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Preliminary evaluation of application of a 3-dimensional network structure of siloxanes Dergall preparation on chick embryo development and microbiological status of eggshells

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ABSTRACT The spatial network structure of Dergall is based on substances nontoxic to humans and the environment which, when applied on solid surfaces, creates a coating that reduces bacterial cell adhesion. The bacteriostatic properties of siloxanes are based on a purely physical action mechanism which excludes development of drug-resistant microorganisms. The aims of the present study were to 1) evaluate a Dergall layer formed on the eggshell surface regarding the potential harmful effects on the chick embryo; 2) evaluate antimicrobial activity and estimate the prolongation time of Dergall's potential antimicrobial activity. Dergall at a concentration of 0.6% formed a layer on the eggshell surface. In vitro testing of the potential harmful effects of Dergall by means of a hen embryo test of the chorioallantoic membrane showed no irritation reaction at a concentration of 3% and lower. The hatchability of the groups sprayed with a Dergall water solution with a concentration of 0 to 5% was 89.1 to 93.8% for fertilized eggs ($P > 0.05$) but decreased to 63.7% ($P < 0.05$) in the group sprayed with a 6% concentration of the solution. This phenomenon was caused by embryo mortality in the first week of incubation. At the concentration of 0.6%, Dergall exhibited strong antibacterial properties against bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, *Shigella flexneri*, and *Salmonella typhimurium*. For *Streptococcus pyogenes*, the highest antibacterial activity of Dergall was reported in the concentrations of 100 and 50%. For *Pseudomonas aeruginosa*, no antibacterial activity of Dergall was generally observed, but in vivo testing showed a strong decrease of all gram-negative bacteria growth. Moreover, a prolonged antimicrobial effect lasting until 3 D after disinfection was observed, which makes Dergall a safe and efficient disinfectant.

Key words: silicon, antibacterial effect, HET-CAM, chicken

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INTRODUCTION

The chicken eggshell is naturally colonized by various bacterial genera, especially *Enterobacteriaceae* and gram-positive cocci such as *Staphylococcus* spp., *Streptococcus* spp., and *Micrococcus* spp. (Mayes and Takeballi, 1983; Musgrove et al., 2004) although this kind of contamination can affect the chicken embryo by breaking the eggshell barrier which may result in interrupted development.

Disinfection of hatching eggs is essential to ensure high-quality production of broilers (Olsen et al., 2017). A good disinfectant and pest control agent is characterized by high broad-spectrum efficacy and no toxicity to animals, humans, and the environment. Therefore, the health risks associated with formaldehyde, the most popular substance used in hatchery practice (Cadirci, 2009), make economical alternatives highly desirable (Keita et al., 2016). At the present, different kinds of disinfectants which could completely replace hatching egg formaldehyde fumigation are being tested, such as, for example,

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spraying hydrogen peroxide or peracetic acid (Sheldon and Brake, 1991; Melo et al., 2019), ultraviolet light-C irradiation (Coufal et al., 2003; Melo et al., 2019), fogging with electrolyzed oxidizing (Bialka et al., 2004; Fasenko, 2007; Keita et al., 2016), fumigation with ozone (Melo et al., 2019) or chlorine dioxide (Chung et al., 2018), and more.

A relatively new method of disinfection in medical and veterinary practice is using silicones and siloxanes to make antibacterial surfaces (Silva et al., 2018).

Siloxanes (silicones) constitute a group of low molecular weight compounds, organosilicon oligomers, and polymers. They are commonly considered to be either nontoxic to humans and the environment or toxic to a very limited extent (Mojsiewicz-Pieńkowska et al., 2016); therefore, they are often an integral part of innovative methods of treatment, health care, and nursing (Mojsiewicz-Pieńkowska et al. 2015, 2016; Schalau et al., 2018; Bračič et al., 2018; Kaliyathan et al., 2018). Organomodified siloxanes applied on solid surfaces create, according to the Stöber mechanism, a 3D Immobilizing Polymeric Network Structure (3D IPNS, Dergall) (Han et al., 2017). Thanks to the effectiveness of these substances, currently, most new skin care products contain at least one type of silicone (Luo et al., 2016; Kaliyathan et al., 2018). This is also due to the fact that when the silicone layer coats biomaterial (e.g., skin), it reduces bacterial cell adhesion (Mojsiewicz-Pieńkowska et al., 2015; Rauner et al., 2018; Silva et al., 2018; Sankaran et al., 2019). More importantly, the bacteriostatic properties of Dergall are based on a purely physical mechanism, which excludes development of drug-resistant microorganisms.

It is known that the increasing drug and antibiotic resistance of pathogenic microorganisms is undoubtedly the biggest question of recent microbiology. Recently, the multidrug-resistant staphylococci and gram-negative bacteria are the biggest challenges for clinicians (Chambers and DeLeo, 2010; Schwartz and Morris, 2018). This phenomenon has been reported worldwide; moreover, it impacts various disciplines, and not only the treatment of infectious diseases in humans or animals. Use of antibacterial medicines is rapidly increasing in many countries in multiple fields of social life and the economy. Those engaged in farming or consumers of animal products are under risk of infection by antibiotic-resistant microorganisms. Moreover, both human and veterinary doctors, as well as the farm staff responsible for hygiene and high sanitary conditions, are losing antibiotics as viable strong tools against pathogens (Mazinska and Hryniewicz, 2017). Therefore, it is necessary to search for new chemical compounds which exhibit antibacterial activity. It seems that siloxanes might present a new solution for disinfecting hatching eggs.

