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Original Paper

Eggshell Microbiota of Eggs from Three Different Laying Hen Rearing Systems

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Penetration of microorganisms is likely to be affected by microbial contamination. The aim of this study was to compare bacterial contamination of eggshell in deep litter, enriched cages and ordinary aviaries. It was investigated how the housing system affected the eggshell's early bacterial infection. There were eight extended experiments conducted. Periodically, the total count of bacteria, coliform bacteria, and *Salmonella* spp. were used to measure the level of bacterial contamination in eggshells found in aviaries, deep litter, and enriched cages. We log-transformed the results. Compared to enriched cage systems, the total number of bacteria contaminating eggs from the deep litter was higher. After 21 days, the second instance of microbial contamination was examined. Day 0 of every experiment showed the highest overall count of bacteria. Microbial species were identified using MALDI TOF MS Biotyper mass spectrometry. The two most isolated species across all housing systems were *Ralstonia pikettii* and *Staphylococcus equorum*.

Keywords: total count of bacteria, coliform bacteria, eggs surface, identification of microorganisms

1 Introduction

Foods of animal origin, such as meat and chicken egg products, are considered to be the main cause of foodborne infections caused by microorganisms (Behravesh et al., 2012; Sabarinath et al., 2009). Bacterial contamination of eggs and commodities can affect their quality. This can result in the spread of pathogens, spoilage, and consumer illness or food poisoning. According to Indhu et al. (2014), microbial contamination of eggs often occurs a few seconds after egg laying during transport and until consumption. Eggs can become infected in three different ways: vertically by microorganisms found in the blood of the digestive tract; horizontally by a variety of organisms (e.g. streptococcus and coli-acrogens during artificial insemination); and horizontally by cloacal contact with nest and litter material during oviposition. Dust in barns and warehouses, hygiene or shell structure (cracks, presence of cuticle and membrane quality), season and storage conditions are other factors that can also influence bacterial contamination (Mallet et al., 2010).

Faeces are the main source of microbial contamination of eggshells. Each time an egg is laid, the egg can become contaminated with faeces and bacteria can pass through the membranes and shell due to the vacuum effect created by the subsequent heat loss of the egg. Under inappropriate conditions of long-term storage and transport, microorganisms can enter the egg contents (Chi et al., 2023). When raw or undercooked contaminated eggs and products are consumed, they pose a significant risk to human health. High levels of contamination can have a negative impact on food safety and the shelf life of eggs. Consumer perceptions of healthy eggs now focus more on microbiological uniformity than shell hygiene and physical characteristics due to increasing consumer knowledge of food safety concerns (Yenilmez, 2020).

A variety of bacteria have been identified on table eggshells including *Escherichia*, *Micrococcus*, *Salmonella*, *Streptococcus*, *Staphylococcus*, *Aeromonas*, *Enterobacter*, *Proteus* and *Pseudomonas*. The same has been found

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for yeasts and moulds (Musgrove et al., 2004; Ricke et al., 2001). However, the Enterobacteriaceae group, which is Gram-negative, has been identified as a significant contamination of commercial hen eggs (Sabarinath et al., 2009). *Salmonella* is a member of the Gram-negative, selectively anaerobic Enterobacteriaceae group. In addition, one of the items that most commonly cause illness caused by *Salmonella* is eggs (Whiley & Ross, 2015).

Bacteria that can colonize the eggshell and enter the egg through its pores are a major source of contamination of the egg-laying environment for the yolk sac of the embryo and neonate (Cortés et al., 2004). Bacteria belonging to the genera *Enterobacter*, *Klebsiella*, *Staphylococcus* and *Streptococcus* have already been identified and isolated as causative agents of yolk sac infection in broiler chickens (Cortés et al., 2004). Even eggs from clean nests contain certain bacteria on the eggshell that could, under the right circumstances, compromise the health and hatchability of newborn chicks. According to Coufal et al. (2003; Zeweil et al. (2015), the total number of aerobic mesophilic bacteria on the shell of these eggs can range from 3.75 to 7.07 log10 colony forming units (CFU) per egg. Accordingly, reducing the microbial load on the eggshell through disinfection methods would increase the quality of incubated eggs and reduce the risk of bacterial infections of developing embryos and newborn chicks (Fasseas et al., 2008).

The aim of our study was to analyze the eggshell microbiota of eggs from three different egg-laying systems and to identify them by mass spectrometry during 21 days of storage at 10 °C.

2 Material and methods

2.1 Study animals

The research was carried out on the poultry farm Babičkin dvor a.s., Veľký Krtíš with a hybrid line of production laying hens Bovans Brown. Layers of the hybrid line Bovans Brown, reared in three different systems were studied: 30,892 birds in enriched cages, 11,130 birds on deep litter and 27,958 birds in aviaries at the beginning of the experiment. A total of 243 eggs from 8 trials from three different locations (A, B, C) were evaluated on days 0. and 21. of storage in the laboratory. The temperature in the laboratory was constant at 10 °C for all 21 days.

2.1.1 Breeding system in enriched cages on the farm

5ON04R cage breeding equipment is used on the farm. The cages are 4-story in 6 batteries. On the front side of the hall there are adapters for collecting eggs, and on the back side of the hall there is a device for removing droppings. The cages include a central power supply with a water control gauge on each floor and medication dispensers.

2.1.2 Deep litter laying hen rearing system on the farm

Laying nests, feeders and nipple waterers are placed in the center of the hall's slatted floor. Dry sand with a thickness of 3 cm is used for bedding. There are 9 laying hens per m^2 of the floor area of the hall. The droppings from the entire hall and from the litter area are removed at the end of the laying cycle after the hens have been removed. The grid floor is placed 500 mm above the floor, so that the accumulated droppings do not exceed the height of the grid at the end of the laying cycle after the hens are removed. Nipple drinkers are located near the laying nests. The slatted area of the floor is used by laying hens to clean the runners when entering the nest.

