Assessment of the microbiological status of two Hungarian ostrich farms

DÓRA LILI BRASSÓ[1,2*] ⓘ, RENÁTA KNOP[2],
ZSÓFIA VÁRSZEGI[2], PÉTER BÁRSONY[2], ISTVÁN KOMLÓSI[2],
ÁRPÁD BACSADI[3] and ANDREA BISTYÁK[3]

[1] Doctoral School of Animal Science, University of Debrecen, Böszörményi Street 138, 4032, Debrecen, Hungary

[2] Department of Animal Husbandry, University of Debrecen, Böszörményi Street 138, 4032, Debrecen, Hungary

[3] Laboratory of the Mammalian, Wild and Poultry Diseases, Veterinary Diagnostic Directorate of the National Food Chain Safety Office, Bornemissza Street 3–5, 4032, Debrecen, Hungary

RESEARCH ARTICLE

ABSTRACT

The aim of this study was to estimate the occurrence of bacterial infection and contamination in two ostrich-producing farms. Compared to other poultry species, the hatchability of ostrich eggs is especially low. In a quest to identify factors that may affect hatchability, we collected faecal samples from adult birds, as well as eggs with dead-in-shell embryos, dead chicks and swab samples from the surface of the eggs and from the environment. The samples were screened for the presence of bacteria by routine bacteriological culture methods. The most prevalent bacteria, detected in the samples, were *Escherichia coli, Bacillus* spp. and coliform bacteria, whereas *Pseudomonas* spp. were less frequently found. The intensity and species compositon of the bacterial contamination was comparable in the two farms. Our results revealed that the bacteria, present in the environment, may likely be transmitted to the surface of the eggs. If they are able to penetrate the shell then the embryos and chicks become infected easily. These findings draw the attention to the special importance of enforcing efficient decontamination and disinfection measures to keep the environment and egg surface free from germs. Besides the appropriate egg treatment procedure, the incubation and hatching technology should also be kept under control.

INTRODUCTION

The hatchability of ostrich eggs is known to be extremely low, i.e. under 40%, especially compared to that of chicken eggs reaching 80–90% (Hastings and Farell, 1991; Deeming, 1995, 1996; Badley, 1997; Van Zyl, 1997; Horbañczuk and Sales, 1999; Cooper, 2001). The hatching results are also low (40–50%) in the ostrich farms in Hungary (Brassó et al., 2021). The mortality of ostrich chicks is also high, i.e. until four weeks of age the mortality rate can reach 46.6% (Brassó et al., 2022). Hatching failure can often be caused by bacteria penetration into the egg due to poor eggshell strength and structure. The hatchability of ostrich eggs is influenced by many factors, such as year of production, sex ratio, stocking density, female age, trios and individuals (Lambrechts et al., 2004; Brassó et al., 2021), nutrition, egg treatment (collection, disinfection, storage time), eggshell structure and thickness, initial egg weight, weight loss of eggs during storage and incubation, and incubation technology (Deeming, 1995; Gonzalez et al., 1999).

Ostrich egg contamination by *Escherichia coli, Pseudomonas, Klebsiella* and *Salmonella* species may cause embryonic death (Perelman, 2009). Other authors mention *Aeromonas,*

*Corresponding author.
Tel.: +36703310903.
E-mail: brasso.dora@agr.unideb.hu

Enterobacter, Acinetobacter, Citrobacter, Staphylococcus and *Achromobacter* species as well as *Bacillus licheniformis* and *Enterococcus faecalis,* as the main concerns of embryonic mortality in ostrich (Cabassi et al., 2004; Mushi et al., 2008). *E. coli* is known to be the prime cause of embryo mortality and chick infection, despite the fact that it is the natural component of the faeces and infected birds do not show clinical signs. However, when *E. coli* penetrates the eggs and infects the developing embryo, the effect is hatching failure, or infected hatched chicks with low vitality. *Salmonella* species, which cause parathyphus can contaminate eggs both inside and externally, to severely harm the developing embryo thus greatly reducing hatchability (Rezaee et al., 2021). *Pseudomonas, Klebsiella, Staphylococcus, Streptococcus, Enterococcus* and *Bacillus* species are also responsible for yolk sac infection (Cortés et al., 2004).