The aim of the present study was to evaluate the siloxanes layer formed on the eggshell surface for potential harmful effects on the chick embryo and to evaluate the antimicrobial activity of the Dergall substance. The second approach was to estimate the prolongation time of the potential antimicrobial activity.

MATERIALS AND METHODS

Chemicals

The water solution of a mixture of organomodified trisiloxanes (Dergall) was used as a test material (Dergall, ICB Pharma, Jaworzno, Poland; Patent No. WO 2016/061259).

The formation of the aforementioned structure was confirmed by the determination of the amount of silicon from the siloxane chain to the silicon present in the trisiloxane structure by the use of a Varian 500-MS mass spectrometer in the following conditions: positive ionization, drying gas temperature 150°C, drying gas pressure 25 psi, capillary voltage 70 V, needle voltage 5 kV.

Eggs

Broiler hatching eggs (Ross 308, Aviagen) obtained from a 40-week-old parental stock (Sławomir Domagała, Poultry Farm, Gołaczewy, Poland) were used in all presented experiments.

Microscopic Evaluation of Organomodified Trisiloxanes and a Sol-Gel Precursor Layer on the Eggshell Surface

The eggs were treated with aqueous solutions of Dergall at concentrations of 0 (untreated control), 1.0, 3.0, and 6.0% by spraying or dipping (10 repetitions, n = 10 embryos/concentration/repetition). The control group was not treated. After drying (about 30 min after treating), the eggshell samples (ca. 25 mm^2) were cut and stocked on to metal tables with a double-sided carbon-based tape and sprayed with a thin layer of gold and palladium. The tables were placed in the chamber of a Hitachi S-3000N scanning microscope (SEM). SEM images were taken under high vacuum conditions using a secondary electron detector at 18 kV acceleration.

In Ovo Testing of Potential Harmful Effects of Dergall by HET-CAM

The potential harmful effects of the mixture of organomodified trisiloxanes and a sol-gel precursor were assessed via in ovo testing by HET-CAM (Luepke, 1985) as an alternative to the Draize's test (Draize et al., 1944; Scheel et al., 2011). The following concentrations of Dergall water solution were selected for testing: 100%, 50%, 25%, 6%, 3%, 1%, 0.6%, 0.3%, and 0% (control). Although the 0.6% concentration of Dergall is recommended, the authors checked higher concentrations in all experiments to determine their efficiency and toxicity. In order to reevaluate the

recommended concentrations, the goal was to determine whether higher concentrations would be more efficient under nontoxic conditions.

Hatching eggs (61.4 ± 3.63 g, n = 100 eggs) were incubated in Masalles 65 incubators at a temperature (T) of 37.8°C and 50% relative humidity for 8 D. Then the eggs were candled, and unfertilized and damaged eggs and those containing dead embryos were discarded. In the remaining eggs, an opening was made above the air chamber, with a diameter of about 20 mm, through which the internal eggshell membrane was moistened with 0.9% NaCl and placed in the incubator for 20 min. Then the eggs were removed from the incubator, the remaining physiological fluid was pipetted, and the internal eggshell membrane was removed, exposing the chorioallantoic membrane (CAM). Subsequently, 200 µL of each tested Dergall solution was dropped on only the intact CAM prepared in this manner (n = 10 embryonated eggs/tested solution). The potential harmful effects were evaluated on a 21-point scale (0–0.9 points, no reaction; 1.0–4.9, weak reaction; 5.0–8.9, moderate reaction; 9.0–21 points, strong reaction) based on degrees of congestion, hemorrhage, and coagulation of CAM's blood vessels after 30 s, 2 min, and 5 min (Luepke, 1985, Table 1). The observations were made at room temperature using a magnifying glass. The HET-CAM was repeated twice.

In Vivo Testing of Potential Harmful Effects of Dergall

This part of the study was performed in a commercial hatchery (ZWD Woldrob, Wolbrom, Poland). The potential embryotoxic effects of Dergall were investigated using 4,000 eggs (egg weight 62.1 ± 5.42 g) from 46-week-old parental stock. The eggs were stored about 48 h in 17 ± 0.5°C and 70% relative humidity and gradually heated to 25°C for 12 h before the planned start of incubation. Next they were divided into 8 experimental groups composed of 10 trays with a capacity of 50 eggs (n = 10 trays × 50 eggs = 500 eggs/group). Using a hand sprayer, a large dose of one of the following was applied to the surface of the eggshells (ca 20 mL/egg): aqueous solutions of Dergall at the concentration of pure water (blank sample), 1%, 2%, 3%, 4%, 5%, and 6%. The control group was not treated. The temperature of the water and/or solutions was about 37°C. After drying (ca 30 min), the trays with the eggs were placed randomly into one trolley of a multistage setter incubator type TL-115 (JARTOM Tomasz Wabiński, Poland) and then the hatcher TK-196 (JARTOM Tomasz Wabiński, Poland) and incubated according to the incubation program used. The eggs were candled at E7, and E19 and the unfertilized or dead eggs were rejected. All rejected and unhatched eggs were breakout analyzed to note the fertilization, developmental stage of the dead embryo, malformations and malpositions, and any infections. The weight of the eggs was determined with an accuracy of 0.1 g before setting and at E19, and the percentage of egg weight loss was calculated. Moreover, all hatched one-day chicks were weighed and navel healing was evaluated on a 6-point scale (1 pt. = completely healed navel; 6 pts. = open navel).