2.1.3 System of rearing laying hens in aviaries on the farm

This breeding system makes it possible to increase the number of laying hens per m^2. There are three-story aviary structures in the hall. In the aisles between the rows of the structure and below them, there is a litter of sand. The litter is used for raking laying hens and also as a dust bath.

2.2 Sample collection and processing

The surface bacterial count was determined with a swab. Before being diluted with regular saline, the entire egg's surface was aseptically swabbed with a sterile cotton swab.

2.3 Microbiological analyses

One hundred µL of each dilution of the samples was applied to the plate count agar (PCA) surface (Oxoid, Basingstoke, UK), Violet red bile agar with lactose (VRBL) surface (Oxoid, Basingstoke, UK) and Xylose Lysine Deoxycholate agar (XLD) surface (Oxoid, Basingstoke, UK), after they had been serially diluted further.

The following were measured: total bacterial count (TBC), coliform bacteria (CB), and number of *Salmonella* spp. (SS). The quantitative CFU (Colony Forming Units) counts determination of the corresponding groups of microorganisms in logarithms of eggshells surface was done using the plate diluting method. For the CFU segregation of TBC, CB, and SS, Plate count agar, Violet red bile agar with lactose, Xylose Lysine Deoxycholate agar were employed (incubation 48–72 h at 30 °C for TCB and 37 °C for CB and SS, aerobic cultivation method).

The microbial colonies were incubated for 18 to 24 hours at 37 °C on TSA agar (Tryptone Soya Agar, Oxoid, UK) prior to detection. A colony was established using eight distinct strains of bacteria. Kačániová et al. (2019) state that the MALDI TOF-MS Biotyper was then used to carry out the identification.

The Excel program was used for the statistical evaluation of the results. The results were evaluated in the form of arithmetic mean, and standard deviation.

3 Results and discussion

3.1 Microbiota of eggshell in 0 day

Total bacterial counts on day 0. are shown in Table 1. In our study, total bacterial counts, coliform counts, and *Salmonella* spp. were analyzed. Our study shows that only total bacterial count (TCB) was present on eggshell. TCB ranged from 2.18 ±2.34 in enriched cages to 2.98 ±2.34 log CFU.eggshell^{-1} in deep bedding in the first experiment, and from 2.16 ±1.34 in enriched cages to 3 in the second experiment, 3.02 ±1.45 log CFU.eggshell^{-1} in aviaries, in the third from 1.95 ±1.45 in deep litter to 3.22 ±1.34 log CFU.eggshell^{-1} in aviaries, in the fourth from 1.12 ±0.34 in deep litter to 2.31 ±1.21 log CFU.shell^{-1} in aviaries, in the fifth from 1.48 ±2.34 in enriched cages to 2.38 ±2.32 log CFU.shell^{-1} in aviaries, in the sixth from 1.12 ±1.45 in enriched cages to 2.32 ±1.12 log CFU.shell^{-1} in deep litter, in the seventh from 1.14 ±2.21 in enriched cages to 2.70 ±1.18 log CFU.shell^{-1} in aviaries, and in the eighth from 1.23 ±2.54 in enriched cages to 2.42 ±0.78 log CFU.shell^{-1} in aviaries.

In total 282 isolates were identified from of eggshell from enriched cages on 0 day (Table 2). Totally 13 family, 15 genera and 31 species were isolated from eggshell samples. The most isolated species was *Staphylococcus equorum* (11%). The other most isolated bacterial species were *Ralstonia picketii* (10%) and *Staphylococcus epidermidis* (5%).

A total of 315 isolates were found in the deep litter 0 day eggshell (Table 3). From eggshell samples, a total of 15 families, 21 genera, and 37 species were isolated. *Escherichia coli* and *Staphylococcus equorum* accounted for 8% of the most isolated species. *Staphylococcus equorum* subsp. *equorum* (5%) and *Ralstonia picketii* (7%), on the other hand, were the other most isolated bacterial species.

In the aviaries on 0 day eggshell, 297 isolates in total were discovered (Table 3). A total of 16 families, 20 genera, and 33 species were separated from eggshell samples. 8% resp. 6 % of the most isolated species were *Staphylococcus equorum* and *Staphylococcus equorum* subsp. *equorum*. The other most isolated bacterial species were, however, *Ralstonia picketii*, *Staphylococcus haemoliticus* (6%), *Ralstonia mannitolilytica* and *Pseudomonas luteola* (5%).

3.2 Microbiota of eggshell in 21 day

Table 5 shows the total number of microorganisms counted on day 21. In our study the total number of bacteria, coliform bacteria, and number of *Salmonella* spp. were evaluated. Our study shows that on eggshell were only total count of bacteria (TCB). TCB in first experiment ranged from 1.60 ±1.07 in aviaries to 3.28 ±1.34 log CFU.eggshell^{-1} in deep litter, in second experiment from 1.12 ±0.56 in enriched cages to 1.70 ±2.34 log CFU.eggshell^{-1} in enriched cages, in third from 1.13 ±1.67 in enriched cages to 2.43 ±1.32 log CFU.eggshell^{-1} in deep litter, in fourth from 1.14 ±0.05 in enriched cages to 2.36 ±1.56 log CFU.eggshell^{-1} in aviaries, in fifth from 1.30 ±1.23 in enriched cages to 2.81 ±1.56 log CFU.eggshell^{-1} in deep litter, in sixth from 1.16 ±0.13 in deep litter to 2.28 ±0.34 log CFU.eggshell^{-1} in aviaries, in seventh from 1.12 ±0.75 in aviaries to 2.04 ±1.43 log CFU.eggshell^{-1} in deep litter and in eight from 1.12 ±1.13 in enriched cages to 1.39 ±0.37 log CFU.eggshell^{-1} in deep litter.