Among parasites, members of the genus *Heterakis* are the most common nonpathogenic nematodes inhabiting the cecum of birds. The parasite ova develop in the body of earthworms (Marchiondo, 2019). The presence of nematodes depends on the hygiene and the stocking density of birds (Chang et al., 2001). Their frequency is also higher in the free-range system compared to closed, artificial nursing (Dingle and Shanawany, 1999). Infestation of birds with nematodes can cause signs ranging in severity from loss of appetite and ruffled feathers to death (Dahl et al., 2002).

Acknowledging the significance of hygiene in ostrich egg production and hatchability, this study aimed to investigate the bacterial and parasitological status of two major Hungarian ostrich farms. The study involved examining the microbial health status of the birds, observing egg handling and disinfection technologies and determining the ratio of specific bacteria per sample type in each farm. The results of this study will provide insight into the aetiology of embryonic and chick death. Further, we gain valuable information about the relationships between the contamination of the environment and the faecal, egg and chick samples, and the differences and similarities between farms in the level of contamination.

MATERIALS AND METHODS

Description of the farms

Samples were obtained from two farms in the East and the Middle of Hungary, referred to as Farm A and Farm B, respectively. Both companies work with around fifty breeder birds kept in trios (one male with two females) and harems (two males with seven females or three males with eight females) on Farm A and only in trios on Farm B in a semi-intensive, semi-free range husbandry system. The soil is grassland on Farm A, whereas Farm B has sandy soil. The breeder birds are not vaccinated nor treated otherwise on any of the farms.

The annual egg production was an average of 1,300–1,500 eggs, with 40–60% mean annual hatchability for both farms. Eggs were collected once a day, in the evenings.

On Farm A, wooden baskets, and on Farm B, plastic boxes filled with sponges were used for egg collection. On Farm A, after collection, eggs were washed in a warm chlorine solution of 42 °C. On Farm B, eggs were sprayed with Virocid 1% disinfectant and before incubation, they were wiped with a sponge dipped in 42 °C warm water and Virocid 1% solution. The storage room temperature was 16 °C on both farms and the eggs were stored on shelves and turned 45° hourly for a maximum of one week. On Farm A, eggs were incubated in a 180-egg-capacity cabinet incubator, while Farm B used a room cabinet. On Farm A, the incubation was conducted at 36.5 °C and 23% humidity and on Farm B, 36.6 °C and 27% relative humidity were applied for 38 days. Eggs were turned 45° hourly. On the 38th day of incubation, eggs with developing embryos were transported to the hatchery at 35 °C and chicks spent a few days here to dry before being transferred to the nursing area. Incubators and hatcheries were washed and disinfected only before the first incubation of the breeding season. On Farm A, until the age of four weeks, and on Farm B, until two weeks of age, chicks were kept in battery cages at 28–30 °C in groups of ten birds, then transferred into larger groups separated by age.

Faecal samples

Faecal samples were from healthy birds, without any clinical signs of illness. Samples included mixed faeces from each pen. On Farm A, faecal samples were collected from four pens, two from trios and the other two from harems. On Farm B, the samples were derived from eight pens of trios. Sample collection was carried out monthly throughout the whole breeding season, from the end of March until the end of August. On each occasion, at least 200–250 g faecal sample was collected per pen. In total, 53 faecal samples were investigated from the two farms.

Dead-in-shell-eggs

On Farm A, eggs were candled on the 10th, 21st and 38th day of incubation, while on Farm B, candling was carried out weekly. The eggs showed normal shape, shell formation, and porosity. When the egg contained an embryo that died at a stage during its development, it was culled and removed from the incubator. All dead-in-shell eggs were transported in sterile bags to the place of the examination without opening. The eggs originated from different trios on both farms. On Farm A, the eggs were unmarked, on Farm B, the number of pens where the eggs were laid was known.

Dead chicks

Chick mortality most frequently occurs between 0 and 21 days of age, so dead chicks of this age were evaluated. No dead chicks were investigated on Farm A. From Farm B, 15 chick carcasses were examined, of which four were freshly hatched, four were one week old, six were two weeks old, and one was three weeks old. Until the examination, the dead chicks were stored in the freezer in nylon bags and then transported to the laboratory.