Table 1. The point scale of harmful effects used in Luepke's hen egg test of the chorioallantoic membrane (1985).

	Observation period		
Harmful effects	0.5 min	2 min	5 min
Congestion	5 points	3 points	1 points
Hemorrhage	7 points	5 points	3 points
Coagulation	9 points	7 points	5 points

In Vitro Testing of Antimicrobial Activity of Dergall

In this study, the *Salmonella* and *Shigella* genera were represented by 2 reference strains each and were provided from the PCM Polish Collection of Microorganisms at the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław, Poland (Table 2). The *Staphylococcus*, *Streptococcus*, *Escherichia*, and *Pseudomonas* genera were represented by 2 strains each; one strain was reference ATCC (American Type Collection Culture), and the other one was a wild strain from the Centre of Microbiological Research and Autovaccines in Kraków, Poland. All the strains were preserved under deep freeze before the experiments. The inocula for the experiments were harvested from a few of the same colonies of each species, originating from fresh overnight cultures. The cells were suspended in sterile 0.9% NaCl to obtain suspensions of 0.5 on the McFarland scale, which ranges from 1 to 2 × 10^8 CFU/1 mL. The densities of suspensions were nephelometrically controlled using a colorimeter.

A culture-diffusion method based on diffuse migration of the tested substance in a particular microbiological medium was used. The inocula were introduced on the surfaces of Mueller-Hinton solid media in Petri dishes. Metal cylinders were placed on the media and then volumes of 50 µL of the tested substance suspensions in water in concentrations of 100%, 75%, 50%, 10%, and 0.6% were introduced to the different cylinders. The cultures were incubated overnight at 37°C. The antibacterial activity of the tested substance was evaluated based on the diameter of the transparent zone in the medium around the cylinder. The transparencies revealed the degrees to which the bacteria cultures were inhibited by the tested substance (EUCAST, KORLD). Control experiments were also performed. Both negative and positive tests using all reagents and buffers, and also 4 commercial antibiotics, chloramphenicol, gentamycin, streptomycin, sulfamethoxazole/trimethoprim, and novobiocin, were used (Wasyl and Osek, 2008). Experiments were performed

Table 2. List of the microorganisms used in the in vitro testing of the bacteriostatic potential of the Dergall, a mixture of organomodified trisiloxanes and a sol-gel precursor (tetraethoxysilane).

Species	Number of the strain	Type of the strain	Source of the strain
Escherichia coli	ATCC 25922	Reference	Reference collection
Escherichia coli	EC/CBMiA	Wild	CBMiA
Pseudomonas aeruginosa	ATCC 27853	Reference	Reference collection
Pseudomonas aeruginosa	PAR/CBMiA	Wild	CBMiA
Salmonella typhimurium	PCM 2565	Reference	Reference collection
Salmonella typhimurium	PCM 2259	Reference	Reference collection
Shigella dysenteriae	PCM 134	Reference	Reference collection
Shigella flexneri	PCM 1793	Reference	Reference collection
Staphylococcus aureus	ATCC 25923	Reference	Reference collection
Staphylococcus aureus	K/7757	Wild	Dept. Microbiol. JU

Abbreviations: ATCC, American Type Collection Culture; JU, Jagiellonian University, Kraków, Poland; CBMiA, Center of Microbiological Research and Autovaccine in Kraków; PCM, Polish Collection of Microorganisms, Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wrocław, Poland.

in 4 repetitions, and antibacterial activity for every repetition was tested in duplicate for each concentration of the substance, and for each bacterial species.

In Vivo Testing of Antimicrobial Activity of Dergall

A series of chicken embryo shells were disinfected using the Dergall substance in 2 concentrations: 0.6%, according to manufacturer's instructions, and additionally 3%. As a control, a group of eggshells was treated with water, and another group was not treated. The experiment included eggshell swabs from a 3 cm^2 area from 6 eggshells of each disinfected group, and 3 of each control group. The swabs were collected before and 1 h after the disinfection to compare the results. All the samples were then cultured on specific media to identify the bacterial genus or species. The media included blood agar (5% sheep blood), (Graso Biotech, Starogard Gdanski, Poland), which made it possible to culture different bacterial species. Then the isolates were cultured for 24 h at 37°C. These cultures were used for morphological analysis of the colonies and standard phenotypic identification. Nonclassified isolates were analyzed with the MALDI-TOF (matrix-assisted laser desorption and ionization) method. All the collected isolates were also cultured on MacConkey, SS, and Baird-Parker media (Graso Biotech), particularly for *Enterobacteriaceae* identification, *Salmonella* or *Shigella* species identification, and staphylococcal species identification, respectively. The collected samples were evaluated as pathogenic or opportunistic bacteria which can be present in food products. Samples were also collected 3, 7, and 14 D after the disinfection to estimate the prolonged antimicrobial activity of the Dergall. To identify the bacterial species which could potentially interrupt the experiment, additional samples were collected from chickens, and from equipment present in the hatcher, as well as from the poultry house. Several samples were also collected from dead chicken embryos in the same farm to specify which bacterial species were etiological agents of infections in the poultry house.