Table 1 The number of total count of bacteria on eggshell in 0 day in log CFU.eggshell^{-1}

Day	Sample	1.	2.	3.	4.	5.	6.	7.	8.
0	ECA	2.51 ±1.34	2.16 ±1.34	2.83 ±1.23	1.85 ±1.23	2.04 ±1.34	1.60 ±2.34	1.14 ±2.21	1.23 ±2.54
0	ECB	2.18 ±2.34	2.36 ±1.23	2.65 ±2.12	1.48 ±2.34	1.70 ±1.23	1.12 ±1.45	2.36 ±1.45	1.78 ±1.43
0	ECC	2.26 ±2.45	2.51 ±1.45	2.04 ±2.25	1.45 ±1.45	1.48 ±2.34	1.17 ±1.06	2.23 ±1.12	2.09 ±0.78
0	DLA	2.89 ±1.45	2.32 ±2.47	1.95 ±1.45	1.71 ±1.08	1.78 ±2.61	1.48 ±0.32	1.85 ±0.34	1.48 ±1.18
0	DLB	2.98 ±2.34	2.42 ±2.63	3.18 ±2.48	1.32 ±0.12	2.15 ±1.54	1.72 ±0.56	1.70 ±0.34	2.04 ±1.34
0	DLC	2.92 ±1.45	2.34 ±1.67	3.18 ±1.34	1.37 ±0.45	2.26 ±1.24	2.32 ±1.12	1.30 ±2.45	1.70 ±1.15
0	AA	2.49 ±1.36	2.23 ±1.34	2.54 ±1.45	1.12 ±0.34	2.38 ±2.32	2.18 ±1.34	2.15 ±1.45	2.42 ±0.78
0	AB	2.52 ±2.17	3.02 ±1.45	3.22 ±1.34	1.17 ±0.56	1.90 ±1.06	1.71 ±0.13	2.70 ±1.18	2.08 ±0.65
0	AC	2.47 ±2.09	3.00 ±1.16	2.92 ±1.13	2.31 ±1.21	2.04 ±1.56	2.23 ±0.45	2.28 ±1.54	2.15 ±1.14

EC – enriched cages, DL – deep litter, A – aviaries

Table 2 Isolated family, genera and species of microorganisms of eggshell from enriched cages on 0 day

Family	Genera	Species	Number of isolates
Comamonadaceae	*Acidovorax*	*Acidovorax* temperans	7
Moraxellaceae	*Acinetobacter*	*Acinetobacter lwoffii*	8
Bacillaceae	*Bacillus*	*Bacillus cereus*	6
Bacillaceae	*Bacillus*	*Bacillus subtilis*	6
Bacillaceae	*Bacillus*	*Bacillus subtilis* subsp. *subtilis*	8
Sphingomonadaceae	*Blastomonas*	*Blastomonas ursincola*	8
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium glutamicum*	7
Enterococcaceae	*Enterococcus*	*Enterococcus faecium*	8
Lactobacillaceae	*Lacticaseibacillus*	*Lacticaseibacillus paracasei* subsp. *tolerans*	9
Lactobacillaceae	*Lactiplantibacillus*	*Lactiplantibacillus plantarum*	13
Microbacteriaceae	*Microbacterium*	*Microbacterium lacticum*	7
Burkholderiaceae	*Paraburkholderia*	*Paraburkholderia phenazinium*	8
Pichiaceae	*Pichia*	*Pichia occidentalis*	9
Burkholderiaceae	*Ralstonia*	*Ralstonia pickettii*	28
Shewanellaceae	*Shewanella*	*Shewanella profunda*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus aureus* subsp. *aureus*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus cohnii* subsp. *urealyticus*	9
Staphylococcaceae	*Staphylococcus*	*Staphylococcus condimenti*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus epidermidis*	15
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum*	32
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum* subsp. *equorum*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus hominis*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus hominis* subsp. *novobiosepticus*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus chromogenes*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus intermedius*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus kloosii*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus lentus*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus saprophyticus* subsp. *saprophyticus*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus sciuri* subsp. *rodentium*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus xylosus*	7
Streptomycetaceae	*Streptomyces*	*Streptomyces* griseus	6
Total			282

