Alternative sanitizers to paraformaldehyde for incubation of fertile eggs

Gabriel da S. Oliveira,[*] Vinícius M. dos Santos,[†,1] Sheila T. Nascimento,[*] and Jullyana C. Rodrigues[†]

[*]*Faculty of Agronomy and Veterinary (FAV), University of Brasília, Brasília 70.910-900, DF, Brazil; and*
[†]*Laboratory of Poultry Science, Federal Institute of Brasília - Campus Planaltina, Brasília 73.380-900, DF, Brazil*

ABSTRACT The aim of this study was to evaluate an ethanolic extract of propolis and clove essential oil as a substitute for paraformaldehyde for the sanitation of fertile eggs. In total, 1,800 hatching eggs (from 40-week-old CPK [Pesadão Vermelho] breeder hens) were randomly distributed among the treatments (grain alcohol, clove essential oil, ethanolic extract of propolis, and paraformaldehyde). Spraying was the application method for all treatments except for paraformaldehyde, for which fumigation was used. The experimental design was a randomized block design with 4 treatments. Analysis of the incubation parameters was based on 6 replications per treatment. The egg weight loss was lower in the eggs treated with ethanolic extract of propolis (8.59 ± 3.34%) than in the eggs treated with grain alcohol (13.40 ± 2.87%), clove essential oil (12.96 ± 3.33%), and paraformaldehyde (13.05 ± 3.24%). The hatchability of the fertile eggs (51.39 ± 5.81%) and the hatchability of the set eggs (44.74 ± 6.79%) were negatively affected by the application of ethanolic extract of propolis. Late mortality of eggs treated was higher than early mortality in the grain alcohol (12.14 ± 4.72%; 2.86 ± 3.30%), clove essential oil (4.60 ± 5.95%; 3.03 ± 3.50%), and ethanolic extract of propolis (36.63 ± 6.60%, 11.98 ± 4.30%) treatments. The eggs treated with clove essential oil (67.90 ± 1.87%), paraformaldehyde (67.80 ± 1.85%), or grain alcohol (67.50 ± 1.92%) presented chick yields as expected. However, due to the high yield of eggs treated with ethanolic extract of propolis (69.25 ± 1.68%), its application at the concentration used in the present research is not recommended. Clove essential oil, when sprayed on fertile eggs as a sanitizing agent, did not differ from paraformaldehyde in relation to hatchery performance parameters.

Key words: clove essential oil, fertile eggs, grain alcohol, hatching results, propolis

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INTRODUCTION

The proper farm biosecurity conditions can reduce eggs contamination after oviposition. Excessive contamination of these eggs can lead to a decrease in incubation capacity, quality, growth, and performance of the chicks (Scott et al., 1993). Thus, using an effective sanitizer on the eggshell surface is important for reducing the potential for external and internal contamination. However, inadequate application of sanitizers allows microorganisms to penetrate the eggshell pores and reach the embryo (Araújo and Albino, 2011). These microorganisms disrupt the embryo and consequently reduce the incubation efficiency (de Faria et al., 2014).

Hatching eggs are conventionally sanitized mainly by paraformaldehyde fumigation (Kusstatscher et al., 2017). This technique efficiently reduces potentially pathogenic microorganisms (Rui et al., 2011) but uses a product that adversely affects the embryos and is harmful to the health of the farm and hatchery professionals (Zeweil et al., 2015; Kusstatscher et al., 2017). Therefore, alternative products are needed that can provide satisfactory sanitization without reducing the incubation efficiency of the embryos or harming the professionals involved in the process.

Propolis, a resinous substance produced from plant exudates harvested by bees, is a candidate sanitizer (Salgueiro and Castro, 2016). Propolis is very promising because it is composed of flavonoids and phenolic compounds (Afrouzan et al., 2018), has antimicrobial activity (Aguiar et al., 2018), presents lipophilic behavior, has a hard and brittle consistency (Marcucci, 1995), and has low innate toxicity (Pinto et al., 2011).

Other alternatives to paraformaldehyde are essential oils. These compounds comprise volatile and lipophilic

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[1]Corresponding author: vinicius.santos@ifb.edu.br

substances (de Morais, 2009). According to Puškárová et al. (2017), clove essential oil has a strong antimicrobial effect; is characterized by high volatility; and contains eugenol, β-caryophyllene, and eugenyl acetate. Eugenol is the major compound in clove essential oil and is responsible for most of the oil's pharmacological effects (Pombo et al., 2018), in addition to having low toxicity (Leal-Cardoso et al., 2002).

Commercial hatcheries still use products that are highly carcinogenic and unhealthy (Rui et al., 2011). Therefore, this study evaluated the effects of replacing paraformaldehyde with an ethanolic extract of propolis and clove essential oil to sanitize hatching eggs on incubation efficiency parameters.

MATERIALS AND METHODS

All the procedures of this study were approved by the Committee of Ethics of Animal Use of the University of Brasília under protocol no. 48/2018.

In total, 1,800 brown fertile eggs of average weight (59.98 ± 4.78 g) from 40-week-old CPK (Pesadão Vermelho) breeder hens were incubated in a multistage setter (CASP MG 62HT; Amparo, São Paulo, Brazil) with a capacity of 61,920 eggs. CPK is a commercial broiler breeder line specifically used in alternative rearing systems. The eggs were randomly distributed among the sanitization treatments (grain alcohol, clove essential oil, ethanolic extract of propolis, and paraformaldehyde) as shown in Table 1. The same number of eggs was used in each treatment to ensure sample homogeneity.