Statistical Analyses

The results were analyzed by 2-way analysis of variance and Tukey's test using the SigmaStat 3.5.

RESULTS

Microscopic Evaluation of Organomodified Trisiloxanes and a Sol-Gel Precursor Layer on the Eggshell Surface

Spraying with organomodified trisiloxanes and a sol-gel precursor at a concentration of 3.0% made a layer on the eggshell surface. Sealing of eggshell pores was also observed (Figure 1C). Moreover, dipping the eggshells in Dergall solution or spraying them with it left the natural cuticle layer undamaged (Figure 1, A–C). Sealing of eggshell pores was also observed (Figure 1C).

In Ovo Testing of Potential Harmful Effects of Dergall by HET-CAM

HET-CAM showed a gradual reduction of irritation correlating to a decreased concentration of the active substance ($P \leq 0.05$). The application of Dergall concentrate (100%) caused a moderate reaction (16 and 5 Luepke scale points in the consecutive test repetitions) while no reaction was observed at a concentration of 3% (Figures 2 and 3).

In Vivo Testing of Potential Harmful Effects of Dergall

The lowest hatchability (mean and SD) 63.7 ± 14.15% of fertilized eggs was observed in the group sprayed with 6% Dergall water solution in comparison to the other groups ($P \leq 0.05$), where this parameter ranged from 89.1 to 93.8% ($P > 0.05$) for eggs treated with 5 and 3% solutions, respectively. This phenomenon was mainly caused by a rapid increase in embryo mortality in the first week of incubation from c.a. 6% to 25.5% (Table 3). There were no differences

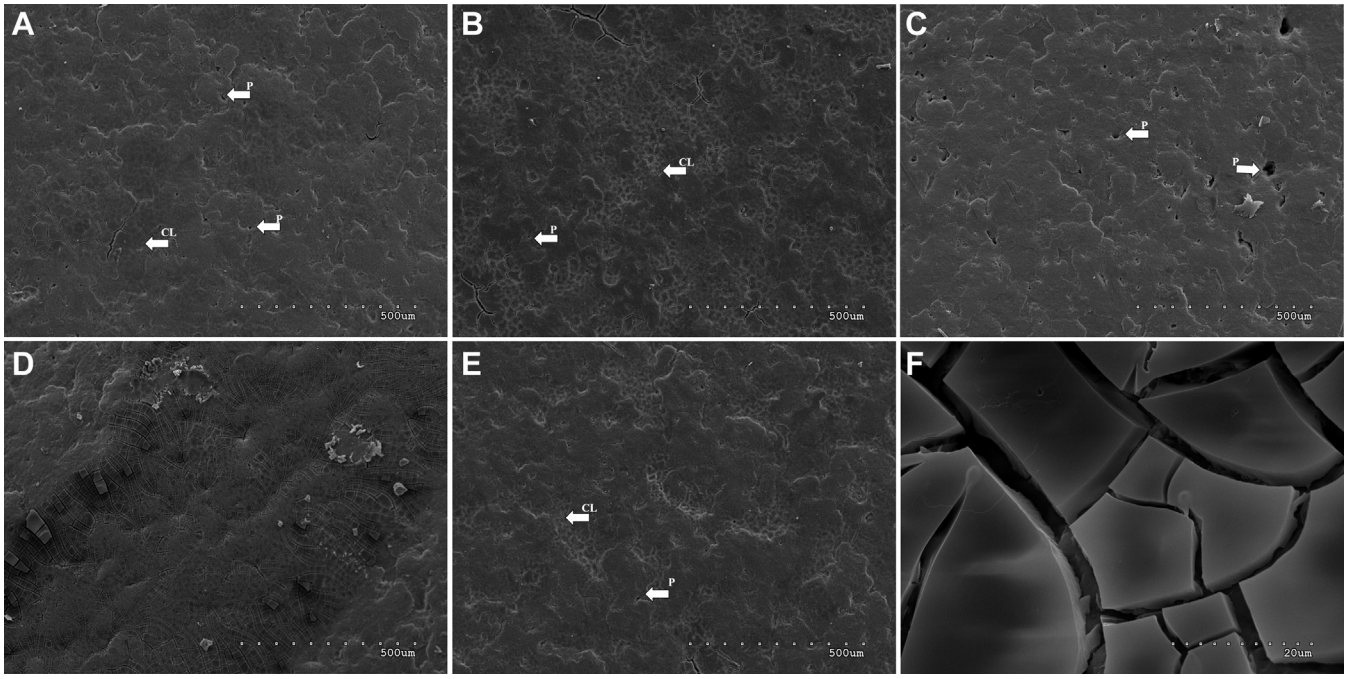

Figure 1. Scanned microscope images of eggshell surface treated by (A) spraying with and (B) dipping in 1% Dergall solution and (C) spraying and (D) dipping with 3% Dergall solution and (E) untreated (bar marker 500 μm) and (F) close-up of the surface of the Dergall layer (bar marker 500 μm). Marks on the images: p—eggshell pore in the shell; sp—eggshell pore in the shell sealed by Dergall; cl—coticule layer; dl—Dergall layer.