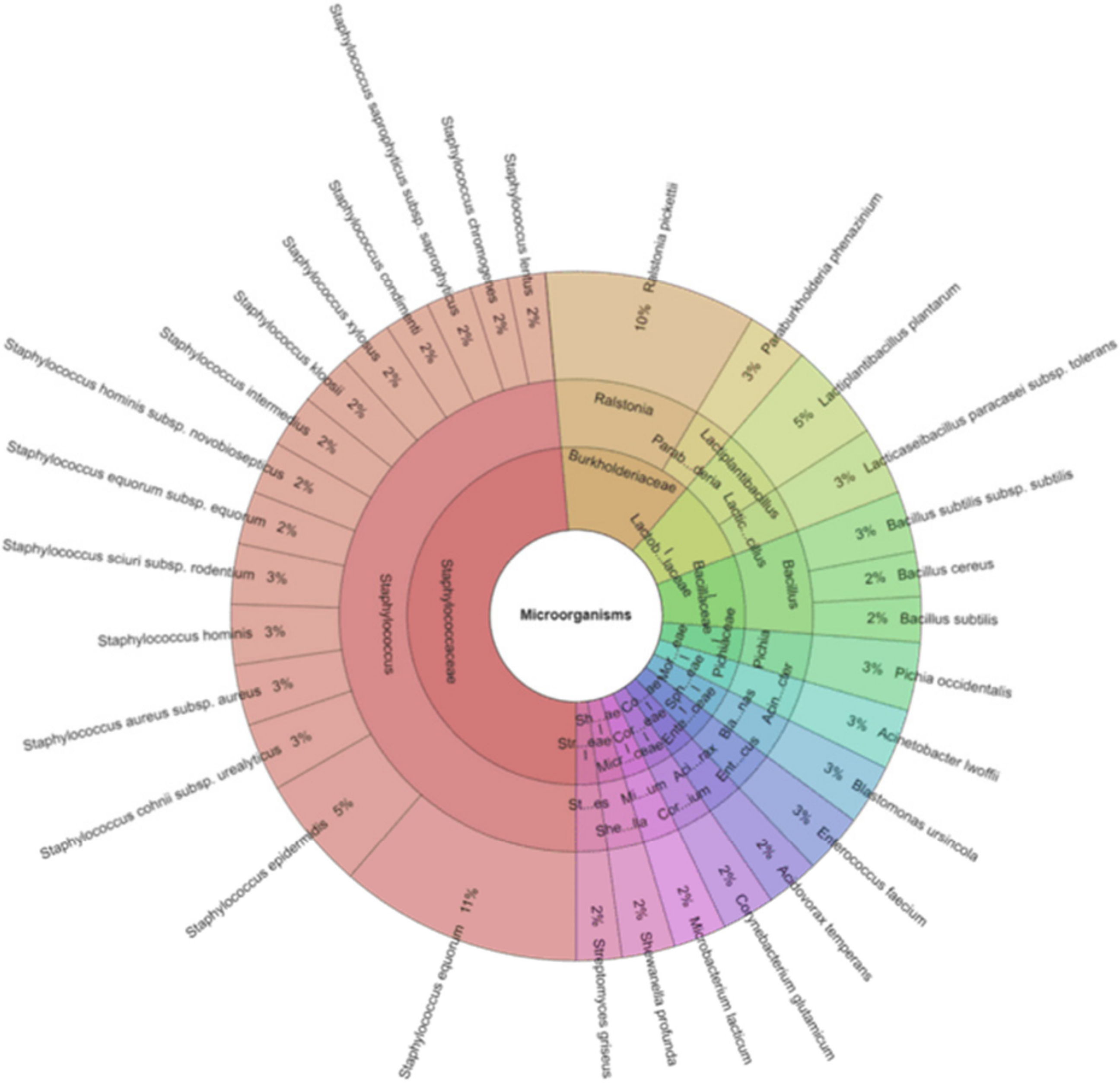

Figure 1 Krona chart: Isolated species of microorganisms of eggshell from enriched cages on 0 day

Table 3 Isolated family, genera and species of microorganisms of eggshell from deep litter on 0 day

Family	Genera	Species	Number of isolates
Moraxellaceae	*Acinetobacter*	*Acinetobacter lwoffii*	8
Aerococcaceae	*Aerococcus*	*Aerococcus viridans*	9
Bacillaceae	*Bacillus*	*Bacillus cereus*	7
Bacillaceae	*Bacillus*	*Bacillus subtilis* subsp. *spizizenii*	8
Brevibacteriaceae	*Brevibacterium*	*Brevibacterium avium*	6
Carnobacteriaceae	*Carnobacterium*	*Carnobacterium maltaromaticum*	7
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium glutamicum*	8
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium kroppenstedtii*	9
Enterobacteriaceae	*Escherichia*	*Escherichia coli*	24
Micrococcaceae	*Glutamicibacter*	*Glutamicibacter creatinolyticus*	9
Weeksellaceae	*Chryseobacterium*	*Chryseobacterium gleum*	8
Micrococcaceae	*Kocuria*	*Kocuria carniphila*	5
Lactobacillaceae	*Amylolactobacillus*	*Amylolactobacillus amylotrophicus*	6
Lactobacillaceae	*Limosilactobacillus*	*Limosilactobacillus gastricus*	4
Lactobacillaceae	*Lentilactobacillus*	*Lentilactobacillus kefiri*	6
Lactobacillaceae	*Lentilactobacillus*	*Lentilactobacillus parabuchneri*	4
Lactobacillaceae	*Lacticaseibacillus*	*Lacticaseibacillus paracasei* subsp. *paracasei*	7
Lactobacillaceae	*Schleiferilactobacillus*	*Schleiferilactobacillus perolens*	7
Burkholderiaceae	*Ralstonia*	*Ralstonia pickettii*	23
Rhizobiaceae	*Rhizobium*	*Rhizobium radiobacter*	7
Micrococcaceae	*Sinomonas*	*Sinomonas atrocyanea*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus arlettae*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus carnosus* subsp. *utilis*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus cohnii* subsp. *Cohnii*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus condimenti*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum*	26
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum* subsp. *equorum*	17
Staphylococcaceae	*Staphylococcus*	*Staphylococcus felis*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus lentus*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus nepalensis*	9
Staphylococcaceae	*Staphylococcus*	*Staphylococcus sciuri* subsp. *sciuri*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus vitulinus*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus xylosus*	7
Streptococcaceae	*Streptococcus*	*Streptococcus suis*	8
Streptomycetaceae	*Streptomyces*	*Streptomyces phaeochromogenes*	9
Zoogloeaceae	*Thauera*	*Thauera aminoaromatica*	6
Comamonadaceae	*Variovorax*	*Variovorax paradoxus*	3
Total			315