Swab samples

A total of 14 swab samples were collected on Farm A, of which seven were derived from the surface of stored and incubated eggs, and also seven swab samples were taken from the storage room shelf, incubator walls, egg trays and incubator doors. The investigations were done on sanitised eggs. The ratio of bacteria was calculated separately for egg and facility surfaces. All examinations were based on *Salmonella* isolation, followed by separating other bacteria using different specialised media. On Farm B, we did not have a chance to obtain swab samples.

Range of bacteria tested for

Using standard laboratory protocols, the presence of members of the following bacterial genera was examined: *Bacillus, Enterococcus, Escherichia, Klebsiella, Pseudomonas, Salmonella, Staphylococcus* and *Streptococcus*. All types of samples were analysed for bacteria.

Bacterium isolation

The bacteriological tests were carried out according to the standard practice in diagnostics to detect the most common infectious microbes in Hungary. As a routine procedure, samples were taken from the parenchymal organs of the corpses (liver and heart blood), the contents of the egg, and the parenchymal organs of the embryo, under sterile conditions. The organ pieces thus obtained were streaked on Columbia blood agar (Biolab), Coliform chromocult (Biolab) and Klimmer (Biolab) agar plates. The cultures were incubated at 37 °C for 24 h, and the grown bacterial flora was further examined. The components of the mixed flora were isolated by preparing colour cultures. Additional differentiation media were selected according to Gram staining. *Enterococci* were isolated using Slanetz-Bartley agar (Biolab), which was incubated at 37 °C for 24 h and then for 48 h. The biochemical probes for the differentiation of bacterial strains were carried out via the ATB system (Biomerieux).

Salmonella enrichment method

Salmonella enrichment was performed according to the MSZ EN ISO 6579-1:2017 standard. The goal was to obtain a culture from *Salmonella* even if the bacteria are present in small quantities in the sample according to the monitoring controls applied in poultry breeding. The sensitivity of the technique allows the indication of a minimum of a thousand bacteria/g samples. After 24-h incubation of the peptone water (Bak-test), the ability to draw was tested on MSRV medium (locally prepared) and streaked on XLD (Biolab) and Rambach (Biolab) differentiation agar plates. Anti-Salmonella polyvalent reagent (Sifin) and biochemical probes were applied to identify the suspected colonies. For the quick identification of the motile cultures belonging to the *Salmonella* genus, *Salmonella Enteritidis* O9 and *Salmonella Typhimurium* O4 monovalent reagent (Sifin) were used in the slide agglutination process. After the exclusion of the two above-mentioned serotypes, we applied the *Salmonella* spp. classification since their veterinary and pathological assessment is the same.

Mycological test

Mycological examinations were performed with fertile eggs, based on the preliminary dissections. Samples were taken from the inner shell membrane and from the air chamber membrane of eggs with developing embryos. Fungus isolation was attempted on Sabouraud agar plates (Biolab). The cultures were kept at 24 °C for five days.

Parasitology test

The flotation method with saline solution was used to determine the presence of parasites in the faeces. The parasitologic evaluation was carried out on mixed and homogenised faeces. Faecal samples were washed through a filter paper with supersaturated saline solution after having been mashed in a mortar. A cup with the mashed and washed faeces was filled up with the solution. The parasite eggs were collected with a glass slide from the surface of the liquid, and the eggs were examined with light microscope for their number, size and shape.

RESULTS

Bacterium isolation from the faecal samples

The number and classification of bacteria in the samples is presented in Table 1. On Farm A, 19 faecal samples were found positive, and some of these contained more than one bacterial species. All samples from Farm B were positive. On Farm A, more than 80% of the evaluated samples were contaminated with *E. coli* and nearly half of the samples with *Bacillus* spp. The same two bacteria were the most prevalent on Farm B, where more than 90% of the samples showed the presence of *E. coli*, while *Bacillus* spp. was present in one-fourth of the faecal samples. *Klebsiella* spp. was detected with lower frequency.

