50 to 60%). Digital thermometers Emos E8860 were used for the checking of required conditions. The temperature and relative humidity were controlled and noted 2 times a day. All laboratory

analyses were realized in the laboratory of the Department of Animal Science of the Faculty of Agrobiology, Food and Natural Resources of Czech University of Life Sciences Prague.

Eggshell Quality Analysis

Analysis of eggshell quality included evaluation of eggshell proportion (**ESP**), thickness (**EST**), strength (**ESST**), surface (**ESS**), and index (**ESI**). Eggshell quality analysis and measurements were made according to Kraus and Zita (2019). The ESST was assessed by Instron device (Instron Universal Testing Machine; model 3342; Instron Ltd., Norwood, MA), which calculates necessary force (in N/cm^2) for eggshell breakage. The eggshell surface was calculated by the formula $ESS = 4.68 \times EW^{2/3}$ (cm^2), where EW is the egg weight (**EW**) in g (Kraus et al., 2019). The ESP was calculated by the formula ESP = ESW/EW (in g) × 100 [%], where ESW symbolizes eggshell weight in g and EW symbolizes EW in g. The EST was determined by a digital micrometer (Digimatic Outside Micrometre, Milutoyo Corporation, Japan) with 0.001 mm precision. The EST was measured without eggshell membranes at the center of the eggshell. Duplication of each measurement was made. According to Ahmed et al. (2005), the ESI was calculated as ESI ($g/100\ cm^2$) = (ESW/ESS) × 100, where ESW is the eggshell weight (g) and ESS is the eggshell surface (cm^2). Furthermore, the EW was analyzed by laboratory scale Ohaus (Model: Traveler TA502, Parsippany, NJ 07054) with 0.01 g precision.

Microbiological Analysis

Microbiological testing consisted of counting of colony-forming units (**CFU**) of *Escherichia coli* (**EC**), *Enterococcus* (**ENT**), and total number of microorganisms (**TNM**) using the standard plate count method. The microbiological contamination was determined in the eggshell surface, eggshell membranes, and thin albumen. The laboratory evaluation was performed in sterile equipment. Every egg for microbial testing of eggshell was placed into sterile plastic bag, where was 10 mL of sterile saline with peptone (Sigma-Aldrich, Saint Louis, MO). The 10 mL of saline peptone consisted of 9 g of sodium chloride, 1 g of peptone, and 1,000 mL of distilled water. The bags were then finely scrubbed for 3 min to cover the entire surface of egg. Next, eggs were removed and 1 mL of neat (10^0) or diluted (10^{-1}–10^{-5}) sterile saline with peptone was filled by pipette to Petri dishes, where standard plate count agar (Oxoid, Basingstoke, UK) was then added to obtain the TNM. The determination of microbial contamination of eggshell membranes and albumen was done in the same way as the determination of eggshell was done. Considering the eggshell and albumen preparation, the eggshell was first cleaned under the water and then sterile by ethanol to obtain sterile conditions for removing eggshell membranes and albumen. The dilution series were performed in accordance with dilutions for eggshells with difference of membranes or albumen, respectively, being a part of dilution 10^0. The count of EC was found using Mac-Conkey agar (Oxoid), the count of ENT using Slanetz Bartley agar (Oxoid). They were both incubated at 37°C for 48 h. Standard plate count agar was in incubator for 120 h at 30°C. Every sample was analyzed in duplicate. After the incubation, typical CFU on eggshell, eggshell membranes, and albumen were calculated on Petri plates per egg. The final value of microbial contamination of each sample was calculated by the formula of standard plate count method.

Statistical Analysis

All statistical analyses were carried out using a commercially available software application SAS 9.4 package (SAS, 2011). Data were tested for normality with univariate plot normal procedure of SAS and subsequently subjected to a 3-way ANOVA in a 3 (genotype—guinea fowl, Leghorn, or commercial hybrid) × 3 (storage time—0, 14 or 28 d) × 3 (storage temperature—fresh, 5°C or 20°C) factorial arrangement of treatments using the Tukey test by the PROC MIX procedure of SAS. A P-value < 0.05 was considered to indicate a statistically significant difference. The tables show the average values of each treatment, interactions among these treatments, and the standard error of the mean. The two-way interaction effects and their mean values were not reported in the tables but discussed in the text when significant.