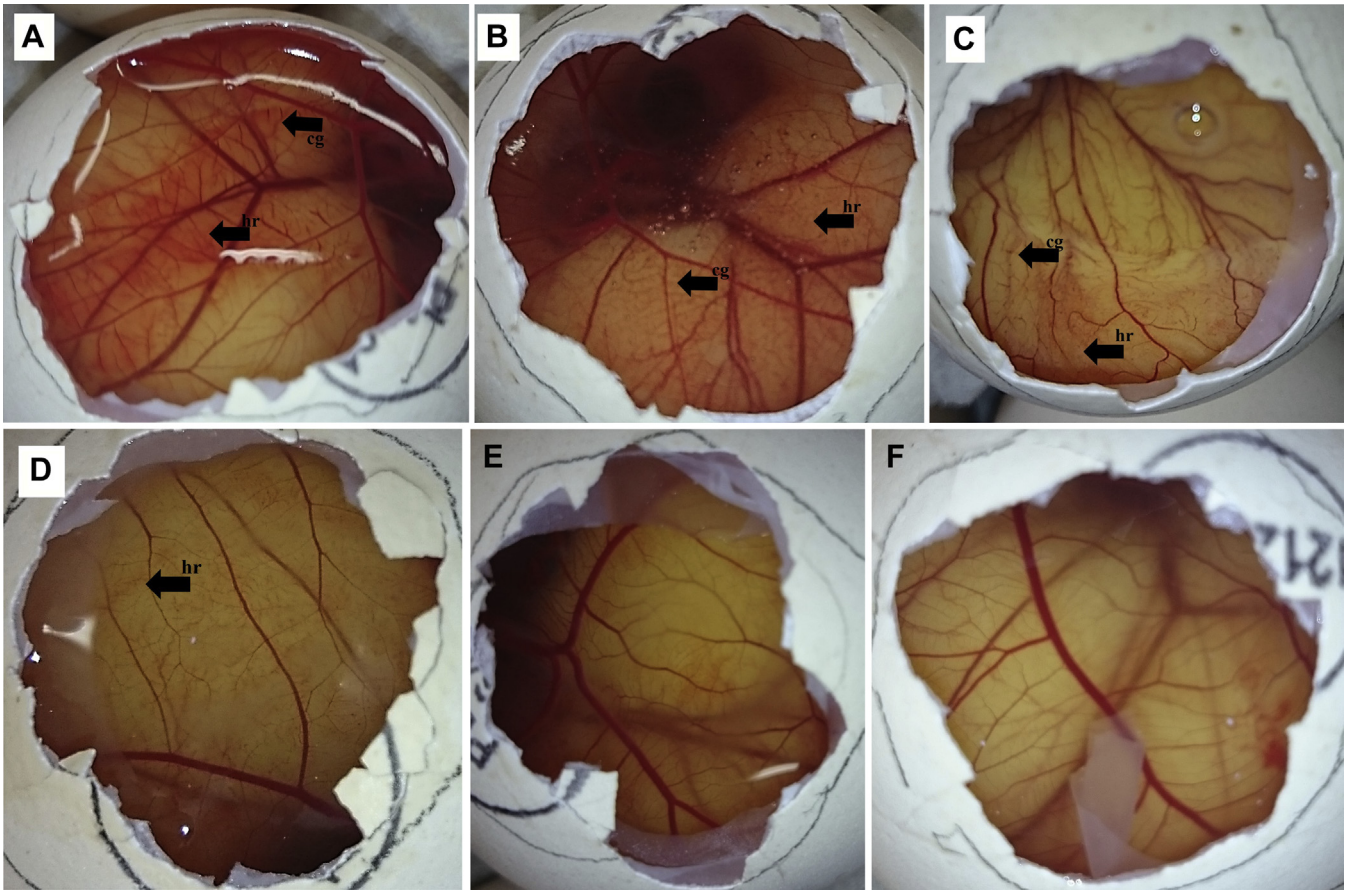

Figure 2. Images of the harmful effects on the chorioallantoic membrane (congestion, hemorrhage, and coagulation) of the Dergall water solution (organomodified trisiloxanes) in concentrations of (A) 100, (B) 50, (C) 10, (D) 5, (E) 3, and (F) 0% (control). Marks on the images: hr—hemorrhage from capillaries; ga—coagulation in blood vasculars.

