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The influence of different methods of decontamination of microbial biofilms formed on eggshells

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ABSTRACT

According to "food legislation" requirements, all eggs entering the production of egg products must be disinfected. Therefore, developing technologies for decontaminating chicken eggs before use for food purposes is a promising work direction in chicken egg production and storage. This research aimed to identify the microbiota of chicken eggs with varying degrees of shell contamination and determine the influence of different methods of decontaminating microbial biofilms formed on eggshells. It was set up that the quantitative content of microorganisms on the surface of chicken eggs ranged from 10^3 CFU to 10^6 CFU/ml of washing and depended on the contamination of the shell with droppings. *Lactobacillus* spp., *Bacillus* spp., *Corynebacterium*, *Staphylococcus* were among the genera of bacteria that prevailed on the clean chicken shell, which were isolated in 30-50% of cases, and gram-negative microbiota was practically absent. The constant release of gram-positive bacteria is noted on the contaminated eggshell, and the frequency of identification of gram-negative microbiota of the *Enterobacteriaceae* genus and non-fermenting genera *Pseudomonas* and *Psychrobacter* increases. That is, the microbial scape of the microbiota of the chicken shell depends on its cleanliness, and the presence of a dirty surface increases the frequency of allocation of the resident microflora of the gastrointestinal tract. It was found that the working solution of the disinfectant Vircon S destroyed planktonic bacteria applied to the eggshell in an average of 2 minutes of exposure, stabilised water ozone for 1 minute, gaseous ozone for 3 minutes, and the action of ultraviolet rays with a length of 253.7 nm for 25-30 min. At the same time, using these disinfection methods on bacteria formed in a biofilm on the eggshell did not cause a bactericidal action during this time. To significantly reduce bacteria in the biofilm using these methods, it is necessary to increase the exposure time of the biocide by 2-3 times. Therefore, the complex structure of the eggshell and the multi-layered matrix of biofilms provide better protection for bacteria against the influence of the investigated disinfection methods.

Keywords: chicken egg, disinfection, biofilm, eggshell microbiota, planktonic bacteria

INTRODUCTION

In the world, the issue of long-term storage of edible eggs and egg products with guaranteed quality and safety characteristics is relevant. After all, the safety of manufactured egg products directly depends on the safety of eggs **[1]**, **[2]**, **[3]**. If technological storage modes have long been included in world standards, then the issue of sanitary tilling of eggs before processing remains debatable **[4]**, **[5]**.

A significant source of bacterial insemination of edible eggs in the process of getting is considered to be the low sanitary condition of premises, equipment **[6]**, apparatus **[7]**, inventory workers' overalls **[8]**, **[9]**, **[10]** etc. In

particular, the researchers found that the microbial contamination of chicken eggs ranges from 10^2 CFU to 10^6 CFU, which depends on the contamination of the surface of the shell with droppings **[4]**.

Egg products (melange, egg powder) are widely used in the food industry and public food. Egg products are made from edible chicken eggs using a technology that involves washing and disinfecting them in various ways **[8]**. Using molecular genetic methods, researchers isolated a variety of microflora from the surface of clean and contaminated chicken eggs, including genera *Lactobacillus*, *Staphylococcus*, *Psychrobacter*, *Pseudomonas*, *Salinicoccus*, *Clostridium*, *Bacteroides*, *Micobacterium*, *Aerococcus*, and others **[4]**, **[11]**, **[12]**. In addition, it is reported that the composition of the surface microbiota of eggs and the frequency of their release depends on surface contamination and sanitary conditions of getting and storing eggs **[13]**, **[14]**, **[15]**, **[16]**. Therefore, to reduce the contamination of egg products with microflora, disinfection of eggs with various biocidal preparations (based on formalin, quaternary ammonium compounds, polyhexabioguanidine, calcium hypochlorite, glutaraldehyde, hydrogen peroxide, chlorine-containing, etc.) is used **[17]**, **[18]**, **[19]**, **[20]**, **[21]**. The disadvantages of the significant use of disinfectants include the formation and spread of resistant microorganisms and the possible accumulation of biocide residues in products **[4]**, **[17]**, **[22]**, **[23]**. Researchers indicate that one of the mechanisms of survival of microbiota on the surface of the shell of chicken eggs is the presence of pores and channels through which bacteria quickly penetrate the middle of the membranes, especially if they are contaminated with faeces **[24]**. This prevents contact of biocidal preparations with bacterial cells. In addition, a contributing factor to the resistance of bacteria in the middle of shell membranes is their ability to form biofilms **[25]**, which further protect cells from disinfectants.

In addition to chemical means for decontaminating chicken eggs, researchers report other methods of reducing egg microbial contamination, in particular, ultrasonic washing [26], gaseous ozone [28], ultraviolet radiation [27], and activated plasma **[29]**, **[30]**. However, these methods have not yet been found to be of sufficient industrial use.

Despite the considerable arsenal of methods of decontaminating chicken eggs, manufactured egg products always contain microflora **[4]**, **[5]**. Therefore, the rapid reproduction of residual microflora begins during the thawing of the melange. Therefore, the quality of the sanitary processing of eggs and compliance with the sanitary and hygienic regime significantly influence the level of seeding of egg products with microorganisms. Considering the above, a promising direction in the technology of production of egg products with minimal microbial contamination is the development and use of simple, cheap, ecological and effective methods of disinfection of eggs.

The work aimed to identify the microbiota of chicken eggs at different levels of shell contamination and investigate the influence of four methods of disinfection of microbial biofilms formed on the eggshell.

Scientific Hypothesis

The use of Vircon S disinfectant, gaseous ozone, stabilized water ozone, and ultraviolet radiation will significantly reduce the microbial load in biofilms on eggshells, as compared to untreated eggshells, thereby enhancing the safety and shelf-life of egg products.

MATERIAL AND METHODOLOGY

Selection of chicken eggs was carried out at the Ternopil Poultry Factory (Ternopil, Ukraine), and microbiological studies were carried out in the laboratories of the Podillia State University (Kamianets-Podilskyi, Ukraine).

Samples

The microflora of 33 chicken eggs, which were clean (shell without visible signs of mechanical contamination), was investigated; 33 eggs – conditionally clean (traces of mechanical contamination on the shell); 33 – contaminated (the surface of the shell contained visible traces of contamination up to 30% of the entire area).