RESULTS AND DISCUSSION

Eggshell Quality Analysis

The results of present study are showed in the tables. Eggshell quality is presented in Table 1, eggshell microbial contamination in Table 2, whereas eggshell membrane contamination is presented in Table 3 and albumen microbial contamination in Table 4. Regarding EW and eggshell quality analysis, commercial hybrid and Leghorn had significantly ($P = 0.0001$) heavier eggs than guinea fowls by +14.41 g and by +14.19 g, respectively, that is in reason of lower EW in guinea fowls in general (Kgwatalala et al., 2013) and selective pressure on hybrid genotypes (Silversides et al., 2006), which resulted in heavier eggs in commercial hybrid. Concerning storage time, it affected ($P = 0.0001$) the EW reasoning in decline of the weight during the time of storage, when fresh eggs were heavier by +2.14 g than eggs, which were stored 28 d. Also, the storage temperature influenced this parameter significantly ($P = 0.0001$). Fresh eggs and eggs stored at the temperature of 5°C were heavier than eggs from 20°C (by +2.10 g and +1.98 g, resp.). Similar trend was observed in study of Samli et al. (2005), who reported decreasing of EW due to increasing of storage time and temperature. Their results are in line with present study in context of no significant weight changes between fresh eggs and 10-day-

Table 1. Effect of genotype, storage time, and temperature on selected eggshell quality parameters.

Genotype	Storage time (days)	Storage temperature (°C)	EW (g)	ESP (%)	EST (mm)	ESST (N/cm^2)	ESS (cm^2)	ESI (g/100 cm^2)
Guinea fowl			43.44^b	16.03^a	0.463^a	112.17^a	57.80^b	12.03^a
Leghorn			57.63^a	8.90^c	0.277^c	33.86^c	69.78^a	7.34^c
Commercial hybrid			57.85^a	9.24^b	0.306^b	38.32^b	69.98^a	7.63^b
	0		54.32^a	10.91^b	0.352^a	58.74	66.97^a	8.69^b
	14		54.23^a	10.98^b	0.333^b	58.77	66.91^a	8.75^b
	28		52.18^b	11.49^a	0.350^a	59.31	65.21^b	9.04^a
		Fresh	54.32^a	10.91^b	0.352	58.74	66.97^a	8.69^b
		5	54.20^a	11.02^b	0.339	58.73	66.88^a	8.77^b
		20	52.22^b	11.44^a	0.344	59.34	65.26^b	9.02^a
Guinea fowl	0	Fresh	44.02	15.94	0.485	110.69	58.32	12.01
	14	5	43.46	16.20	0.446	120.55	57.83	12.16
		20	44.37	15.75	0.459	109.19	58.62	11.91
	28	5	43.70	15.85	0.452	106.70	58.02	11.91
		20	41.55	16.41	0.471	113.92	56.13	12.14
Leghorn	0	Fresh	57.47	8.42	0.281	31.29	69.65	6.94
	14	5	59.82	8.83	0.276	34.80	71.56	7.37
		20	57.75	8.97	0.277	33.24	69.89	7.40
	28	5	57.96	8.81	0.269	34.24	70.05	7.28
		20	54.98	9.54	0.282	36.07	67.65	7.74
Commercial hybrid	0	Fresh	60.22	9.04	0.308	41.16	71.89	7.57
	14	5	58.84	8.75	0.300	35.54	70.79	7.27
		20	57.53	9.05	0.277	37.52	69.73	7.47
	28	5	57.95	9.22	0.317	37.23	70.06	7.62
		20	54.72	10.15	0.329	40.14	67.43	8.23
P-value								
Genotype			0.0001	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
Storage time			**0.0001**	**0.0008**	**0.0008**	0.7972	**0.0001**	0.0167
Storage temperature			0.0001	0.0022	0.1677	0.9186	0.0001	0.0224
Genotype × storage time			0.6644	0.0622	**0.0016**	0.2119	0.7445	0.2072
Genotype × storage temperature			0.1922	0.1615	0.1049	0.5263	0.2273	0.1892
Storage time × storage temperature			0.0302	0.0022	0.0374	0.0211	0.0260	0.0179
Genotype × storage time × storage temperature			0.6424	0.7400	0.2958	0.0673	0.5831	0.9879
SEM			0.462	0.207	0.005	2.296	0.389	0.137