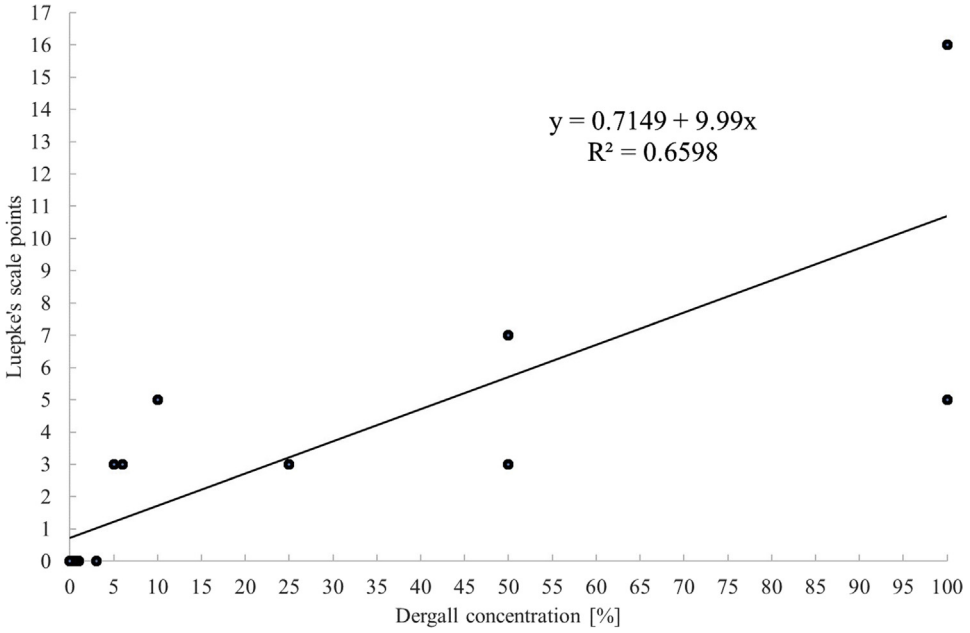

Figure 3. Change of irritation potential evaluated by hen's egg chorioallantoic membrane test (HET-CAM) in Luepke's 21-point scale (y) depending on the concentration (x) of the Dergall water solution (a mixture of organomodified trisiloxanes). Luepke's point scale: 0–0.9 points = no reaction; 1.0–4.9 = weak reaction; 5.0–8.9 = moderate reaction; 9.0–21 points = strong reaction).

between groups in terms of egg weight loss during incubation (7.81 ± 2.88%, $P > 0.05$), the weight of 1 D chicks (48.4 ± 3.57 g, $P \leq 0.05$), and the navel healing score (1.2 ± 1.03 point, median 1 point) (results not shown).

In Vitro Testing of Antimicrobial Activity of Dergall

The performed experiments showed that the analyzed Dergall substance exhibited antibacterial activity in the concentrations used against the enteropathogenic species *Salmonella* and *Shigella*. For *Salmonella typhimurium* PCM2565 and for *S. typhimurium* PCM2259, moderate and low antibacterial activity of the 0.6% Dergall was reported. For genus *Shigella*, the Dergall activity was higher than for *Salmonella*, particularly in the higher concentrations of the substance. Although both *Shigella* species were highly susceptible to 0.6% Dergall, for *Shi. flexneri* PCM1793, moderate antibacterial activity was observed in the concentrations of 100, 75, and 50%, but the most effective results were reported for *Shi. dysenteriae* PCM134 in the same concentrations (Table 4). Also, the analyses showed that for *S. typhimurium* PCM2565 Dergall at 0.6% exhibited much higher activity (a larger transparent zone). Dergall at 0.6% showed low activity (a smaller transparent zone) for *S. typhimurium* PCM2259, but it was still significantly active. The analyzed strains from genus *Shigella* were much more susceptible to 0.6% Dergall. Dergall at the concentration of 0.6% exhibited antibacterial activity for both *Shi. flexneri* PCM1793 and *Shi. dysenteriae* PCM134 in all the tests of all repetitions.

Dergall showed antimicrobial activity against both reference and wild strains of *Staphylococcus aureus*,

Table 3. Results (mean ± SD) of hatchability from chicken eggs treated with a water solution of a mixture of Dergall (organomodified trisiloxanes preparation) of different concentrations 0 (water), 1, 2, 3, 4, 5, and 6%.

Item		Control (not treated)	Dergall 0% (water)	Dergall 1%	Dergall 2%	Dergall 3%	Dergall 4%	Dergall 5%	Dergall 6%
Fertilized eggs per group [N]		49.0 ± 1.15	48.6 ± 0.89	48.8 ± 1.23	47.8 ± 1.87	48.8 1.40	48.5 1.27	48.5 ± 1.58	46.3 ± 2.43
Dead embryos	E1-E6	3.6 ± 2.65^{a}	4.3 ± 3.48^{a}	4.3 ± 1.83^{a}	3.5 ± 2.17^{a}	2.7 ± 3.45^{a}	4.9 ± 3.15^{a}	5.6 ± 4.31^{a}	25.5 ± 11.47^{b}
	E7-E17	2.4 ± 2.52	0.4 ± 0.96	1.4 ± 2.15	1.0 ± 1.09	1.2 ± 1.07	0.4 ± 0.84	1.7 ± 1.89	2.1 ± 1.60
	E18-E21	1.4 ± 2.15	2.9 ± 1.89	3.3 ± 3.03	4.8 ± 4.09	2.1 ± 1.36	2.0 ± 2.36	3.5 ± 2.44	5.3 ± 4.68
	Total	7.5 ± 4.14^{a,b}	7.6 ± 3.71^{a,b}	9.0 ± 4.39^{a,b}	9.4 ± 4.93^{a,b}	5.9 ± 2.98^{a}	7.4 ± 5.43^{a,b}	10.7 ± 5.54^{b}	32.9 ± 13.81^{c}
Malformations		1.2 ± 1.44	1.0 ± 1.07	0.8 ± 1.42	0.8 ± 1.45	0.0 0.00	0.4 ± 0.86	1.4 ± 2.51	1.5 ± 2.38
Microbiological contaminations		0.4 ± 0.88	0.4 ± 0.95	0.2 ± 0.65	0.6 ± 1.03	0.2 0.67	0.0 ± 0.00	0.2 ± 0.63	1.9 ± 1.44
Hatched chicks		92.1 ± 4.21^{a}	92.0 ± 3.99^{a}	90.8 ± 4.39^{a}	90.0 ± 4.89^{a}	93.8 ± 3.25^{a}	92.6 ± 5.43^{a}	89.1 ± 5.55^{a}	63.7 ± 14.15^{b}

^{a,b}Values in rows marked various letters differ significantly ($P \leq 0.05$).