Chemicals

Virkon S disinfectant (Lanxess, Cologne, Germany) was used in the experiment; nutrient media: meat peptone agar, meat peptone broth, Saburo, Endo, Enterococcus-agar, Kesler (Pharmaktiv, Ukraine), MRS-agar, Bile Esculin Azide agar, *Streptococcus Selective agar* (HiMedia, India); Baird-Parker agar, cetramide agar (Merck KGaA, Germany).

Animals and Biological Material

The bacterial strains *S. aureus* ATCC 25923, *P. aeruginosa* 27/99, *E. coli* 055K59 No. 3912/41, *and Enterococcus faecalis ATCC 19433 were obtained from the State Scientific and Control Institute of Biotechnology and Strains of Microorganisms* (Kyiv, Ukraine). The bacterial culture was obtained by reviving the lyophilisates in the liquid nutrient broth after 24-48 h of incubation at 37 °C.

Instruments

Gaseous ozone produced by an (ATWFS, China), ozone generator, stabilized water ozone (Baerain, China), and a 253.7 nm ultraviolet lamp (laminar cabinet model AC2-4E8, ESCO, Singapore), were used in the experiment. Multiskan FC microbiological spectrophotometer (Thermo Scientifis, Finland). Collection of washings with the help of a sterile disposable cotton pad, soaked in a peptone-saline solution, the egg's surface was wiped, to which a 2×2 cm stencil was applied.

Laboratory Methods

Determination of the number of aerobic and facultatively anaerobic microorganisms

To determine the number of microorganisms, the selected washes in the amount of 1ml and their tenfold dilutions were sown in the meat peptone agar medium, incubated at a temperature of +30 °C for 72 hours, the number of colonies was counted and the average amount in 1 ml of washings was determined.

To determine the generic and species composition of the microbiota of chicken eggs, the selected washings were sown on selective media for a certain type of bacteria, and the isolated colonies were identified using commercial API test systems (bioMerieux, France).

Determination of the effect of biocides on planktonic bacteria

To determine the influence of the investigated biocides on planktonic bacteria applied to the eggshell, a suspension of test strains of microorganisms was prepared in the amount of 10^7 CFU/ml, applied 0.1 ml per 1 cm^2 of the shell surface, distributed evenly over the entire surface, processed with biocides, kept for a certain time and washings were collected and inoculated into storage medium meat peptone broth with glucose (Pharmaktiv, Ukraine) (Figure 1).

Figure 1 Study of the effect of biocides on planktonic bacteria on eggshells.

Determining the number of bacteria in biofilms on eggshells after exposure to biocides

It was carried out on daily microbial biofilms grown on chicken eggshells in Petri dishes. To do this, 1 ml of a suspension of test bacteria in peptone water with glucose was applied to 1 cm^2 of the area of the chicken shell, kept in a thermostat at a temperature of + 37 °C for 24 hours, after which the unattached bacteria were washed off with a sterile phosphate buffer, the shell was dried for 15-20 min and processed with biocides, kept for a specific time, the biocide was washed off with phosphate buffer and the washings was collected, which was sown on elective media to count the surviving bacteria.

Determination of the density of the formed biofilms on the eggshell after the influence of biocides

1 ml of a suspension of test bacteria in peptone water with glucose was applied to 1 cm^2 of the area of the chicken shell and kept in a thermostat at a temperature of + 37 °C for 24 hours after that, the unattached bacteria were washed off with a sterile phosphate buffer, the shell was dried for 15-20 minutes and processed with biocides, kept for a certain time, washed off the biocide with phosphate buffer, dried the shell, processed with ethyl alcohol for 10 min, dried, stained with crystal violet, then the shell was placed in the well of the tablet, ethyl alcohol was added, crystal violet was washed off, and the density of the solution was measured on a Multiskan FC (Thermo Scientific, Finland) multichannel microbiological spectrophotometer at a wavelength of 584 nm **[31]**. The results were interpreted as the average arithmetic value of the optical density of 3 experimental wells (Figure 2).

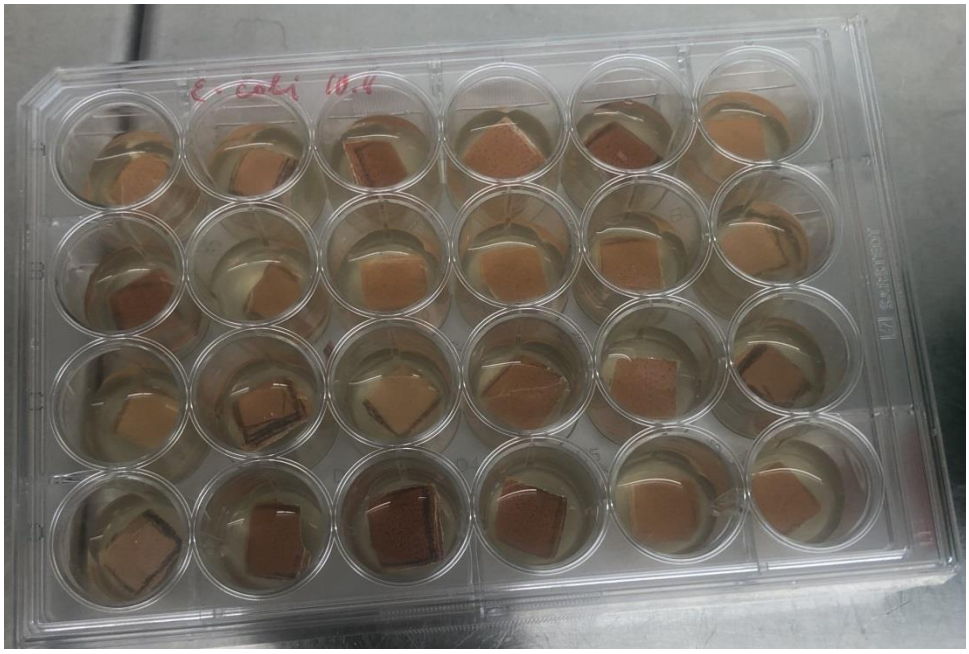

Figure 2 Study of the density of the formed biofilms on the eggshell.

Description of the Experiment

Sample preparation: Freshly laid chicken eggs were collected from poultry houses with caged chickens the eggs were visually evaluated for contamination, placed in a plastic container, and delivered to the microbiological laboratory for research within 2-4 hours at a temperature of +4 to +6 °C.

Number of samples analyzed: We analyzed 99 samples.

Number of repeated analyses: All measurements of instrument readings were performed three times.

Number of experiment replication: The number of repetitions of each experiment to determine one value was three times.