[a,b,c]Values marked with different superscript letters in each column are significantly different ($P \leq 0.05$).

Abbreviations: EW, egg weight; ESP, eggshell proportion; EST, eggshell thickness; ESST, eggshell strength; ESS, eggshell surface; ESI, eggshell index; SEM, standard error of mean.

old eggs, when our study showed this trend until 14 d of storing at 5°C. However, Lee et al. (2016) detected changes in EW from the 12th day. This could indicate that the weight of eggs starts to decrease between 10 and 14 d in general with respect to storing conditions. Losing weight or greater losses of weight during the increasing storage time was also observed by Scott and Silversides (2000). The two-way interaction (P = 0.0302) between storage time and storage temperature, which influenced EW, was found in present study. The heaviest eggs were 14 d stored at temperature of 5°C, whereas the lightest eggs were stored 28 d at 20°C and their difference was 3.63 g. The similar trends were showed by interaction presented in study of Samli et al. (2005), where the highest losses were observed in eggs stored 10 d (the longest tested time of storage) during temperature of 21 and 29°C. It is obvious that higher temperatures than 20°C make the process of weight loss quicker.

The eggshell proportion was then significantly influenced by genotype (P = 0.0001), storage time (P = 0.0008), and storage temperature (P = 0.0022). The eggshell proportion was significantly higher in guinea fowls than in commercial hybrid and Leghorn (16.03 vs. 9.24 vs. 8.90%, resp.). Moreover, the storage time had significant effect on proportion of eggshell, when the highest value was found in the end of observation. That is consistent with Scott and Silversides (2000), who observed the highest proportion of shell at last day of measuring. The eggshell proportion also linearly increase depending on increasing of storage temperature and the interaction between storage time and storage temperature was found (P = 0.0022). The highest proportion of eggshell was in eggs, which were stored 28 d at 20°C, while eggs stored at 5°C for 14 d had the lowest values. These results could be supported by statement of Decuypere et al. (2001), who reported modifications of some egg characteristics (water loss, carbon dioxide, and subsequent increase of the pH of the albumen) on account of storage. The highest (P = 0.0001) values for eggshell thickness were for guinea fowls as well as eggshell strength (P = 0.0001) and eggshell index (P = 0.0001), the differences were significant among all of groups. These results are really important because it reflects the mean value of the whole eggshell (Tyler and Geake, 1961). The highest value of thickness in favor of guinea fowl compared with pheasant, chukar, and quail was found by Song et al. (2000). Their study

Table 2. Effect of genotype, storage time, and temperature on eggshell microbial contamination (CFU).