Each experimental group was composed with 10 repetitions of 50 eggs. The results of hatchability showed such as "percent of fertilized eggs." Chick embryo morality was analyzed in periods: first–sixth day of incubation (E1–E6); 7th–17th D of incubation (E7–E17) and 18th–21st D of incubation (E18–E21).

Table 4. Results of antimicrobial activity testing in vitro.

Strain name	Experiment no.	Concentrations				
		100%	75%	50%	10%	0.6%
Salmonella typhimurium PCM 2565	1	−	+	−	+	+
	2	+	+	+	−	+
Salmonella typhimurium PCM 2259	1	−	−	+	−	+
	2	+	−	+	−	+
Shigella flexneri PCM 1793	1	+	+	+	+	+
	2	−	+	+	+	+
Shigella dysenteriae PCM 134	1	+	+	+	+	+
	2	+	+	+	+	+
Staphylococcus aureus ATCC 25923	1	+	−	+	−	+
	2	+	−	+	−	+
Staphylococcus aureus K/7757	1	+	−	−	+	+
	2	+	−	−	+	+
Escherichia coli ATCC 25922	1	+	+	+	+	+
	2	+	+	+	+	+
Escherichia coli EC/CBM	1	+	+	+	+	+
	2	+	+	+	+	+
Streptococcus pyogenes ATCC 19645	1	+	+	+	−	−
	2	+	+	+	−	−
Streptococcus pyogenes S45	1	+	+	+	−	−
	2	+	+	+	−	−
Pseudomonas aeruginosa ATCC 27853	1	−	−	−	−	−
	2	−	−	−	−	−
Pseudomonas aeruginosa PAR/CBM	1	−	−	−	−	−
	2	−	−	−	−	−

Tests were performed in duplicates. + decreased growth of microorganisms, − no effect on microorganisms' growth.

Escherichia coli, and *Streptococcus pyogenes*. The highest activity of Dergall was noted for *S. aureus* (in concentrations of 100 and 0.6%), for *E. coli* (in all concentrations), and then for *Str. pyogenes* (but only in concentrations of 100 and 50%). Much lower antibacterial activity was observed against *Pseudomonas aeruginosa* and was considered a negative result (Table 4). The most reliable results were observed in 0.6% Dergall concentrations because the substance in 75% concentration transforms into gel consistency and it is difficult to uptake and to introduce to cylinders, so careful measurement is not warranted.

In Vivo Testing of Antimicrobial Activity of Dergall

Before disinfection, the chicken embryo shells were highly colonized by gram-positive *Staphylococcus* sp., and *Streptococcus* sp. In a minority, they were colonized by gram-positive *Bacillus* sp., and *Corynebacterium* sp., and also gram-negative *Pseudomonas* sp., and *Proteus* sp. Dergall was 100% efficient for gram-negative bacteria and highly reduced the growth of gram-positive ones. Bacteria that persisted despite the disinfection were identified as *Staphylococcus* or *Streptococcus* species. Results from the cultures collected 1 h after disinfection by particularly 0.6, and 3% concentrations of Dergall showed a reduced number of colonies in most of the samples. The control samples showed high similarity to the samples collected before the disinfection. Three days after disinfection, the results were similar to the results obtained 1 h after disinfection, and the only difference was the presence of the individual colonies of *Bacillus* sp., and *Corynebacterium* sp. Similarity of the results performed 1 h and 3 D after the disinfection suggest prolonged antimicrobial activity of Dergall. Seven days after disinfection, the 3% concentration of Dergall still presented antimicrobial activity, although samples treated with the 0.6% concentration showed a high number of isolates, especially belonging to coagulase-negative staphylococci, *Bacillus* sp., and *Enterobacteriaceae*. The last isolates were collected 14 D after disinfection, but correct identification of bacterial isolates could not be performed because of the excessive number of *Proteus* sp. colonies. The MALDI-TOF MS analysis identified these colonies as *Proteus mirabilis*. Samples from chickens, walls, equipment present at the hatcher, and the poultry house showed a similar bacteriological profile to the eggshell results. The hatcher wall swabs showed the presence of singular colonies of coagulase-positive staphylococci (CoPS) and gram-negative rods on the selected media. A similar observation was shown on samples from healthy chickens' umbilical cords and feathers, which suggests strong colonization by CoPS and coagulase-negative staphylococci (CoNS). In one chicken, *Proteus* sp. and other gram-negative rods were isolated. In addition, we performed a series of swabs from the chicken embryos autopsy. Analysis of isolates originating from dead chicken embryos showed a very high number of *P. mirabilis* colonies.