Design of the experiment: To determine the quantitative content of mesophilic microorganisms on the surface of the shell of chicken eggs, depending on their contamination; to determine the generic composition of the microbiota of chicken eggs depending on their contamination; to investigate the time of the bactericidal action of the Vircon S disinfectant, gaseous and stabilized water ozone, and ultraviolet radiation on planktonic and bioplic strains of bacteria on eggshells.

Statistical Analysis

Statistical processing of the results was carried out using methods of variation statistics using Statistica 9.0 (StatSoft Inc., USA). Non-parametric methods of research were used (Wilcoxon-Mann-Whitney test). The arithmetic mean (x) and the mean (SE) standard error were determined. The difference between the comparable values was considered significant for $p < 0.05$.

RESULTS AND DISCUSSION

During the investigation of the quantitative contamination of the surface of the shell of chicken eggs, we conditionally divided the eggs into three groups: the first – clean (shell without visible signs of mechanical contamination); the second - conditionally clean (traces of mechanical contamination on the shell); the third is contaminated (the surface of the shell contained clearly visible traces of contamination up to 30% of the entire area). Quantitative determination of the mesophilic microbiota of the shell of chicken eggs at different degrees of contamination revealed (Table 1) the naturally lowest content of microorganisms on the surface of the shell of the first group. In particular, the number of bacteria on the shell of these eggs was $7.3 \pm 0.3 \times 10^3$ CFU/cm^3 of washings. In the presence of minor contamination of the shell surface (the second group), an increase of one order of magnitude in the microbial number of the mesophilic microflora is noted compared to the first group.

Table 1 Microbial contamination of the surface of chicken eggs with different purity, x ±SE.

Conditional groups of egg purity	The number of examined eggs, n	The number of aerobic and facultative anaerobic microorganisms, CFU/cm^3 of washings
The first	7	$7.3 \pm 0.3 \times 10^3$
The second	7	$8.6 \pm 0.2 \times 10^{4*}$
The third	7	$4.7 \pm 0.2 \times 10^{6*}$

Note: * – $p < 0.05$ to the number in the first group.

In the third group, the eggshells were dirty, respectively, in the washings from the surface, the largest number of mesophilic bacteria was found – $4.7 \pm 0.2 \times 10^6$ CFU/cm^3, which is approximately two orders of magnitude higher than in the shells of the second group and three orders of magnitude higher, comparing with the first group.

Therefore, to reduce the microbiota in egg products, it is necessary to use eggs with the least contaminated shell and to use various safe methods to neutralize the microbiota on the surface.

The cleanliness of the surface of the egg shell, which is subjected to various methods of technological processing (washing, disinfection) before use, significantly influences the formation of the microbiota of egg products. The actual investigation of microbiota of the surface of chicken eggs is a necessary condition in developing a technology to disinfect them for the production of safe egg products. During the investigation of various methods of disinfection of chicken eggs, at the first stage, the microflora of the eggshell was investigated (Figure 3).

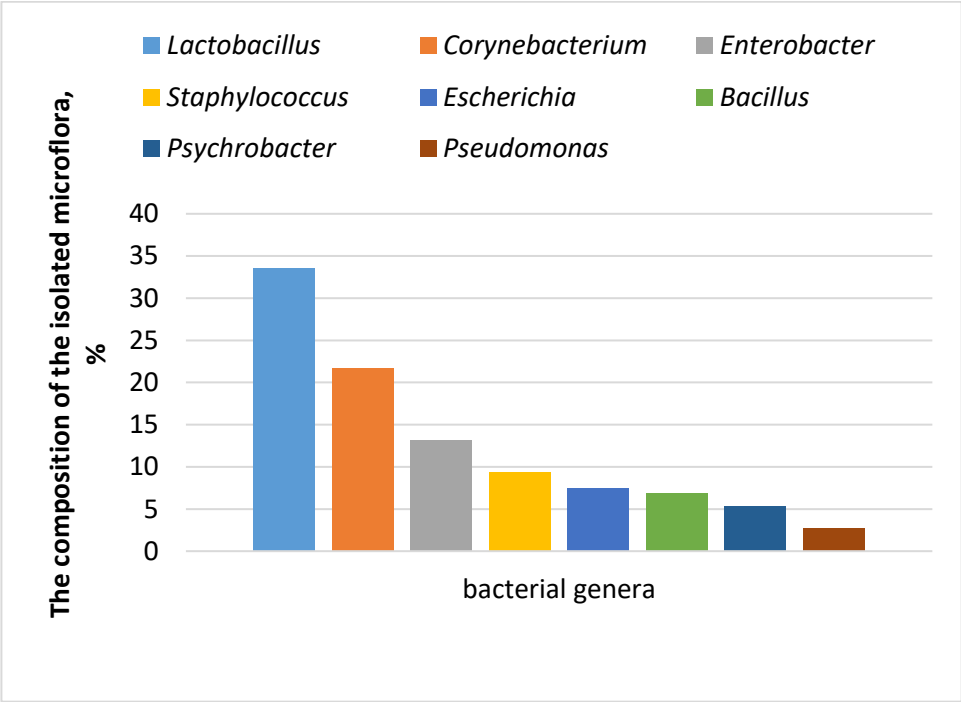

Figure 3 Genus composition of the microbiota identified on the shell of chicken eggs.

It was found that the microbiota of eggs is represented by genera of bacteria that usually belong to the resident microflora of the gastrointestinal tract of poultry. In particular, the largest share was made up of bacteria of the genus *Lactobacillus* spp. – 33.5 ±0.8% among the isolated microorganisms. In the second place were representatives of the genus *Corynebacterium* spp., which accounted for 21.7 ±0.5% of the studied bacteria. Ubiquitous bacteria of the genus *Staphylococcus* spp. in this biotope was 9.4 ±0.3% among the investigated aerobic and facultatively anaerobic bacteria, and aerobic spore-forming bacilli were 6.8 ±0.2%.

The representatives of the *Enterobacteriaceae* genus (*Enterobacter* spp. and *Escherichia* spp.) comprised 20.8 ±0.5% of the microbial population of the eggshell surface. At the same time, the share of *Escherichia* spp. was 1.8 times less than *Enterobacter* spp. Non-fermenting aerobic representatives of the genera *Psychrobacter* spp. and *Pseudomonas* spp. Occupied the smallest niche among the representatives of the eggshell surface microbiota – 5.3 ±0.2 and 2.7 ±0.1%, respectively.

The investigations on the frequency of isolation of different genera of bacteria from the surface of the eggshell were of interest since not all detected genera of bacteria are of equal importance, both from the point of view of

safety and compliance with sanitation and from the point of view of the presence of technically harmful microbiota that causes defects in egg products.