Parasite examination in faeces

All faecal samples from Farm A were found negative. On Farm B, eggs of *Ascaridia, Heterakis* and *Capillaria* spp. were suspected based on their morphology. Exact species determination was not performed. The ratio of *Ascaridia* eggs was slightly higher but all of them were below 10%.

Bacterium isolation from the swab samples

Of the swabs taken from the eggshell surface, 71.4% contained *Bacillus* spp., while 28.6% were negative (Table 1). *Salmonella* enrichment was negative. *Bacillus* spp. were also isolated from 57.1% of swab samples from the storage room and the incubator and 28.6% of the samples contained coliforms.

Table 1. Results of the bacterium isolation attempts with the number of positive samples for each bacterium. *Several samples contained more than one of the tested microbe species

Bacteria	Faecal samples		Eggshell surface swabs	Storage room & incubator swabs	Dead-in-shell eggs		Dead chicks*	Sum
Farm (number of samples)	A (n = 20)*	B (n = 33)*	A (n = 7)	A (n = 7)	A (n = 50)	B (n = 43)*	B (n = 15)	175
Bacillus spp.	9	8	5	4	1	2	-	29
Coliform bacteria	-	-	-	2	14	9	2	27
Enterococcus faecalis	-	-	-	-	1	-	5	6
Escherichia coli	17	32	-	-	8	3	2	72
Klebsiella spp.	-	1	-	-	1	1	-	3
Pseudomonas spp.	-	-	-	-	3	3	1	7
Salmonella spp.	-	-	-	-	-	5	-	5
Staphylococcus spp.	-	-	-	-	-	3	-	3
Streptococcus spp.	-	-	-	-	-	-	-	-
Positive	19	33	5	6	28	26	8	120
Negative	1	-	2	1	22	22	7	55

Bacterium and fungus isolation from dead-in-shell eggs

Isolation of bacteria was attempted from 93 eggs, of which 23 (24.7%) were infertile. Seven eggs (7.5%) were infected with moulds and contained dead embryos. The quality of the eggshells was optimal without any leaks, cracks or deformation. On both farms, half of the samples were found to be contaminated. Coliform bacteria were present in the highest percentage (Table 1). *Salmonella* was isolated only on Farm B with 10% frequency. *Bacillus, Klebsiella, Pseudomonas, Staphylococcus* species and *Enterococcus faecalis* were detected in one to three samples, thus each of these bacteria were present in less than 7% of the examined eggs. *Bacillus, Klebsiella* and *Pseudomonas* species were found on both farms. Four samples contained coliform bacteria and *Salmonella* spp. simultaneously while one was contaminated with *Staphylococcus* along with *Salmonella*. All five dead-in-shell eggs on Farm B, which showed the presence of *Salmonella*, had been laid in July. Two of the embryos, in these five eggs, died on the second week of incubation, one on the third week, whereas the other two at the latest stage of development immediately before hatching. We did not possess data on the overall number of dead-in-shell eggs.

Bacterium isolation from dead chicks

All dead chicks originated from Farm B. No clinical signs, i.e. lesions or developmental disorders were seen, however, most of them were weak at hatching and had a reduced body weight. A few days prior to death, the chicks refused the feed and lost weight. The neck curved in an S-shape indicating discomfort. Until the last day, chicks were able to walk but a few hours before death, they just laid on the ground. Upon autopsy, pieces of straw from the bedding could be found in the gizzard, the lungs were seriously oedematous, the hearts were pale and flabby, the kidneys were oedematous, and the yolk sac was swollen. A large amount of residual egg yolk was in the abdominal cavity. The digestive tract was empty and the stomach and intestines were catarrhal in every chick necropsied.

Half of the samples were bacteriologically positive (Table 1). *Enterococcus faecalis* was present in one-third of the dead chicks. *Escherichia coli* and coliform bacteria were also found in the same two samples, while *Pseudomonas* was present only in dead chick accounting less than 7% of the positive samples. The result of the *Salmonella* enrichment was negative. We did not have data on the allover number of dead chicks.