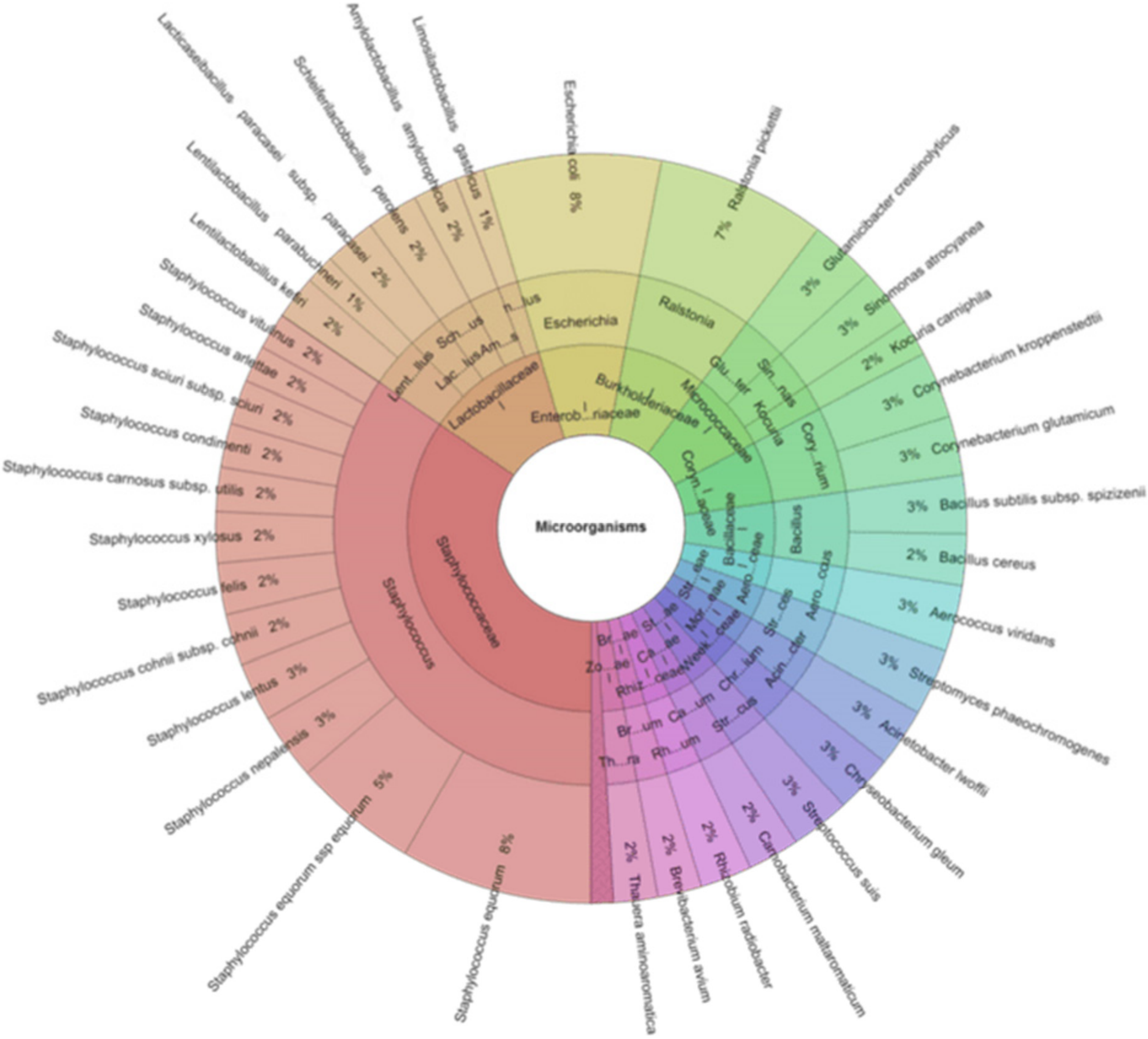

Figure 2 Krona chart: Isolated species of microorganisms of eggshell from deep litter on 0 day

Table 4 Isolated family, genera and species of microorganisms of eggshell from aviaries on 0 day

Family	Genera	Species	Number of isolates
Aerococcaceae	*Aerococcus*	*Aerococcus viridans*	7
Brevibacteriaceae	*Brevibacterium*	*Brevibacterium linens*	8
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium testudinoris*	6
Micrococcaceae	*Glutamicibacter*	*Glutamicibacter creatinolyticus*	5
Streptomycetaceae	*Kitasatospora*	*Kitasatospora phosalacinea*	8
Micrococcaceae	*Kocuria*	*Kocuria carniphila*	8
Lactobacillaceae	*Ligilactobacillus*	*Ligilactobacillus acidipiscis*	8
Lactobacillaceae	*Paucilactobacillus*	*Paucilactobacillus oligofermentans*	8
Lactobacillaceae	*Latilactobacillus*	*Latilactobacillus sakei* subsp. *carnosus*	6
Lactobacillaceae	*Ligilactobacillus*	*Ligilactobacillus salivarius*	6
Methylobacteriaceae	*Methylobacterium*	*Methylobacterium fujisawaense*	7
Methylobacteriaceae	*Methylobacterium*	*Methylobacterium* spp.	4
Microbacteriaceae	*Microbacterium*	*Microbacterium maritypicum*	5
Brucellaceae	*Pseudochrobactrum*	*Pseudochrobactrum asaccharolyticum*	4
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas luteola*	15
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas viridiflava*	8
Burkholderiaceae	*Ralstonia*	*Ralstonia mannitolilytica*	16
Burkholderiaceae	*Ralstonia*	*Ralstonia pickettii*	19
Micrococcaceae	*Rothia*	*Rothia endophytica*	8
Micrococcaceae	*Rothia*	*Rothia nasimurium*	9
Sphingomonadaceae	*Sphingomonas*	*Sphingomonas faeni*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus arlettae*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus arlettaeickettii*	9
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum*	28
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum* subsp. *equorum*	19
Staphylococcaceae	*Staphylococcus*	*Staphylococcus haemolyticus*	17
Staphylococcaceae	*Staphylococcus*	*Staphylococcus chromogenes*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus lentus*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus lugdunensis*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus nepalensis*	9
Staphylococcaceae	*Staphylococcus*	*Staphylococcus xylosus*	5
Tsukamurellaceae	*Tsukamurella*	*Tsukamurella spumae*	5
Weeksellaceae	*Weeksella*	*Weeksella virosa*	4
Total			297