The frequency of bacterial release from the shell of chicken eggs at different degrees of contamination (Figure 4) revealed a significantly more diverse microbial population on the surface of a dirty shell than on a clean one. In addition, from the shells of clean eggs, much less bacteria were identified than those present on dirtier surfaces. In particular, bacteria of the genus *Lactobacillus* spp. and *Bacillus* spp. were revealed from clean surfaces in an average of 50% of cases. At the same time, the frequency of allocation of these genera of bacteria from the conditionally clean shell was increased by approximately 10 to 63.6%. These genera were identified from dirty shells in 100% of cases.

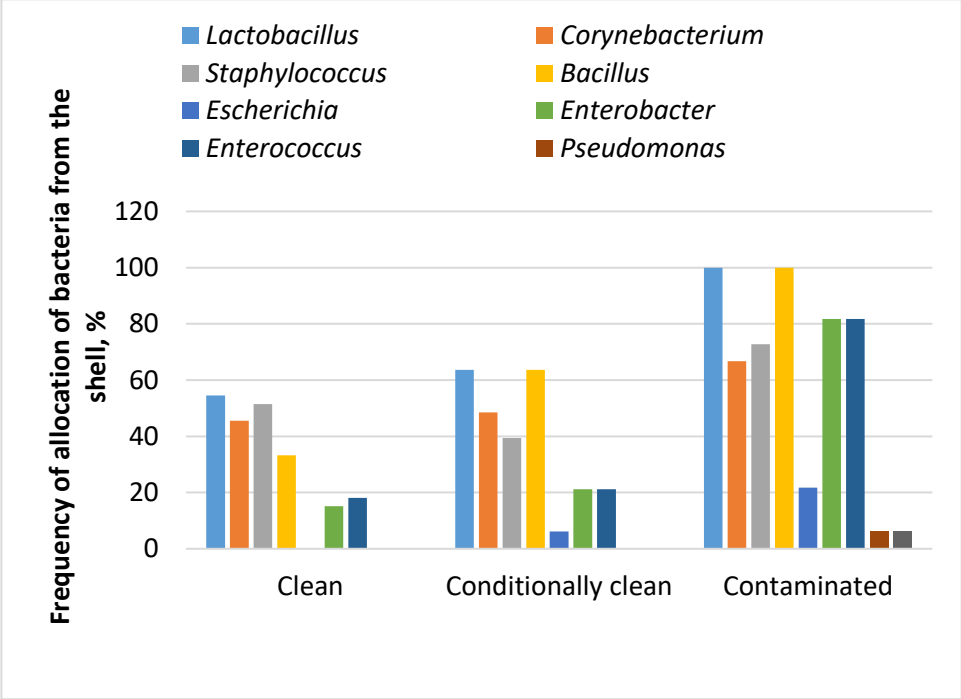

Figure 4 Identification of bacteria from the shell of chicken eggs at different degrees of contamination, n = 33.

The frequency of allocation of bacteria of the genus *Corynebacterium* from clean and conditionally clean shells was approximately the same and amounted to 45.5 and 48.5%, respectively. On the contaminated eggshell, the allocation frequency of corynebacteria was increased by 1.5 and 1.4 times, respectively. A similar tendency about the frequency of allocation was observed in bacteria of the genus *Staphylococcus* spp., which were released from the shell of eggs of the first and second groups in 33.3 and 39.4% of cases and from the surface of eggs of the third group in 2.2 and 1.8 times, respectively more often.

The frequency of identification of bacteria of the genus *Escherichia* depended on the cleanliness of the eggshell since they were not isolated from the surface of the first group of eggs, from the second group only in 6.1% of cases, and from the third (dirty) group the most – in 21.7% of cases. The frequency of identification of *Enterobacter* spp. and *Enterococcus* spp. from pure eggshells was insignificant and amounted to 15.1-18.1%, respectively, and from conditionally clean eggshells, a slight increase was noted to 21.2%. At the same time, the frequency of isolation of these types of bacteria from the dirty surface of the eggs was increased by an average of 4 times compared to the conditionally clean shell.

Gram-negative non-fermenting bacteria of the genera *Pseudomonas* and *Psychrobacter* were not identified on clean and conditionally clean shells, and the frequency of isolation from the dirty egg surface was 6.1% for the two genera.

Therefore, the microbial landscape of the chicken shell's microbiota depended on its cleanliness. In the presence of a dirty surface, the frequency of isolation of different genera of bacteria increases.

At the next research stage, the influence of disinfecting the shell of chicken eggs with Vircon S disinfectant, two forms of ozone: gaseous and stabilised water and ultraviolet rays, was determined. First, the time of the bactericidal action of selected biocides and ultraviolet rays at the concentration recommended by the instructions for use was investigated on planktonic forms of strains of bacteria and then on biofilms formed on the shell of chicken eggs.

It was set up (Table 2) that for the destruction of test strains of bacteria applied to the shell of chicken eggs in the amount of 10^6 CFU/cm^2 of the area, the Vircon S disinfectant in a working concentration of 1% must act for

an average of 2 minutes. The bactericidal action of stabilized aqueous ozone at a concentration of 0.0023% on these strains of bacteria on the chicken shell appeared after 1 minute of washing. Gaseous ozone at a concentration of 60 mg/h had a bactericidal action on plankton cultures of strains after three minutes of the influence. The longest time was required to achieve a bactericidal effect on planktonic bacteria using ultraviolet irradiation, an average of 30 min.

Table 2 The influence of the investigated biocides on planktonic bacteria applied to the eggshell, x ±SE.

Bacteria	The number of bacteria (CFU) in 1 cm^3 of washing from the surface before processing with a biocide	Disinfectant exposure time for which the bactericidal action was appeared, min			
		Vircon S (1% solution)	SAO (0.0023% conc. ozone)	gaseous ozone (60 mg/h)	ultraviolet radiation, 253.7 nm
S. aureus	$3.7 \pm 0.1 \times 10^6$	2 min	1 min	3 min	30 min
E. coli	$2.5 \pm 0.1 \times 10^6$	1 min	1 min	3 min	25 min
P. aeruginosa	$3.3 \pm 0.1 \times 10^6$	2 min	1 min	3 min	30 min
E. faecalis	$4.1 \pm 0.2 \times 10^6$	2 min	1 min	3 min	30 min

Therefore, the received results regarding the exposure time of disinfectants during which the bactericidal action on planktonic bacteria on the eggshell was used to investigate the influence of disinfection modes on biofilm forms of bacteria.