DISCUSSION

The microscopic pictures show that eggshell Dergall spraying or dipping covers the surface with a thin layer. Naturally, fresh eggshell is covered by a cuticle (mucin

layer) which decreases gas diffusion (Tullett, 1990; D'Alba et al., 2017) and pathogen penetration (Cook et al., 2003; Gole et al., 2014; D'Alba et al., 2017; Bain et al., 2018). This cuticle is naturally removed during egg incubation (Peebles et al., 1987; D'Alba et al., 2017) but also artificially during egg washing (Gole et al., 2014). This improves gas exchange during late stages of embryo development (Burton and Tullett, 1985; Deeming, 1987), but on the other hand, horizontal contaminations can enter (Peebles et al., 1987; Gole et al., 2014). Therefore, some authors have tested artificial layers to prolong storage (Hutchison et al., 2003) and egg hatching storage periods (Brake et al., 1997; Fasenko, 2007).

Substances which are in close contact with humans and animals must be tested in terms of toxicity. According to manufacturer instructions, disinfectants' concentration usually falls in the range of 0.6 to 3%. Our studies found that the 3% OTS solution has no irritation impact on the chorion-allantois membrane of chick embryos (0 Luepke points). These results confirm a European Commission report which states that siloxanes had a nonirritant effect on rabbit skin and eyes (Becker et al., 2013; European Commission, 2009). Moreover, Korowiecka et al. (2017) showed that standard concentrations of disinfectants based on quaternary ammonium compounds, glutaraldehyde, and stabilized hydrogen peroxide have weak to moderate irritating effect (3–9 Luepke points) in HET-CAM. These values suggest a stronger irritating effect in contrast to OTS. In addition, the HET-CAM results are reliable in comparison to other tests using laboratory animals. This suggests that HET-CAM is a proper alternative to Draize's test.

The results of HET-CAM enabled estimating safe and effective OTS solutions below 6%. In vivo tests indicated that spraying eggshells with 5% and lower OTS solutions had no influence on chicken embryo development and hatchability while treatment with a 6% Dergall solution resulted in a rapid decrease of hatchability (approx. 25%). This phenomenon can be explained by the high sensitivity of chick embryo to hazard factors in 3–4 D of incubation (the critical phase of chick development) (Romanoff and Romanoff, 1972; Bruggeman et al., 2003; Lis et al., 2006). Another explanation suggests the closing of eggshell pores and embryo suffocation (Meir and Ar, 1996; Sumara et al., 2019). However, this explanation is not supported by the lack of differences between groups in egg weight loss during incubation. It should be also noted that this indicator was lower than the 11% that is considered optimal (Tona et al., 2001).

Our study confirmed the antimicrobial properties of the Dergall. Moreover, the antimicrobial activity was prolonged to 3 D. Similar results were found by Zweil et al. (2015), who examined a set of disinfectants. However, all proposed substances exhibited toxic properties for chick embryos. Interestingly, the disinfection with Dergall inhibited the increase of *P. mirabilis*, known as an actively colonizing bacteria. Eggshells are naturally colonized by multiple species of bacteria (Mayes and Takeballi, 1983; Musgrove et al., 2004; Olsen et al., 2017); therefore, it is essential to practice proper management and high standards of hygiene when hatching eggs, and disinfection is one of the most important factors in these programs (Cadirci, 2009; Olsen et al., 2017).

The most important results are for the observation received for Dergall in the concentration of 0.6% which is recommended by the producer for disinfection procedures. The microorganisms used in the in vitro testing of the bacteriostatic potential of Dergall include representatives and examples of pathogens responsible for severe infections or toxemias such as the genera *Salmonella* and *Shigella*, represented by 4 species, and another 4 species from the genera *Staphylococcus*, *Streptococcus*, *Escherichia*, and *Pseudomonas*, which are known as opportunistic pathogens, although reported to be responsible for dangerous infections of various localizations, manifestations, or courses in humans or animals. All the *Salmonella* and *Shigella* species were susceptible to 0.6% Dergall. Different antibacterial activities of Dergall were observed for the higher concentrations. For *Salmonella*, the activity of Dergall was slightly lower than for *Shigella*. In vitro testing on *S. aureus* and *E. coli* showed high level of susceptibility, although for *S. pyogenes* Dergall presented antimicrobial effect only in high concentrations. On the other hand, in vitro antimicrobial activity against *P. aeruginosa* was not observed. By contrast, in vivo analysis showed strong reduction of growth on gram-negative bacteria, and susceptibility of *E. coli* confirmed these results. The antimicrobial effect against gram-positive bacteria was high in both tests.

In conclusion, the results of in vitro (HET-CAM) and in vivo tests indicate that Dergall is not irritating for chick embryo. Because of the physical mode of action of this preparation, it does not contain any substance classified as active (hazard); therefore, it might be a good alternative to formaldehyde as an egg disinfectant without negatively affecting hatchability and workers and chicks' health. Nontoxic concentration (0.6%) of the Dergall showed strong antimicrobial properties against both gram-negative and gram-positive bacteria. These results were confirmed in vitro and in vivo. Additional property was prolonged antimicrobial effect to 3 D after disinfection, and this feature characterized Dergall as a good candidate to routine eggshell disinfection.

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SUPPLEMENTARY DATA

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