Figure 3 Krona chart: Isolated species of microorganisms of eggshell from aviaries on 0 day

Table 5 The number of total count of bacteria on 21 day in log CFU.eggshell^{-1}

Day	Sample	1.	2.	3.	4.	5.	6.	7.	8.
21	ECA	2.08 ±1.12	1.12 ±0.56	1.23 ±0.56	1.30 ±0.67	1.30 ±1.45	1.30 ±0.45	1.34 ±0.34	1.12 ±1.13
21	ECB	1.78 ±0.43	1.32 ±1.12	1.30 ±2.31	1.30 ±0.45	1.30 ±1.23	1.23 ±0.23	1.23 ±0.56	1.17 ±1.32
21	ECC	1.48 ±0.45	1.70 ±2.34	1.13 ±1.67	1.14 ±0.05	1.34 ±1.45	1.30 ±0.34	1.30 ±0.54	1.34 ±1.51
21	DLA	3.28 ±1.34	1.70 ±1.26	2.43 ±1.32	1.34 ±0.45	2.42 ±1.26	1.16 ±0.13	1.48 ±0.48	1.39 ±0.37
21	DLB	2.62 ±2.11	1.48 ±0.67	1.78 ±1.26	1.27 ±1.16	2.51 ±1.16	1.70 ±0.43	1.23 ±1.06	1.24 ±0.38
21	DLC	2.69 ±2.56	1.12 ±0.45	2.23 ±1.14	1.23 ±2.12	2.81 ±1.56	1.30 ±1.13	2.04 ±1.43	1.30 ±0.65
21	AA	2.28 ±2.17	1.60 ±1.17	1.32 ±2.06	1.90 ±2.14	2.72 ±0.45	2.28 ±0.34	1.12 ±0.75	1.34 ±0.34
21	AB	2.60 ±1.67	1.30 ±1.32	2.08 ±1.45	2.36 ±1.56	2.20 ±0.25	1.95 ±0.54	1.48 ±0.45	1.17 ±0.27
21	AC	1.60 ±1.07	1.48 ±1.34	1.60 ±1.05	1.48 ±1.23	2.46 ±0.43	1.70 ±0.48	1.30 ±0.34	1.14 ±0.17

EC – enriched cages, DL – deep litter, A – aviaries

In total 128 isolates were identified from of eggshell from enriched cages on 21 day (Table 6). Totally 9 family, 13 genera and 19 species were isolated from eggshell samples. The most isolated species was *Ralstonia picketii* (14%). The other most isolated bacterial species were *Staphylococcus equorum* (7%), *Agromyces rhizospherae* and *Staphylococcus sciuri* subsp. *carnaticus* (6%).

A total of 222 isolates were found in the deep litter on 21 day eggshell (Table 7). From eggshell samples, a total of 13 families, 14 genera, and 26 species were isolated. *Ralstonia picketii* accounted for 15% of the most isolated species. *Staphylococcus equorum* subsp. *equorum* (7%) and *Staphylococcus xylosus* (5%), on the other hand, were the other most isolated bacterial species.

In the aviaries on 21 day eggshell, 279 isolates in total were discovered (Table 8). A total of 13 families, 17 genera, and 26 species were separated from eggshell samples. 12%, 10%, resp. 9% of the most isolated species were *Ralstonia pickettii*, *Staphylococcus equorum* and *Staphylococcus equorum* subsp. *equorum*. The other most isolated bacterial species were, however, *Ralstonia insidiosa*, and *Staphylococcus lentus* (6%).

Table 6 Isolated family, genera and species of microorganisms of eggshell from enriched cages 21 day

Family	Genera	Species	Number of isolates
Microbacteriaceae	*Agromyces*	*Agromyces rhizospherae*	8
Bacillaceae	*Bacillus*	*Bacillus subtilis*	6
Debaryomycetaceae	*Candida*	*Candida glabrata*	5
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium glutamicum*	4
Lactobacillaceae	*Loigolactobacillus*	*Loigolactobacillus coryniformis* subsp. *torquens*	7
Lactobacillaceae	*Lactobacillus*	*Lactobacillus delbrueckii* subsp. *lactis*	7
Microbacteriaceae	*Microbacterium*	*Microbacterium testaceum*	5
Nocardiaceae	*Nocardia*	*Nocardia kruczakiae*	6
Micrococcaceae	*Paeniglutamicibacter*	*Paeniglutamicibacter kerguelensis*	7
Bacillaceae	*Priestia*	*Priestia megaterium*	4
Micrococcaceae	*Pseudarthrobacter*	*Pseudarthrobacter polychromogenes*	5
Burkholderiaceae	*Ralstonia*	*Ralstonia pickettii*	18
Staphylococcaceae	*Staphylococcus*	*Staphylococcus capitis*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus epidermidis*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum*	9
Staphylococcaceae	*Staphylococcus*	*Staphylococcus hominis* subsp. *novobiosepticus*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus lentus*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus sciuri* subsp. *carnaticus*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus xylosus*	6
Total			128