Since the relief of the eggshell is heterogeneous, it was essential to compare the time of bactericidal action of disinfectants on planktonic forms with biofilms formed on the surface of the shell.

Table 3 The influence of disinfectant Vircon S (1%) on bacteria in biofilms formed on eggshells, x ±SE.

Bacteria	The number of bacteria (CFU) in 1 cm^3 of washing from the surface before processing with a biocide	The number of bacteria (CFU) in the biofilm after the action of the biocide during, min			
		1	2	3	4
S. aureus	$4.7 \pm 0.2 \times 10^8$	$5.6 \pm 0.2 \times 10^4$	$8.4 \pm 0.3 \times 10^2$	$2.8 \pm 0.1 \times 10^1$	4.2 ± 0.2
E. coli	$5.8 \pm 0.2 \times 10^8$	$3.7 \pm 0.2 \times 10^3$	$2.6 \pm 0.1 \times 10^2$	$1.4 \pm 0.1 \times 10^1$	–
P. aeruginosa	$6.5 \pm 0.2 \times 10^8$	$4.1 \pm 0.2 \times 10^3$	$5.1 \pm 0.2 \times 10^2$	$1.7 \pm 0.1 \times 10^1$	1.8 ± 0.1
E. faecalis	$8.4 \pm 0.3 \times 10^8$	$5.1 \pm 0.2 \times 10^3$	$3.7 \pm 0.1 \times 10^2$	$2.0 \pm 0.1 \times 10^1$	2.1 ± 0.1

It was found (Table 3) that the disinfectant Vircon S in the concentration according to the instructions for use within one minute of the influence reduced the number of cells in the biofilm formed by *S. aureus* by four orders of magnitude. In the biofilms formed by *E. coli, P. aeruginosa* and *E. faecalis* the number of viable cells was reduced by five orders of magnitude, compared to the content before processing. During the next minute of action of the disinfectant, the bactericidal effect on the bacterial cells in the biofilm was less pronounced, as their number was decreased by only one order of magnitude. Three minutes later, after exposure to Vircon S on eggshells with microbial biofilms, a decrease in the number of bacteria by one order of magnitude was noted, compared to the two-minute action of the biocide. At the same time, during the five-minute exposure to the disinfectant, no live cells were detected from the biofilms formed by *E. coli,* and from the biofilms of *P. aeruginosa* and *E. faecalis,* the number of bacteria was, on average, 2.0 ±0.1 CFU/cm^3 of washing. The most cells were isolated after 5 minutes of Vircon S action from the staphylococcal biofilm – 4.2 ±0.2 CFU. Therefore, the bactericidal action of the Vircon S disinfectant against bacteria in biofilms on the eggshell is approximately 5 minutes, against 1 – 2 minutes for planktonic forms. In addition, single cells were released from the biofilms formed by *S. aureus, P. aeruginosa,* and *E. faecalis* even within 5 minutes of the biocide action.

The investigation of the anti-biofilm action of stabilised aqueous ozone is shown in Table 4.

Table 4 The influence of stabilized aqueous ozone (0.0023%) on bacteria in biofilms formed on eggshell, x ±SE.

Bacteria	The number of bacteria (CFU) in 1 cm^3 of washing from the surface before processing with a biocide	The number of bacteria (CFU) in the biofilm after the action of the biocide during, min			
		1	2	3	4
S. aureus	$5.1 \pm 0.2 \times 10^8$	$6.2 \pm 0.2 \times 10^3$	$8.4 \pm 0.3 \times 10^2$	$6.3 \pm 0.2 \times 10^1$	8.7 ± 0.3
E. coli	$5.9 \pm 0.2 \times 10^8$	$4.1 \pm 0.1 \times 10^3$	$5.4 \pm 0.2 \times 10^2$	$3.8 \pm 0.1 \times 10^1$	–
P. aeruginosa	$6.4 \pm 0.2 \times 10^8$	$5.4 \pm 0.2 \times 10^3$	$7.3 \pm 0.2 \times 10^2$	$5.4 \pm 0.2 \times 10^1$	–
E. faecalis	$8.2 \pm 0.3 \times 10^8$	$4.3 \pm 0.2 \times 10^3$	$7.7 \pm 0.3 \times 10^2$	$5.9 \pm 0.2 \times 10^1$	–

Stabilised water ozone in the concentration produced by the ozonator penetrated well into the pores of the eggshell and the matrix of the biofilm since within one minute of exposure, the number of viable cells was decreased by five orders of magnitude to 10^3 CFU/cm^3 of washing. During the next two minutes of action of stabilised water ozone, the number of bacteria was decreased by two orders of magnitude, on average to $5.0 \pm 0.2 \times 10^1$ CFU/cm^3 washed from the shell surface. At the same time, five minutes after the beginning of the processing of the eggshell with ozone, viable bacteria were isolated only from the biofilm formed by *S. aureus* in the amount of 8.7 ± 0.3 CFU/cm^3 of washing.

Thus, the bactericidal action of stabilised aqueous ozone at a concentration of 0.0023% on the biofilm forms of bacteria formed on the eggshell mainly appeared within 5 minutes of exposure. At the same time, bacterial cells in staphylococcal biofilms were more protected since even after 5 min of exposure, single viable bacteria were isolated.

Along with stabilised water ozone, the bactericidal action on biofilm forms of gaseous was determined (Table 5).

Table 5 The influence of gaseous ozone (60 mg/h) on bacteria in biofilms formed on eggshells, x ±SE.