Genotype	Storage time (days)	Storage temperature (°C)	EC	ENT	TNM
Guinea fowl			3.90[b]	2.01	4.13[b]
Leghorn			3.72[b]	1.43	4.01[b]
Commercial hybrid			5.05[a]	2.06	5.72[a]
	0		4.50	2.61[a]	5.01
	14		3.99	1.92[a,b]	4.82
	28		4.32	1.36[b]	4.22
		Fresh	4.50	2.61[a]	5.01
		5	4.28	1.96[a,b]	4.68
		20	4.03	1.32[b]	4.36
Guinea fowl	0	Fresh	3.78	2.16	3.70
	14	5	3.95	1.80	4.07
		20	2.97	1.21	4.38
	28	5	4.47	2.90	4.73
		20	4.32	1.95	3.78
Leghorn	0	Fresh	3.98	2.74	4.94
	14	5	4.11	2.90	4.73
		20	3.65	1.02	4.59
	28	5	3.69	0.51	3.48
		20	3.18	0	2.30
Commercial hybrid	0	Fresh	5.72	2.92	6.40
	14	5	4.55	2.79	5.40
		20	4.69	1.80	5.72
	28	5	4.90	0.84	5.66
		20	5.38	1.93	5.39
P-value					
Genotype			0.0001	0.1821	0.0001
Storage time			0.0785	0.0026	0.0729
Storage temperature			0.1916	0.0015	0.3277
Genotype × storage time			0.0148	0.0545	0.0458
Genotype × storage temperature			0.1213	0.4979	0.6779
Storage time × storage temperature			0.3259	0.2428	0.1423
Genotype × storage time x storage temperature			0.6360	0.5083	0.9092
SEM			0.130	0.211	0.193

[a,b,c]Values marked with different superscript letters in each column are significantly different ($P \leq 0.05$).

Abbreviations: CFU, colony-forming units; EC, *Escherichia coli*; ENT, *Enterococcus*; TNM, total number of micro-organisms; SEM, standard error mean.

reported almost same value for this parameter (462.8 µm) as our study (0.463 mm). Higher values of thickness in guinea fowls are caused by +2.5 h longer shell deposition process than in chickens (Panheleux et al., 1999). The highest values of thickness and strength in guinea fowls report a great quality of their eggshell also in context that Kibala et al. (2015) stated positive correlation between shell thickness and breaking strength, which means much less likely to form cracks. The eggshell thickness was also affected ($P = 0.0008$) by storage time, when fresh eggs and 28-day-old eggs had the highest values (0.35, 0.35 mm, respectively) and the lowest values were for 14-day-old eggs (0.33 mm). These results are controversy and in contrast with study of Samli et al. (2005) and Lee et al. (2016), who found the lowest values at the end of observations. Differences between previous mentioned studies and the present study are in reason of a really high value of eggshell thickness at 28 d and low value at 14 d in eggs of commercial hybrid, which caused the major differences due to counting eggs as an average. Contrary to eggs of hybrid hens, the rest of eggs met a declining trend of decreasing shell thickness with increasing storage time. The interaction between genotype and storage time and the interaction between storage time and storage temperature was found significant ($P = 0.0016$, $P = 0.0374$, resp.) for thickness, when guinea fowls fresh eggs and fresh eggs in general had the highest values of eggshell thickness. Furthermore, the significant interaction ($P = 0.0211$) for eggshell strength was discovered between storage time and storage temperature, when 14-day-old eggs stored at 5°C had the strongest eggshell and the thinnest eggshell was found in 28-day-old eggs stored at 5 °C. Concerning eggshell strength, similar results was observed by Sert et al. (2011), who found higher strength in eggs stored for 10 d at 5°C than in eggs stored at 22°C for the same time. Genotype, storage time, and storage temperature significantly ($P = 0.0001$, $P = 0.0001$, $P = 0.0001$, respectively) affected eggshell surface area. Commercial hybrid had bigger surface area than guinea fowls and Leghorn by +12.18 cm^2 and by +11.98 cm^2. This trend is caused by the size of eggs or their weight, respectively, and that is why these results are not surprising due to selective process of hybrids in selection programmes, which are focused on EW, besides and due to losing weight during the storing process (shell surface is calculated by formula, where the weight is also counted). Surface area linearly decreased

Table 3. Effect of genotype, storage time, and temperature on microbial penetration into the egg content—eggshell membranes (CFU).