The general ratio of bacteria in all samples

Regarding all 120 positive samples (Table 1), *E. coli, Bacillus* spp. and coliform bacteria were the most frequently isolated microbes. The other bacteria were detected in very low number, individually accounting for less than 5% of all samples. *Bacillus* spp. were detected in 32% of faecal samples, whereas. *E. coli* was present in almost every faecal sample, as well as in dead-in-shell eggs and in the blood of

dead chicks. Coliform bacteria occurred in the third highest percentage (15.4%) of all samples, but were not obtained from the faeces. They were most prevalent in dead-in-shell eggs, and dead chicks and were isolated from the swabs samples from the incubators. *Bacillus* spp. were discovered in all types of sample except for dead chicks. The presence of *Salmonella* was revealed only in dead-in-shell eggs. *Pseudomonas* spp. and *Enterococcus faecalis* were observed in dead-in-shell eggs and dead chicks. *Klebsiella* was found in both faeces and dead-in-shell eggs but in small amounts only. *Staphylococci* were detected only in dead-in-shell eggs in a low ratio, whereas no *Streptococci* were isolated at all.

DISCUSSION

The general low hatchability of ostrich eggs (Cooper, 2001) results in great economic losses. The appropriate hatchability requires precise and accurate implementation of feeding, husbandry and egg treatment technologies, including hygiene, as well. As with the fertility and hatchability of eggs, the vitality and survival of chicks are also influenced by pathogens. In this regard, the assessment of the microbiological status of farms can be an important aspect in responding to infections and improving hatchability and chick survival. Monitoring the microbiological condition of the farms is of utmost importance given the current relevance of biosecurity. By selling breeding eggs, infectious agents can spread from one farm to another, infecting the chicks and the associated products. Ostrich eggs can also be consumed, so the presence of bacteria in the eggs can pose a health risk to humans as well. Microbiological evaluation helps to reveal the causes of the embryo and chick mortality as an indicator of hygiene in all production stages.

The advantage of sandy ground was imperceptible since there were more bacterium strains obtained from Farm B than from Farm A, though the ratio of *Bacillus* spp. was lower. Hassan et al. (2016) have found a wide range of bacteria in ostrich faeces, including *E. coli, Corynebacterium, Enterobacter, Proteus, Pseudomonas* and *Salmonella species* being the most prevalent ones. Although the farm conditions have not been described in that study, the birds were considered healthy with no transmission of subclinical infection. Asmaa et al. (2016) have reported that the environment (including feed, water, soil and faeces) of ostrich farms with adult birds or chicks contained *E. coli* and *Salmonella* species in the highest number of all examined bacteria. Adults were kept on sandy ground and chicks had cement ground. The prevalence of *E. coli* in the faeces was 60% for chicks and 65.3% for adults. In soil samples, *E. coli* was present in 53% in chick yards and 56.3% in adult pens. Timur et al. (2009) have reported a lower ratio of 40.7% of *E. coli* present in the faeces of healthy ostriches. Asmaa et al. (2016) have also detected *Salmonella* in the faeces of chicks (25%) and adults (28%). These authors have pointed out that the 3%-difference could be attributed to the more controlled environment of chicks compared to the open yards of adults. Although in our case, wild birds were present in the open

yards of adult ostriches, we could not isolate *Salmonella* from their faeces. Differences between the literature data and our results can be explained by the different climate conditions and husbandry technology, as well as the exposure of the breeding area to small mammals (e.g. rodents) or wild birds. However, the literature did not provide enough information for proper comparison.

Nematode eggs could be detected only on Farm B, and the prevalence was low, below 10%. The occasional occurrence of *Heterakis* in Iran has been described (Eslami et al., 2007). Gordo et al. (2002) have found *Capillaria* in ostrich faeces in Spain but only from one bird, and no other nematodes have been found. Ederli and Oliveira (2015) have reported the detection of *Codiostomum struthionis* and *Lybiostrongylus* species in ostrich faeces but the husbandry system of the examined farms has not been discussed in detail. In our case, there could be a correlation between the presence of parasites and the husbandry conditions. The occurrence of small mammals and birds on the farm can influence the parasitological status of pens. However, our investigation did not include the examination of the faeces of wild animals on or near the farms.