Bacteria	The number of bacteria (CFU) in 1 cm^3 of washing from the surface before processing with a biocide	The number of bacteria (CFU) in the biofilm after the action of the biocide during, min			
		1	2	3	4
S. aureus	$5.0 \pm 0.2 \times 10^8$	$7.8 \pm 0.3 \times 10^5$	$8.4 \pm 0.3 \times 10^3$	$9.2 \pm 0.3 \times 10^1$	9.7 ± 0.3
E. coli	$6.0 \pm 0.2 \times 10^8$	$3.4 \pm 0.2 \times 10^5$	$3.6 \pm 0.2 \times 10^3$	$7.7 \pm 0.2 \times 10^1$	6.9 ± 0.2
P. aeruginosa	$6.4 \pm 0.2 \times 10^8$	$4.7 \pm 0.2 \times 10^5$	$4.1 \pm 0.2 \times 10^3$	$8.5 \pm 0.3 \times 10^1$	7.3 ± 0.3
E. faecalis	$7.9 \pm 0.3 \times 10^8$	$5.9 \pm 0.3 \times 10^5$	$6.5 \pm 0.3 \times 10^3$	$7.1 \pm 0.3 \times 10^1$	7.1 ± 0.3

It was set up that the bactericidal action of gaseous ozone was weaker on microbial biofilms formed on the eggshell than the influence of stabilized aqueous ozone. In particular, during one minute of exposure to gaseous ozone at a concentration of 60 mg/h, the number of bacteria in biofilms was decreased by approximately three orders of magnitude to 10^5 CFU/cm^3 of washing. Increasing the ozone exposure time to two minutes led to an increase in the bactericidal effect, since two orders of magnitude decreased the number of viable cells in the biofilm and was, on average, 10^3 CFU/cm^3 of washing from the shell. Within three minutes of biofilm processing with gaseous ozone, microbial cells were destroyed significantly, as the number of living bacteria was $8.1 \pm 0.3 \times 10^1$ CFU/cm^3 of washing.

It should be noted that when gaseous ozone acts on the planktonic forms of bacteria on the eggshell, they are destroyed after three minutes. After 5 min of exposure to gaseous ozone on the biofilm forms on the eggshell, up to 10 living cells were isolated from all biofilms. It was also noted that the largest number of viable cells after a three-minute exposure to gaseous ozone was in biofilms formed by *S. aureus* – 9.7 ± 0.3 CFU/cm^3.

So, gaseous ozone at a concentration of 60 mg/h effectively destroyed bacterial cells in the biofilm on the eggshell within 5 minutes of exposure, as 99.99% of living bacteria die during this time.

Ultraviolet rays are widely used to disinfect the surfaces of objects in various sectors of the national economy. The research on the influence of ultraviolet rays on microbial biofilms formed on eggshells (Table 6) found that the cells in the biofilm were much more resistant to the action of irradiation compared to their planktonic forms.

Table 6 The influence of gaseous ozone (60 mg/h) on bacteria in biofilms formed on eggshells, x ±SE.

Bacteria	The number of bacteria (CFU) in 1 cm^3 of washing from the surface before processing with a biocide	The number of bacteria (CFU) in the biofilm after the action of the biocide during, min			
		30	40	50	60
S. aureus	$4.9 \pm 0.2 \times 10^8$	$4.5 \pm 0.2 \times 10^5$	$7.0 \pm 0.2 \times 10^4$	$3.1 \pm 0.2 \times 10^4$	$9.3 \pm 0.3 \times 10^3$
E. coli	$5.9 \pm 0.2 \times 10^8$	$4.7 \pm 0.2 \times 10^5$	$5.7 \pm 0.2 \times 10^4$	$1.8 \pm 0.3 \times 10^4$	$8.1 \pm 0.3 \times 10^3$
P. aeruginosa	$6.3 \pm 0.2 \times 10^8$	$5.6 \pm 0.2 \times 10^5$	$6.8 \pm 0.2 \times 10^4$	$3.4 \pm 0.2 \times 10^4$	$9.1 \pm 0.3 \times 10^3$
E. faecalis	$8.3 \pm 0.3 \times 10^8$	$7.5 \pm 0.3 \times 10^5$	$7.2 \pm 0.3 \times 10^4$	$4.3 \pm 0.3 \times 10^4$	$9.5 \pm 0.3 \times 10^3$

In particular, the action of ultraviolet rays destroyed planktonic forms after approximately 30 minutes of exposure (Table 2), and when acting on biofilms during this time, the number of bacteria, although reduced by three orders of magnitude, was still quite significant (10^5 CFU/cm^3 of washing from the shell). Continuation of the surface processing with ultraviolet rays, even for 60 minutes, did not significantly destroy bacteria in biofilms since an average of $9.0 \pm 0.3 \times 10^3$ CFU/cm^3 of washing was released from the shell.

So, the research found that surface processing with ultraviolet rays effectively kills planktonic bacteria but weakly influences biofilm forms.

Along with investigating the action of selected biocides and ultraviolet rays on the survival of bacteria in the formed biofilms on the eggshell, we determined the influence on the density (degradation) of the biofilm matrix. After all, the resistance of microbial cells to environmental factors is related to the matrix. The results of the degradation of the matrix of microbial biofilms under the influence of Vircon S disinfectant (Table 7) found that already within 1 minute after surface processing with a biocide, the density of microbial biofilms was decreased by an average of 1.5 times, compared to that before processing. However, the biofilms were still of high density and amounted to about 2.0 units. Continuation of the action of the disinfectant Vircon S for 5 minutes increased the destruction of biofilms, which became, on average, 1.8 times less dense compared to the action for 1 minute. At the same time, the density of such biofilms was, on average, 8 times higher than that of destroyed biofilms on eggshells. This indicates that Vircon S penetrates the biofilm matrix, but does not cause its destruction within 5 min of exposure, during which planktonic bacteria and almost all cells in the biofilm die. In addition, it was found that biofilms formed by *E. coli* were the least dense on the eggshell.

Table 7 The influence of disinfectant Vircon S (1%) on the density of microbial biofilms formed on eggshells, x ±SE.

Bacteria	Density of biofilms (units) before the action of the biocide	Density of biofilms (units) after the action of the biocide during, min			
		1	2	3	5
S. aureus	2.79 ±0.07	1.90 ±0.06*	1.57 ±0.05*	1.30 ±0.04*	1.05 ±0.04*
E. coli	2.71 ±0.06	1.84 ±0.06*	1.51 ±0.06*	1.22 ±0.05*	0.98 ±0.04*
P. aeruginosa	2.98 ±0.09	1.94 ±0.07*	1.65 ±0.07*	1.41 ±0.06*	1.11 ±0.05*
E. faecalis	3.21 ±0.09	2.13 ±0.08*	1.90 ±0.08*	1.63 ±0.07*	1.17 ±0.05*
Control (shell without bacteria)	–	–	–	–	0,14 ±0,02

Note: * – $p > 0.05$, before the biocide action.

The research on the process of degradation of biofilms after processing with stabilised aqueous ozone (Table 8) found a tendency that during the first minute after processing with aqueous ozone, the biofilm density was decreased by an average of 1.6 times, and during the next 5 minutes by 1.7-2.0 times. Stabilised water ozone had an influence on the degradation of microbial biofilms from eggshells, similarly to the disinfectant Vircon S. That is, even within 5 min of action, the biofilm matrix was not destroyed and it was still of significant density, especially when compared to the control.