Genotype	Storage time (days)	Storage temperature (°C)	EC	ENT	TNM
Guinea fowl			0	0	0[b]
Leghorn			0	0	0.25[a]
Commercial hybrid			0	0	0.17[a,b]
	0		0	0	0.41[a]
	14		0	0	0.02[b]
	28		0	0	0.12[a,b]
		Fresh	0	0	0.41[a]
		5	0	0	0.13[a,b]
		20	0	0	0.01[b]
Guinea fowl	0	Fresh	0	0	0
	14	5	0	0	0
		20	0	0	0
	28	5	0	0	0
		20	0	0	0
Leghorn	0	Fresh	0	0	1.24
	14	5	0	0	0
		20	0	0	0
	28	5	0	0	0
		20	0	0	0
Commercial hybrid	0	Fresh	0	0	0
	14	5	0	0	0.07
		20	0	0	0.07
	28	5	0	0	0.72
		20	0	0	0
P-value					
Genotype			-	-	0.0014
Storage time			-	-	0.0027
Storage temperature			-	-	0.0018
Genotype × storage time			-	-	0.3162
Genotype × storage temperature			-	-	0.1849
Storage time × storage temperature			-	-	0.1899
Genotype x storage time × storage temperature			-	-	0.1829
SEM			0	0	0.061

[a,b,c]Values marked with different superscript letters in each column are significantly different ($P \leq 0.05$).

Abbreviations: CFU, colony-forming units; EC, *Escherichia coli*; ENT, *Enterococcus*; TNM, total number of micro-organisms; SEM, standard error mean.

depending on storage time and storage temperature. Likewise, the two-way interaction between storage time and storage temperature was found significant ($P = 0.0260$) for this parameter, where the biggest surface area was for eggs stored at 5°C for 14 d and the smallest for eggs stored at 20°C for 28 d. Eggshell index then linearly increased ($P = 0.0224$) from fresh eggs, which had the lowest values (8.69 g/100 cm^2) to eggs stored at 20°C with values of 9.04 g/100 cm^2. According to Ahmed et al. (2005), who associates the shell index with the size of the crystals, the total value of the shell index affects the value for shell strength. Explicitly, the lower shell index means larger crystals and the lower strength. With our increasing value of shell index, it can be assumed that storage temperature had effect on size of crystals, which size decreased and potentially increased the strength of shell (in our study not significantly, but numerically). Moreover, significant ($P = 0.0179$) interaction between storage time and temperature was also detected. The highest values were in eggs stored at 20°C for 28 d and the lowest were found in fresh eggs and 14-day-old eggs (5°C). These findings support previously mentioned statement, that with increasing temperature, shell index increases (the size of crystals decrease) and with connection with time, which is giving a value to whole process, the space for significant changes is created. These results seem to be important in point of view of shell quality.

Microbiological Analysis

Eggshell Contamination Regarding microbial contamination of eggshell, the present study shows significant effects of genotype, storage time, and temperature. In accordance with the results, genotype significantly ($P = 0.0001$) affected contamination of eggshell by EC, when commercial hybrid had shells more contaminated than Leghorn and guinea fowls by +1.33 log CFU/eggshell and +1.15 log CFU/eggshell. Fresh commercial hybrid eggs were the most contaminated by EC and in comparison with guinea fowls eggs (14 d), they had greater contamination by +2.27 log CFU/eggshell. These results suggest that commercial hybrid hens could have the bacteria intracontamination in oviduct on the highest level or indicate intestinal problems comparing to other animals. To support this statement, EC is a common innocuous commensal, but

Table 4. Effect of genotype, storage time, and temperature on microbial penetration into the egg content—albumen (CFU).