Figure 4 Krona chart: Isolated species of microorganisms of eggshell from enriched cages on 21 day

Table 7 Isolated species of microorganisms of eggshell from deep litter 21 day

Family	Genera	Speices	Number of isolates
Rhizobiaceae	*Agrobacterium*	*Agrobacterium tumefaciens*	5
Microbacteriaceae	*Agromyces*	*Agromyces hippuratus*	7
Microbacteriaceae	*Agromyces*	*Agromyces rhizospherae*	9
Bacillaceae	*Bacillus*	*Bacillus cereus*	7
Bacillaceae	*Bacillus*	*Bacillus pseudomycoides*	7
Brevibacteriaceae	*Brevibacterium*	*Brevibacterium avium*	6
Caulobacteraceae	*Brevundimonas*	*Brevundimonas nasdae*	5
Glomerellaceae	*Colletotrichum*	*Colletotrichum gloeosporioides*	8
Corynebacteriaceae	*Corynebacterium*	*Corynebacterium testudinoris*	7
Cryptococcaceae	*Cryptococcus*	*Cryptococcus neoformans*	5
Enterobacteriaceae	*Klebsiella*	*Klebsiella aerogenes*	7
Lactobacillaceae	*Lentilactobacillus*	*Lentilactobacillus parabuchneri*	9
Microbacteriaceae	*Microbacterium*	*Microbacterium liquefaciens*	7
Micrococcaceae	*Micrococcus*	*Micrococcus luteus*	8
Burkholderiaceae	*Ralstonia*	*Ralstonia pickettii*	34
Staphylococcaceae	*Staphylococcus*	*Staphylococcus arlettae*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus cohnii*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum*	9
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum* subsp. *equorum*	12
Staphylococcaceae	*Staphylococcus*	*Staphylococcus lentus*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus nepalensis*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus saprophyticus*	5
Staphylococcaceae	*Staphylococcus*	*Staphylococcus saprophyticus* subsp. *saprophyticus*	6
Staphylococcaceae	*Staphylococcus*	*Staphylococcus vitulinus*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus xylosus*	16
Streptomycetaceae	*Streptomyces*	*Streptomyces avidinii*	8
Total			222

Figure 5 Krona chart: Isolated species of microorganisms of eggshell from deep litter on 21 day

Table 8 Isolated family, genera and species of microorganisms of eggshell from aviaries 21 day

Family	Genera	Species	Number of isolates
Moraxellaceae	*Acinetobacter*	*Acinetobacter lwoffii*	7
Moraxellaceae	*Acinetobacter*	*Acinetobacter parvus*	8
Micrococcaceae	*Arthrobacter*	*Arthrobacter tecti*	6
Bacillaceae	*Bacillus*	*Bacillus cereus*	8
Saccharomycetaceae	*Kluyveromyces*	*Kluyveromyces lactis*	7
Micrococcaceae	*Kocuria*	*Kocuria rhizophila*	8
Lactobacillaceae	*Loigolactobacillus*	*Loigolactobacillus coryniformis* subsp. *torquens*	5
Lactobacillaceae	*Lactobacillus*	*Lactobacillus crispatus*	6
Lactobacillaceae	*Lactobacillus*	*Lactobacillus oligofermentans*	7
Microbacteriaceae	*Microbacterium*	*Microbacterium liquefaciens*	8
Microbacteriaceae	*Microbacterium*	*Microbacterium maritypicum*	9
Microbacteriaceae	*Microbacterium*	*Microbacterium oxydans*	9
Micrococcaceae	*Micrococcus*	*Micrococcus luteus*	7
Neisseriaceae	*Neisseria*	*Neisseria elongata* subsp. *nitroreducens*	5
Nocardiaceae	*Nocardia*	*Nocardia* spp.	6
Micrococcaceae	*Paeniglutamicibacter*	*Paeniglutamicibacter sulfureus*	6
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas poae*	7
Burkholderiaceae	*Ralstonia*	*Ralstonia insidiosa*	18
Burkholderiaceae	*Ralstonia*	*Ralstonia pickettii*	34
Micrococcaceae	*Rothia*	*Rothia endophytica*	9
Staphylococcaceae	*Staphylococcus*	*Staphylococcus epidermidis*	8
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum*	27
Staphylococcaceae	*Staphylococcus*	*Staphylococcus equorum* subsp. *equorum*	26
Staphylococcaceae	*Staphylococcus*	*Staphylococcus lentus*	18
Staphylococcaceae	*Staphylococcus*	*Staphylococcus succinus* subsp. *succinus*	7
Staphylococcaceae	*Staphylococcus*	*Staphylococcus xylosus*	8
Corynebacteriaceae	*Turicella*	*Turicella otitidis*	5
Total			279

Figure 6 Krona chart: Isolated species of microorganisms of eggshell from aviaries on 21 day