Table 8 The influence of stabilized aqueous ozone (0.0023%) on the density of microbial biofilms formed on eggshells, x ±SE.

Bacteria	The density of biofilms (units) before the action of the biocide	Density of biofilms (units) after the action of the biocide during, min			
		1	2	3	5
S. aureus	2.74 ±0.08	1.66 ±0.06*	1.35 ±0.05*	1.15 ±0.05*	0.96 ±0.04*
E. coli	2.65 ±0.07	1.61 ±0.6*	1.30 ±0.05*	1.08 ±0.05*	0.87 ±0.04*
P. aeruginosa	2.93 ±0.09	1.77 ±0.07*	1.43 ±0.05*	1.24 ±0.05*	1.03 ±0.04*
E. faecalis	3.25 ±0.11	1.98 ±0.08*	1.67 ±0.08*	1.39 ±0.05*	1.10 ±0.05*
Control (shell without bacteria*)*	–	–	–	–	0.15 ±0.02

Note: * – p >0.05, before the biocide action.

The research on the influence of gaseous ozone on the degradation of the matrix of biofilms (Table 9) found a weaker destructive action compared to a stabilised aqueous solution.

Table 9 The influence of gaseous ozone (60 mg/h) on the density of microbial biofilms formed on eggshells, x ±SE.

Bacteria	The density of biofilms (units) before the action of the biocide	Density of biofilms (units) after the action of the biocide during, min			
		1	2	3	5
S. aureus	2.70 ±0.07	2.41 ±0.06	2.00 ±0.06*	1.89 ±0.06*	1.60 ±0.05*
E. coli	2.67 ±0.05	2.38 ±0.06	2.01 ±0.06*	1.82 ±0.06*	1.55 ±0.05*
P. aeruginosa	2.95 ±0.06	2.46 ±0.07	2.09 ±0.06*	1.86 ±0.06*	1.63 ±0.05*
E. faecalis	3.18 ±0.07	2.57 ±0.07	2.21 ±0.07*	1.98 ±0.07	1.72 ±0.06*
Control (shell without bacteria)	–	–	–	–	0.12 ±0.02

Note: * – p >0.05, before the biocide action.

In particular, probable values regarding the decrease in the density of microbial biofilms during processing with gaseous ozone were noted only after two minutes of action. During this time, the density of biofilms was decreased by an average of 1.3 times to 2.00 units. In addition, even after a five-minute influence of gaseous ozone on biofilms, their density did not decrease significantly (1.7-1.8 times), compared to biofilms before processing. This indicates that gaseous ozone, penetrating the matrix, influences the biofilm forms of bacteria but does not cause a significant destructive action.

Ultraviolet rays had an even less destructive influence on the degradation of the microbial biofilm (Table 10).

Table 10 The influence of ultraviolet radiation (253.7 nm) on the density of microbial biofilms formed on eggshells, x ±SE.

Bacteria	The density of biofilms (units) before the action of the biocide	Density of biofilms (units) after the action of the biocide during, min			
		1	2	3	5
S. aureus	2.67 ±0.07	2.60 ±0.07	2.53 ±0.06	2.3 ±0.05	1.96 ±0.05*
E. coli	2.74 ±0.07	2.68 ±0.07	2.51 ±0.06	2.27 ±0.05	1.89 ±0.05*
P. aeruginosa	2.88 ±0.08	2.77 ±0.07	2.65 ±0.06	2.37 ±0.06	2.02 ±0.04*
E. faecalis	3.20 ±0.09	3.12 ±0.08	3.03 ±0.08	2.71 ±0.07	2.35 ±0.06*
Control (shell without bacteria)	–	–	–	–	0.12 ±0.02

Note: * – p >0.05, before the biocide action.

It was found that during the time (30 min), which had a detrimental influence on the planktonic forms of the investigated bacteria, a probable decrease in the density of microbial biofilms was not observed. Probable changes in the biofilm matrix degradation were noted after 60 min of exposure to ultraviolet rays. In particular, only after 1 h of irradiation the density of microbial biofilms was decreased by an average of 1.4 times. However, all of them were still quite dense, especially when compared with the control.

Therefore, ultraviolet rays during 30 minutes of irradiation do not influence biofilms' degradation, and their action during 60 minutes does not significantly reduce their density.

The use of safe methods of reducing microbial contamination of chicken eggs is an issue that has been relevant for a long time **[32]**, **[33]**, **[34]**. It is a guarantee of getting safe egg products is the use of eggs with minimal microbial contamination **[5]**, **[18]**. Therefore, developing technologies for decontaminating chicken eggs before use for food purposes is a promising area of work in the field of chicken egg production and storage. This research aimed to identify the microbiota of chicken eggs and determine the influence of different disinfection methods of microbial biofilms formed on the eggshell. Our research found that the quantitative content of microorganisms and the biodiversity of their generic and species composition on the surface of the eggshell depends on the area of litter contamination. In particular, three orders of magnitude more microorganisms (10^6 CFU) were isolated on the shell contaminated with droppings than on the relatively clean surface of the eggs. Scientists **[16]**, **[17]**, **[35]**, **[36]**, **[37]** also found a significantly higher number of microorganisms on the shell of chicken eggs provided they were in poor sanitary conditions. Therefore, we support the opinion of researchers **[4]**, **[5]**, **[38]** about the need to introduce various methods to get an egg with the cleanest possible surface. Such methods include washing eggs with chlorinated water **[39]**, quaternary ammonium salts **[40]**, hot water, or steaming at a temperature 5-10 °C higher than the surface temperature of the egg **[41]**. Processing with ultrasound, lysozyme **[26]**, pro- and prebiotics **[42]**, bacteriocins **[43]**, hot air **[44]**, steam, and infrared radiation **[45]**, **[46]**.

Therefore, various methods of reducing microorganisms on the surface of the eggshell are actually used because, in the conditions of industrial production, the presence of a certain number of dirty eggs is almost inevitable. Therefore, special attention must be paid to the disinfection of dirty eggs.

The evaluation of the generic composition of the microbiota of the shell of chicken eggs under different contamination found an increase in the frequency of the release of bacteria of faecal origin belonging to the *Enterobacteriaceae* genus. In particular, bacteria of the genus *Escherichia* were isolated 21.7% more often from the contaminated shell, *Enterobacter* and *Enterococcus* 5.0-5.2 times, and bacteria of the genera *Pseudomonas* and *Psychrobacter* were detected in 6.1% of the samples, which were absent on a clean eggshell. Therefore, it can be stated that the microbial contamination of the eggshell in most cases is the result of contact with dirty surfaces contaminated with chicken droppings, which is consistent with other researchers' data **[4]**, **[18]**.