Genotype	Storage time (days)	Storage temperature (°C)	EC	ENT	TNM
Guinea fowl			0	0	0^{b}
Leghorn			0	0	$0.09^{b,c}$
Commercial hybrid			0.06	0	0.21^{a}
	0		0	0	0.16^{a}
	14		0.05	0	0.18^{a}
	28		0	0	0^{b}
		Fresh	0	0	0.16^{a}
		5	0	0	0.18^{a}
		20	0.05	0	0^{b}
Guinea fowl	0	Fresh	0	0	0^{c}
	14	5	0	0	0^{c}
		20	0	0	0^{c}
	28	5	0	0	0^{c}
		20	0	0	0^{c}
Leghorn	0	Fresh	0	0	0.47^{b}
	14	5	0	0	0^{c}
		20	0	0	0^{c}
	28	5	0	0	0^{c}
		20	0	0	0^{c}
Commercial hybrid	0	Fresh	0	0	0^{c}
	14	5	0	0	1.07^{a}
		20	0.28	0	0^{c}
	28	5	0	0	0^{c}
		20	0	0	0^{c}
P-value					
Genotype			0.4867	-	0.0019
Storage time			0.2724	-	0.0260
Storage temperature			0.2724	-	0.0260
Genotype × storage time			0.3010	-	0.0093
Genotype × storage temperature			0.3010	-	0.0093
Storage time × storage temperature			0.2724	-	0.0260
Genotype x storage time × storage temperature			0.3010	-	0.0093
SEM			0.019	-	0.051

[a,b,c]Values marked with different superscript letters in each column are significantly different ($P \leq 0.05$).

Abbreviations: CFU, colony-forming units; EC, *Escherichia coli*; ENT, *Enterococcus*; TNM, total number of micro-organisms; SEM, standard error mean.

its capacity for causing significant diarrheal and extraintestinal diseases is nowadays well known (Croxen et al., 2013). These findings could show that the eggshell contamination does not have to be reasoned just by contaminated litter or housing system in general; however, the contamination is most common after laying (Board, 1966), but it can show the starting gastrointestinal disease in studied flock. That could confront the cleanliness of housing system by feces, but also reflect health status and adaptability of a single genotype and its immunocompetence. The theory of ecoimmunology (the intersection between immunocompetence, pathogen dispersal, and the environment conditions) should be in the future the key factor, how to completely evaluate possible reasons, why some genotypes have the shell more contaminated than others as per housing systems all over the world with respect to climate, different processing practice in Europe or United States or humidity conditions. The idea of ecoimmunology as the key factor how to evaluate all factors is cited from Demas and Nelson (2012). Consequently, to understand how genotype really affects the microbial contamination of eggshell, it is necessary to understand how a single genotype coexists with every housing system and compare environment conditions, which much differ in free range and cages. The study of Jones and Anderson (2013) indicates that the optic, which is commonly used (the contamination is caused by housing system conditions as the major effect), does not to be clear as well as seems to be because they found the genotype of laying hen appears to affect the population of indicator organisms. They observed that every genotype (2 types of Hy-Line and the native breed Barred Plymouth Rock) produced eggs with different contamination in accordance with the same systems of housing, when, for example, Hy-Line Brown hens produced significantly higher contaminated eggs from free range than from cages, but the significance of free-range housing as the main factor of contamination was not observed in other 2 groups of hens. Concerning their study, Hy-Line Silver Brown hens had almost the same numbers of *Enterobacteriaceae* counts in every housing system. Supporting the statement, microbial contamination seems to be multifactorial problem. The review of Holt et al. (2011) summarizes that one of the factors of increased *Salmonella* in a flock is stress, which subsequently leads to rise of *Salmonella*. They presented study of Campo et al. (2008), who demonstrated higher stress response of