Bacillus spp. were detected on eggshell and facility surfaces in more than 60% of the swab samples. In the incubator, coliform bacteria also occurred. *Bacillus* spp. contaminated the walls and door of the incubators, while coliform bacteria were present on the incubator basket. The high prevalence of coliform bacteria in dead-in-shell eggs could be attributed to the contamination through the incubator. More than 60% of stored eggs were infected with *Bacillus* spp. This fact highlights that the disinfection of eggs was not effective enough to kill all the bacteria. Jahantigh (2010) has reported that *Bacillus* (33.4%) was the most prevalent bacterium isolated from swab samples of unhatched ostrich eggs. Other pathogens detected were *Staphylococcus* (29.1%), *E. coli* (12.5%), *Proteus* (12.5%), *Streptococcus* (8.3%), and *Klebsiella* (4.2%). The eggs have been disinfected with formalin and potassium permanganate before incubation, and all the unhatched eggs have shown some bacterial infection. Metawea and El-Shibiny (2013) have investigated environmental swab samples originating from an ostrich hatchery where the eggs have been cleaned first by a dry cloth then wiped with Virucidal extra 0.25% solution. They have found that the highest coliform and other Enterobacteriaceae count was isolated from the floor and walls of the hatchery and the lowest from the egg receiving room. The same bacteria were present on all surfaces but in different ratios. Conversely, in our case, both the type and ratio of bacteria differed by the place of origin. The bacterium count was higher on eggs before sanitation than after sanitation. The relevance of the presence of bacteria on hatchery equipment and surfaces is high due to the risk of contamination of eggs and chicks. Newly hatched chicks are seriously susceptible to bacterial infection until the age of one week. For example, Metawea and El-Shibiny (2013) have reported that regarding all samples, the prevalence of *E. coli* was higher than that of the *Salmonella* spp. The highest ratio of these bacteria has been found on the floor
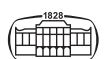
of the hatchery and on the eggs before sanitation, while the lowest was detected on eggs after sanitation. The authors highlighted the significance of the strict application of disinfectants and the implementation of control programs from breeder farms to hatcheries. By meeting the microbiological requirements of farms and the control of the effectiveness of disinfection, the fertility and hatchability of eggs can be improved.

In dead-in-shell eggs, coliform bacteria showed the highest percentage on both farms, though, on Farm A, the ratio was higher. On Farm A, *E. coli,* while on Farm B, *Salmonella* were the second most prevalent bacteria contaminating dead-in-shell eggs. On Farm A, three times more *E. coli* were present than on Farm B. *Salmonella* was detected only on Farm B in a higher percentage than the *E. coli*. The ratios of *Bacillus, Klebsiella, Pseudomonas, Staphylococcus* species and *Enterococcus faecalis,* ranged between 2 and 7%. On Farm B, there was no tendency for bacterial contamination (the type of occurring bacterial strains) of eggs by pen. Around half of the dead-in-shell egg samples did not show any signs of bacterial infection.

When ostriches turn the eggs right after laying, the soil clings to the eggshell since the cuticle has not yet dried and bacteria can easily penetrate the egg. If the disinfection process is not carried out properly, the bacteria can contaminate the developing embryo and can be present in the hatched chicks after penetration. Bacterial infection in hatcheries is a common problem since there are many ways of transmission through incubator facilities and devices (Wales and Davies, 2020). Burger et al. (1995) has stated that sand is an appropriate soil for ostrich breeding since it enables the drainage of urine and puddles from the ground surface, so ostriches are less exposed to bacterial infections. Even though there was sandy ground on Farm B, the bacterial contamination of eggs was as high as on Farm A. In dead-in-shell ostrich eggs, Deeming (1995) has found *Staphylococci, E. coli, Bacillus licheniformis* and *Achromobacter* spp. causing yolk sac infection. In our case, almost all bacteria detected were present inside the eggs. The Gram-negative and motile bacteria that penetrate the eggs before the disinfection are less susceptible to the antimicrobial components of the albumen and are present during the incubation (Silvetti et al., 2017). The bacteria may cause the death of the developing embryo (Yassin et al., 2008) or infection of the newly-hatched chicks (Baron et al., 2014).