The most common procedures used to reduce microbial contamination of eggs in the technological process of production of egg products are washing in tap water followed by soaking in chlorinated water **[4]**, **[5]**. However, this technological operation does not wholly decontaminate the shell from microorganisms **[17]**. We investigated the influence of four methods of decontaminating eggshells from applied strains of bacteria while determining the influence on planktonic bacteria and biofilms that were formed on the shell. It was found that the working solution of the disinfectant Vircon S destroyed planktonic bacteria applied to the eggshell in an average of 2 minutes of exposure, stabilised water ozone for 1 minute, gaseous ozone for 3 minutes, and the action of ultraviolet rays with a length of 253.7 nm – for 25-30 min. At the same time, using these disinfection methods on bacteria formed in a biofilm on the eggshell did not cause a bactericidal action during this time. For a significant reduction in bacteria in the biofilm under the influence of these methods, it is necessary to increase the exposure time of the biocide by 2-3 times. However, even raising the exposure time did not destroy the bacteria in the biofilm. This indicates that the complex structure of the matrix of biofilms **[47]**, **[48]** and not simple topography of the eggshell surface **[49]** provide better protection for bacteria against the influence of these disinfection methods. Literature data indicate that food pathogens such as salmonella **[50]** and pseudomonads **[51]** can produce biofilms on eggshells in a wide range of temperatures (20-37 °C) and thereby pose a danger to consumers. In addition, it is reported **[52]** that *Pseudomonas aeruginosa* formed a dense biofilm on the shell of quail eggs, which was difficult to remove with calcium oxide. At the same time, its degradation was much more accessible from rubber and plastic. However, in addition to the ability to form a biofilm, there are other mechanisms of resistance formation by bacteria to the used biocides on the eggshell **[53]**. In particular, resistance can be acquired in microorganisms, in which certain strains of bacteria survive at biocide concentrations that suppress the bulk of existing microorganisms **[54]**, **[55]**. Scientists note **[56]** that eggs can be a factor in the spread of antibiotic-resistant strains among consumers who consume them raw or unprocessed. In particular, it was found that 73.3% of microbial isolates were isolated from chicken eggs and had multiple medicinal resistance to antibiotics used to treat intestinal infections in consumers. Therefore, we consider that the practical use of biocides for egg disinfection should consider the formation of resistance and conduct monitoring investigations on the effectiveness of such means and regimes.

Researchers from Mexico **[57]** indicated that enterotoxigenic strains of *Bacillus cereus* survived on the shells of chicken eggs sold in the market and supermarkets due to their ability to form biofilms. Therefore, we believe it is necessary to use such eggshell disinfection regimes that affect the bacteria in biofilms, especially for eggs contaminated with droppings. Reliable control of such a regime will guarantee the sale of a safe egg and the production of high-quality, shelf-stable egg products. In addition, our research found that although Vircon S biocides, stabilised water, and gaseous ozone penetrated the microbial biofilms on the shell, they did not wholly destroy the matrix. This is probably due to the multi-layered nature of biofilms, which are intertwined with the pores and channels of the eggshell membranes. Thus, according to the data **[58]**, the shell of a chicken egg contains, on average, 7,000 to 17,000 pores, and the largest of them are visible to the naked eye as small indentations on the surface of the shell. On average, per 1 cm^2 of the surface of chicken eggs, there are about 154 pores, and their total area is 2.3 mm^2. It is precisely in these pores and the formed biofilm matrix that bacteria are more protected from the action of biocides due to the inability of the latter to penetrate deeply. Also, our researchers found that ultraviolet radiation had a bactericidal influence on planktonic bacteria on the eggshell during 30 exposures. At the same time, 10^3 CFU/washing were selected from biofilms even under the influence of ultraviolet rays for 60 min. Such data are consistent with reports **[59]** that ultraviolet radiation does not penetrate the matrix of biofilms well, and only the first few upper layers of microbial cells are exposed to its harmful influence. Therefore, we believe that the action of ultraviolet rays will be effective against planktonic bacteria, and in the case of the formation of biofilms, their survival is possible. This indicates the practicality of combining different methods of disinfection of the microbiota on the eggshell.

In general, the data obtained on the determination of the influence of various methods of disinfection of microbiota on the surface of chicken eggs indicate that bacterial pathogens, which are usually present in chicken droppings, can form dense biofilms on the shell and be the cause of contamination of egg products in the case of the use of ineffective methods of decontamination. In our opinion, it is necessary to combine various methods of reducing the microbial seeding of the egg, as well as chemical disinfectants, ozone, and ultraviolet radiation. At the same time, each disinfection method needs approval in production conditions. In addition, in our opinion, treatment with stabilised water ozone, a biocide, is effective and promising in terms of practical use in production conditions, which is safe both for the edible egg and the service personnel and consumers.

CONCLUSION

The quantitative content of microorganisms on the surface of chicken eggs ranged from 10^3 CFU to 10^6 CFU/ml washing, depending on the shell's contamination with droppings. *Lactobacillus* spp., *Bacillus* spp., *Corynebacterium*, *Staphylococcus* were found among the genera of bacteria that prevailed on the clean chicken shell, which were isolated in 30-50% of cases, and gram-negative microbiota was practically absent. On the contaminated eggshell, there is an almost constant excretion of gram-positive bacteria, and the frequency of identification of gram-negative microbiota of the *Enterobacteriaceae* genus and non-fermenting genera *Pseudomonas* and *Psychrobacter* increases. That is, the microbial landscape of the microbiota of the chicken shell depends on its cleanliness, and the presence of a dirty surface increases the frequency of allocation of the resident microflora of the gastrointestinal tract. It was found that the working solution of the disinfectant Vircon S destroyed planktonic bacteria applied to the eggshell in an average of 2 minutes of exposure, stabilised water ozone for 1 minute, gaseous ozone for 3 minutes, and the action of ultraviolet rays with a length of 253.7 nm – for 25-30 min. At the same time, using these disinfection methods on bacteria formed in a biofilm on the eggshell did not cause a bactericidal action during this time. To significantly reduce bacteria in the biofilm under the influence of these methods, it is necessary to increase the exposure time of the biocide by 2 – 3 times. Therefore, the complex structure of the eggshell and the multi-layered matrix of biofilms provide better protection for bacteria against the influence of the investigated disinfection methods.

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