certain hen genotype to different housing conditions, comparted with other genotypes. In our study, the interaction between genotype and storage time was found significant ($P = 0.0148$), when fresh and 28-day-old commercial hybrid eggs were the most contaminated, whereas guinea fowl eggs (fresh and 14 d old) and Leghorn hens eggs (fresh, 14 and 28 d old) had the lowest level of contamination by EC. Decline in EC contamination regarding to the length of storing was also found by Vlčková et al. (2018). From the results, it is obvious that increased time of storage and the amount of bacteria (genotype effect) on eggshell significantly decreased the amount of bacteria. However, the storage temperature was not found as a significant effect, it can be assumed that the temperature will have some role according to Henry et al. (1962), who described specific growth rate of EC in the range from 35 to 15°C with minimal temperature for growth approximately 8°C. *Enterococcus* contamination was then significantly influenced by storage time ($P = 0.0026$). Fresh eggs had the shell more contaminated than 28-day-old eggs (2.61 vs. 1.4 CFU/eggshell, resp.). Moreover, storage temperature also affected ENT presence on shell, resulting in higher contamination in fresh eggs than in eggs stored at 20°C (2.61 vs. 1.32 log CFU/eggshell; $P = 0.0015$). The effect of temperature on microbial growth was presented in study of Theron et al. (2003). They found that 4-hour cold shocks and consequent storage at 25°C delivered longer egg shelf life with reduced micro-organism presence. On the contrary, high temperature shocks reasoned in higher micro-organism counts. This could support our results that lower temperatures decreased bacterial growth and reduced them. With respect to findings, the amount of ENT on eggshell demonstrates the real load of ENT, but the decline during the storing eggs suggests that the growth rate of these bacteria decreased in time and also due to temperature. However, enterococci grow from 0°C up to 50°C (Gardini et al., 2001); the results of our study could indicate that their growth is really poor at lower temperatures. Considering the presence of TNM on shell, commercial hybrid genotype had egg surface more contaminated than Leghorn and guinea fowl (5.72 vs. 4.00; 5.72 vs. 4.13 log CFU/eggshell; $P = 0.0001$). Likewise, the significant ($P = 0.0458$) interaction between genotype and storage time was found, where fresh commercial hybrid eggs were the most contaminated (6.40 CFU/eggshell), in comparison with guinea fowls eggs (14 d) and Leghorn eggs (28 d), which had the lowest values detected, they had greater contamination by +2.18 log CFU/eggshell and +3.51 log CFU/eggshell.

Eggshell Membrane Contamination In view of eggshell membranes, EC and ENT were not detected. Total number of microorganisms was significantly ($P = 0.0014$) affected by genotype, when guinea fowls had no TNM and Leghorn had 0.25 log CFU/eggshell membranes of TNM. These findings show guinea fowl eggshell quality in connection with no possibility of micro-organism to penetrate in the egg content. The trans-shell penetration needs to be contextualized. Our findings of the thickest eggshell in guinea fowls and no bacterial content in their eggs show the connection, which was mentioned in the study of Bain et al. (2013), who found that the thickness of eggshell and cuticle led to decreased penetration of bacteria to egg content. Depending on storage time, significant ($P = 0.0027$) decline of values from 0.41 log CFU/eggshell membranes to 0.02 log CFU/eggshell membranes in fresh eggs and 14 d stored eggs was observed. Furthermore, fresh eggs had significantly ($P = 0.0018$) higher value of TNM on eggshell membranes than eggs stored at 20°C (0.41 vs. 0.01 log CFU/eggshell membranes, respectively). In contrast to our results, Vlčková et al. (2018) reported the highest penetration of EC and ENT in eggs from free-range systems. They explain their results by increased contamination of eggshell in this housing system compared with cages. Also, De Reu et al. (2007) observed higher microbial penetration in alternative housing system. It can be hypothesized that the penetration of bacteria can also be affected by the size of dirtiness, such as feces, which concentration of bacteria is too high and gives a stronger possibility for bacteria to penetrate.