In Italy, Cabassi et al. (2004), by investigating infertile ostrich eggs, revealed that several bacteria infected almost 20% of egg yolk and albumen. *E. coli* was the most common isolate, however, *Pseudomonas* and *Staphylococcus* have shown high prevalence, too. Dzoma and Dorrenstein (2001) have also drawn the attention to the microbiological infection of incubated ostrich eggs. The authors have reported that 42% of dead-in-shell eggs showed the presence of bacteria of which *E. coli* had the highest ratio. In Iran, *E. coli* has been reported to be the most prevalent bacterium in dead-in-shell ostrich eggs, and it was also present in the meconium, heart blood and liver of the embryos (Rezaei et al., 2013). None of the listed authors have provided

information on egg disinfection to allow drawing more conclusions about the reason for differences. *Staphylococcus* spp. are part of the normal microflora colonising human skin, thus it can be found on hands (Michael, 2010). Egg treatment without gloves can lead to the infection of eggs with *Staphylococcus* and *Pseudomonas* species. Also, by this activity, the water loss of eggs during incubation will be greater than optimal, thereby decreasing hatchability.

Escherichia coli often causes death of the developing embryos in a later stage of incubation or immediately after hatching in the hatchery (Knöbl et al., 2012). The clinical signs include neurological disorder, septicemia, omphalitis and death (Perelman, 2009). In our case, due to the limited number of dead chicks, it was difficult to draw a conclusion regarding the cause of death. Coliform bacteria and *Pseudomonas* species are natural components of soil and groundwater (Daoliang and Shuangyin, 2019), so it is possible that due to the poor eggshell disinfection, ground bacteria could also penetrate the eggs and contribute to the death of embryos. Other authors have detected pathogens inside the ostrich eggs, too. For example, De Reu et al. (2006) have reported that among the other bacteria, *Salmonella enteritidis, Staphylococcus warneri* and *Streptococcus* species could be detected in ostrich egg in four to five days after the infection of the eggshell.

In our study, *Salmonella* was present only in dead-in-shell eggs on Farm B. These eggs were collected from separate pens, so it could not be decided whether the pathogens were transmitted from one pen to the other or whether the infection happened separately. Eggs of the same age were incubated together. Being a semi-free range system, wild birds or rodents from the surroundings of the farm could visit the feeders, drinkers and yards of ostriches. We did not analyse the composition of faeces of other species but it was likely that *Salmonella* spp. originated from them since the faecal samples of parents were found to be free from *Salmonella*. Wet ground or shaded areas that the UV rays of the Sun could not reach to disinfect the surface of eggs, could help the persistence of *Salmonella* spp. The results also indicated that the egg treatment and disinfection procedure was not appropriate, so *Salmonella* from the environment could get into the eggs. Yazeed et al. (2015) have also isolated *Salmonella* from dead-in-shell eggs with a low prevalence (2.1%), however, they have not provided an explanation for their findings. Knöbl et al. (2012) have not found *Salmonella* in dead-in-shell eggs. There have been slight differences in the eggshell disinfection and incubation technology on the examined farms. However, obvious effects of these on the microbiological status have not been revealed.

In dead chicks, *Enterococcus faecalis* was present in one-third of the samples. *Escherichia coli* and coliforms were sporadic, while *Pseudomonas* was isolated from one dead chick only. Mixed infections were also found. Interestingly, almost half of the dead chick samples (7 from 15) were negative. No clinical signs, only post-mortem lesions were observed. *Enterococci* including *Enterococcus faecalis* are opportunistic pathogens inhabiting the intestinal tract of birds (Chadfield et al., 2004), however, the infection can be

destructive for embryos and chicks (Morishita, 2019). Among clinical signs, reduced growth, osteoarthritis and death can be observed (Fertner et al., 2011). The bacterium may have infected weaker chicks when they eat excrements of other chicks, i.e. coprophagy occurs.

The greatest variety of bacterial species was present in dead-in-shell eggs. This can be explained by the fact that incubated eggs are the most exposed to the different environmental effects (microbiological condition of yards, the method of egg collection and the treatment during storage, incubation, and hatching).