3.3 Discussion

The shell protects the egg from internal contamination and mechanical damage in the commercial egg industry. The value of the egg as a food product is compromised if the shell fails for any reason. The financial implications of shell failure are considerable, so egg producers need to be mindful of these problems. All the nutrients have already been invested by the time the shell is formed, so the farmer may suffer a total loss if the nutritional value is lost (Hunton, 2005). The functional quality of the eggshell is influenced by many elements, especially those that occur before the egg is placed. These elements include the load, age and nutrition of the bird, stress, disease, and housing method. As mentioned above, the housing system has a significant influence on the quality of the eggshell. However, the results of the effect of housing systems on eggshell quality remain unclear. A number of factors can be used to determine eggshell quality, including eggshell weight, specific gravity, proportion, thickness, deformation and strength. Lower eggshell strength, which leads to eggshell cracking, is associated with significant financial losses for egg farmers. According to Mertens et al. (2006), eggs reared in aviaries had the strongest shells, while eggs reared outdoors were the weakest. The interaction of housing system, age, genotype, oviposition time and mineral nutrition is associated with inconsistent results, which can be explained by differences in eggshell structure (Ketta & Tůmová, 2016). The aim of our study was evaluated microbiological contamination of eggshell from different laying hen rearing systems. The eggshell and its cuticle, which offer pathogen protection as well as a strong container for the egg's contents, are the first line of defense against adhering and penetrating microorganisms (Rose-Martel et al., 2012; Rose-Martel & Hincke, 2017). Through respiratory apertures that puncture the eggshell, microbial pathogens have the potential to reach the egg (Watson & De Meester, 2015). However, a proteinaceous cuticle that extends into pores as small as 50 µm often covers the outside apertures of the respiratory pores, preventing bacterial pathogens from entering (Sparks & Board, 1985). In order to protect food against viruses like *Salmonella* and *Escherichia coli*, an intact cuticle a physical barrier covering the eggshell is essential (Bain et al., 2013; Gole et al., 2014; Muñoz et al., 2015). In our study was evaluated the total count of bacteria, coliform bacteria and *Salmonella* spp. Count. From the all group of microorganisms was only total count of bacteria identified. The most abundance species in all three laying hen rearing systems were *Staphylococcus equorum* and *Ralstonia pickettii*. The contents of the egg can be contaminated by microorganisms on the egg's surface. According to the findings of a study conducted by De Reu et al. (2006), *Salmonella enteritidis* was the pathogen that penetrated the eggshell the least frequently, followed by *Pseudomonas* spp. And *Alcaligenes* spp. 60, 58, and 43% of the agar-filled egg penetration was attributed to these bacteria, respectively. Higher eggshell contamination increased the likelihood of microbe penetration and contaminated egg content, as demonstrated by studies by (De Reu et al. (2006) and Messens et al. (2007). This finding may be connected to higher egg contamination in alternate housing systems. Among all the microorganism species under observation, free-range eggs had a noticeably higher level of eggshell contamination than cage eggs. Our findings are consistent with a study conducted by Bełkot & Gondek (2014)litter, free ranging, ecological, whose source was from the Lublin voivodeship and obtained during the spring and summer period (April–July, who examined the microbial contamination of eggs from four distinct housing systems and found that the cage system had fewer aerobic bacteria than the litter, free range, and organic systems. According to Vučemilo et al. (2010), the cage is the best system in terms of cleanliness. Cleanliness is most likely connected to a higher level of microbial contamination of eggs (Singh et al., 2009). The current study's findings indicate that storage duration has an impact on the microbiological contamination of eggshells. According to De Reu et al. (2005), the total count of aerobic bacteria and the total count of Gram-negative bacteria significantly decreased within 14 days of storage time (from 4.04 to 3.23 log CFU. eggshell^{-1}). This is consistent with the observation that the number total count of bacteria decreased with storage time. In our trial, the housing system had the biggest impact on the total count of bacteria's penetration. Higher microbial contamination of the eggshells is thought to have an impact on a higher microbial penetration in the free-range eggs, which is consistent with the findings of Messens et al. (2007). Similarly, compared to eggs placed in an enriched cage (1.9%), eggs from an alternate housing system had a higher penetration into the egg content (2.3%), according to Reu et al. (2007). In our experiment the penetration of total count of bacteria was mainly affected by the housing system. A higher microbial penetration in the eggs from free range is assumed to be affected by higher microbial contamination of the eggshells, and this assumption corresponds with Messens et al. (2007). Likewise, Reu et al. (2007) detected a higher penetration into the egg content in eggs from an alternative housing system (2.3%) compared to eggs laid in an enriched cage (1.9%). The effect of storage time on the penetration of different bacterial species was investigated by De Reu et al. (2006). The authors discovered that the most common time for eggshell penetration to be seen was between four and five days, regardless of the strain that was used. Total eggshell penetration reached up to 80% and more than 95% on days 6 and 14, respectively. Microorganism penetration can be influenced by a variety of parameters, including pore density and eggshell quality. De Reu et al. (2006), however, could not find any connection between bacterial eggshell penetration and the area, thickness, or number of holes in the eggshell. These contradicting findings lead one to believe that the type of bacteria and their activity may potentially have an impact on penetration. For example, the study by De Reu et al. (2006) suggested that certain types of microorganisms probably penetrate more easily than others. *Salmonella enteritidis*, *Pseudomonas* spp., and *Alcaligenes* spp. Penetrated more frequently than *Staphylococcus*, *Acinetobacter*, *Serratia*, and *Carnobacterium*.

4 Conclusions

As a result of our experiments, in conclusion that total bacterial counts were lower in enriched cages compared to the other laying hen housing methods. Total bacterial counts were determined at the beginning of the experiment compared to eggs stored on day 21. A final evaluation of all experiments revealed that the same bacteria were present in all storage systems. The species abundance of microorganisms at day 21. was lower compared to the initial identification. A total of 1,523 isolates were identified from all tested samples of eggs with high score of identification. Further research on the impact of housing systems on shell contamination is needed to improve food safety, layer's health, and create a healthier working environment in alternative poultry production facilities.

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