Egg Albumen Contamination No significant effect was found in penetration of EC to albumen, as well as in eggshell membranes, no ENT content was detected. However, a really poor number (0.06 log CFU/albumen) of EC were found in the albumen of commercial hybrid eggs probably due to larger amount of bacteria of different eggs during treatments. On the contrary, Vlčková et al. (2018) detected EC in albumen in enriched cages and also in free-range housing system, but ENT just in eggs from free-range hens, also with higher amount of EC in contrast with our results. In addition, significant effect of genotype ($P = 0.0019$), storage time ($P = 0.0260$), and storage temperature ($P = 0.0260$) was observed with results of higher values of TNM in commercial hybrid (0.21 log CFU/albumen) than in Leghorn, where no micro-organisms were detected. Fresh eggs had 0.16 log CFU/albumen of TNM, meanwhile the 28-day-old eggs had no TNM in albumen and in fresh eggs (0.16 log CFU/albumen) vs. eggs from 20°C storage temperature, where was no TNM. Furthermore, significant interactions were found, genotype × storage time ($P = 0.0093$), genotype × storage temperature ($P = 0.0093$), and storage time × storage temperature ($P = 0.0260$). Significantly highest value ($P = 0.0093$) of TNM in albumen was detected in 14-day-old commercial hybrid eggs than in eggs from Leghorn and guinea fowls, whose albumen was free of bacteria. The highest value for TNM in albumen had also the commercial hybrid eggs, which were stored at 5°C, whereas guinea fowls had the albumen free of TNM ($P = 0.0093$). This interaction seems to be really important in view of evaluation the factors which can affect in tandem the penetration of microorganisms to egg content. In accordance with this statement, Board and Ayres (1965) found there is no strong indication that temperature alone is the major influencer of trans-shell infection. The genotype as a key factor in this interaction could support the literature

findings summarized in the study of Horrocks et al. (2014), who connected the content of lysozyme, which catalyzes the lysis of cell walls of gram-positive bacteria, such as ENT, and ovotransferrin has bactericidal properties and binds iron to make it unavailable for bacterial growth. In connection with that, species-specific content as well as the individuality should be considered (D'Alba and Shawkey, 2015; Bílková et al., 2018). Also, lysozyme and ovotransferrin in the eggshell matrix were detected in hen's eggs (Gaustron et al., 1997) as well as in guinea fowls (Le Roy et al., 2019). In the study of Le Roy et al. (2019), 5 stages of guinea fowl eggshell calcification were described and showed similarities with hen's eggshell on proteomic base with specific activity in guinea fowls. Considering matrix of eggshell, the study of Wellman-Labadie et al. (2008b) described matrix proteins extracted from shell showed antimicrobial activity in vitro against EC, respectively. The resistance against trans-shell bacteria penetration may be according to Hincke et al. (2000) due to the presence of lysozyme, the levels of which are the highest during initiation and rapid calcification phases of shell formation and differ according to species (Wellman-Labadie et al., 2008a) and genotype in serum (Castellini et al., 2016) or in eggs (Lewko and Gornowicz, 2009). In connection with different amount of lysozyme and forming the shell process, when the crystals are formed (Zhang et al., 2019), our results of significantly highest eggshell index in guinea fowls specify smaller crystals and owing to that, higher thickness and strength and indicate different distribution of components such as lysozyme. That could be one of the factors of guinea fowl eggs resistance to intrapenetration of bacteria. Moreover, in present study, the highest value of TNM was detected in 14-day-old eggs, which were stored at 5°C in comparison with significantly ($P = 0.0260$) pure albumen in eggs stored 14 d at 20°C and 28-day-old eggs stored at 5°C and 20°C.

CONCLUSION

Guinea fowls produced eggs with the best eggshell quality in comparison with other hen genotypes. Guinea fowls and Leghorn produced eggs with significantly lower count of microorganism than commercial hybrid. Significant decline of EC depending on storage time was observed with better results of guinea fowl and Leghorn than in commercial hybrid. Moreover, the storage temperature reduced the count of ENT on eggshell in general. Considering the penetration of bacteria to egg content, the present study shows that there are differences in findings regarded to genotype effect and scientific studies among the EU and the US. However, influence of genotype and selective pressure with negative results of protecting egg content against bacteria has already been mentioned in some scientific studies. The environmental conditions need to be considered during the evaluation process because microbial loads on eggshells and trans-shell infection rates are highest in cool, wet, and humid environments. It seems as really difficult to completely evaluate all factors. Our findings could hypothesize that native genotypes (Leghorn and guinea fowl) are more adapted for free-range housing system. Also, guinea fowls can be recommended as an alternative source of safe and quality eggs with regard to results of no penetration of bacteria to egg content.

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DISCLOSURES

There is no conflict of interest.

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