Escherichia coli occurred in faeces, dead-in-shell eggs and dead chicks, of which the greatest percentages were determined in faecal samples on both farms. In dead-in-shell eggs, on Farm B, the ratio of *E. coli* was lower than in the faeces; however, it was several times higher than in dead chicks. *Bacillus* spp. was present in the faeces, dead-in-shell eggs, eggshell surfaces and environment (storage room and incubator) swab samples. Regarding all samples, *Bacillus* spp. had the highest ratio in faeces, half of it was present on the eggshell surface and 2–3% occurred in dead-in-shell eggs and environment samples. *Klebsiella* species could be isolated only from faeces in less than 1% and dead-in-shell eggs in more than 1%. Though coliform bacteria were not detected in faecal samples, they occurred in dead-in-shell eggs in a relatively high ratio and were isolated also from dead chick and environment swab samples in low percentages. *Pseudomonas* spp. were detected in dead-in-shell eggs in fewer samples compared to coliform bacteria and *E. coli*. It was also present in dead chicks but in a small ratio. *Staphylococcus* species occurred only in dead-in-shell eggs in a small percentage. *E. faecalis* was present solely in dead-in-shell eggs and dead chicks. The ratio of *E. faecalis* in dead-in-shell eggs was extremely low. *Salmonella* was isolated only from dead-in-shell eggs in a relatively low ratio. As was supported by Metawea and El-Shibiny (2013), cross-contamination of samples of different origins due to less effective sanitation is a significant problem on ostrich farms. Genetic effects also can be determining factors in embryo mortality (Brand et al., 2017), however, we have not examined these under the scope of this study.

Regarding faecal samples, *E. coli* and *Bacillus* spp. were present on Farm A and also on Farm B but in a relatively different ratio. While the prevalence of *E. coli* was higher on Farm B than on Farm A, the ratio of *Bacillus* spp. was the opposite. On Farm B, *Klebsiella* spp. was also isolated but in a low percentage.

In dead-in-shell eggs, coliform bacteria, *Bacillus, Klebsiella, Pseudomonas* species and *E. coli*, were present on both farms. Coliform bacteria showed the highest ratio in dead-in-shell eggs including both farms; however, its prevalence was higher on Farm A. *Escherichia coli* was the second most prevalent bacteria, contaminating more samples on Farm A than on Farm B. *Salmonella* was present only on Farm B with a relatively high ratio compared to *E. coli*. *Pseudomonas* was isolated from dead-in-shell eggs on both farms in a comparable but low ratio. The prevalence of *Bacillus* spp. in dead-in-shell eggs was almost twice higher on Farm B

than on Farm A. *Staphylococcus* was present only on Farm B in a similar percentage as *Pseudomonas, E. faecalis* occurred only on Farm A, in just one sample. The ratio of *Klebsiella* was very low on both farms. The small differences in the microbiological status of the examined farms may have stemmed from the slightly different egg treatment and egg disinfection protocol.

The presence of *Bacillus* and *Klebsiella* in the faeces indicates that regular and thorough soil disinfection is required. Egg handling with gloves and the application of wide-range solutions and appropriate concentrations of disinfectants can help to mitigate the penetration of unfavourable bacteria into the eggs. Investigating the appropriate concentrations of disinfectants can be a future research topic to obtain more pieces of information on the proper application of the disinfection of egg surfaces and facilities. Regarding chick mortality, the level of hygiene in the nursing area should be improved. Apart from the microbiological issues, the technology of egg treatment (the method of egg collection and storage), incubation and chick nursing should be better performed with strict attention to hygiene and sanitation. Although the purpose of our research was not to determine the causes of death of already-hatched ostrich chicks, the following can be inferred from our findings. The quality of day-old and juvenile birds is decisively influenced by the factors that cause poor development. If the bird is able to break out of the egg, in addition to the non-infectious causes that delay the development of the embryo (technological or machine failure, human failure, etc.), the bacterial damage may result in low vitality. The parasitic contamination in faecal samples was insignificant since more than 90% of the samples were negative. Although, the presence of several parasite ova in the samples of Farm B reveals that the soil hygiene in the pens should be improved. The application of soil disinfectants and nematicides can be suggested.

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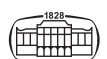
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