The grain alcohol used in the study was Cromoline 93.5% (Cromoline Química Fina, Diadema, São Paulo, Brazil) and served as the carrier vehicle for the propolis and clove essential oil. Its individual effect on the sanitation of fertile eggs without these added compounds was also tested.

Clove essential oil (*Syzygium aromaticum*) was obtained from a commercial clove sample from a local market in Planaltina, Federal District, Brazil. The essential oil was extracted by steam distillation using a Clevenger extractor system (Vidrolabo, Poá, São Paulo, Brazil) according to the procedures recommended by Ascenção and Filho (2013). After the extraction, the clove essential oil was diluted in grain alcohol at 0.6 mg/mL, and its antimicrobial potential was determined according to Silvestri et al. (2015), which demonstrated its efficacy.

The ethanolic extract of propolis was obtained from a commercial apiary in Brasília, Federal District, and had a light brown color and moldable texture. Before the extract was prepared, the propolis sample was cleaned by removing stones, dead bees, and other foreign bodies.

Table 1. Sanitizers and their respective concentrations.

Treatment		Concentration	Application
T1	Grain alcohol	93.5%	Spraying
T2	Clove essential oil	0.6 mg/mL	Spraying
T3	Ethanolic extract of propolis	15%	Spraying
T4	Paraformaldehyde	6 g/m^3	Fumigation

The ethanolic extract of propolis was prepared following a method adapted from the study by Aygun et al. (2012) using 15% raw propolis and 85% grain alcohol at 93.5% under constant stirring at room temperature for 24 h to obtain a homogeneous extract. The extract was then filtered through a Melitta paper filter.

Paraformaldehyde at a concentration of 6 g/m^3 was used for the sanitization. Product burning, fumigation, and gas exhaust proceeded for 20 min in a hermetically sealed chamber. The relative humidity and temperature in the chamber were 70% and 30°C, respectively.

The eggs were sanitized by a research partner at a commercial hatchery in Planaltina, Federal District, Brazil. Before sanitization, the researcher's hands and the bench were disinfected with 70% ethanol. Eggs treated with grain alcohol (T1), clove essential oil (T2), and ethanolic extract of propolis (T3) were placed on metal screen stands for spraying, whereas eggs treated with paraformaldehyde (T4) were fumigated with paraformaldehyde. The estimated time between egg collection and sanitization was 20 min.

Treatments T1, T2, and T3 were sprayed homogeneously over the entire egg surface using hand sprayers. After being sprayed, the eggs remained in the stands to dry at room temperature for 30 min. The paraformaldehyde fumigation (T4) consisted of exposing the eggs to paraformaldehyde sublimation. After sanitization, all eggs were stored for 4 D at 70% relative humidity and at a temperature of 16°C to 20°C.

Each tray was weighed individually before incubation and on day 18 of incubation on a precision scale to evaluate the weight loss throughout incubation. The temperature and relative humidity of the setter were 37.7°C and 60%, respectively. Eggs were turned at an angle of 45° at a frequency of 24 times per day until day 18 of embryonic development.

The eggs were transferred from the setter to the hatcher (CASP G 42HT; Amparo, São Paulo, Brazil) on day 19 of embryonic development. All hatcher baskets were identified according to treatments and randomly distributed within the hatchery. The hatcher temperature was 36.6°C, and the relative humidity was 65%. During this period, the number of chicks hatched and their respective weights were recorded. After 21 D of incubation, the unhatched eggs were counted, opened, and examined to determine the percentage of infertile eggs and the percentage of embryonic deaths (early, 1–18 D; late, 19–21 D).

The 1) egg weight loss (%), 2) fertility rate (%), 3) hatchability of set eggs (%), 4) hatchability of fertile eggs (%), 5) early embryonic mortality (%), 6) late embryonic mortality (%) and 7) chick yield (%) were calculated per the COBB Hatchery Management Guide (2008) and Aviagen (2011) using the following formulas:

1) Egg weight loss (%) = [(initial egg weight − egg weight measured on transfer day)/initial egg weight] × 100.
2) Fertility rate (%) = (number of fertilized eggs / number of eggs set) × 100.

3) Hatchability of set eggs (%) = (number of hatched chicks/total number of set eggs) × 100.
4) Hatchability of fertile eggs (%) = (number of hatched chicks/number of fertile eggs) × 100.
5) Early embryonic mortality (%) = (number of dead embryos on days 0–18 of incubation/number of fertile eggs) × 100.
6) Late embryonic mortality (%) = (number of dead embryos on days 19–21 of incubation/number of fertile eggs) × 100.
7) Chick yield (%) = (chick weight at the day of hatch/initial egg weight) × 100.

After the incubation period, the thickness of each eggshell was measured without removing the internal membranes. Averages were obtained from 3 distinct points in the equatorial region of the shell using a digital caliper with 0.001-mm precision.

The experimental design was a randomized block design with 4 treatments. Analysis of the incubation parameters (egg weight loss, hatchability of set eggs, hatchability of fertile eggs, and early and late embryonic mortality) was based on 6 replications per treatment in which each tray (block) of 75 eggs constituted a replicate. For analysis of eggshell thickness and chick yield, each egg and chick, respectively, were considered a replicate. The data were analyzed by analysis of variance (PROC GLM) using SAS Studio University Edition (Inst. Inc., Cary, NC), and means were compared using Tukey's test at a significance level of 5%.

Variables were analyzed according to the following mathematical model:

$$y_{ij} = m + b_j + t_i + e_{ij}$$

where y_{ij} = observation j of experimental unit subjected to treatment i in block j, m = overall mean, b_j = effect of blocks, t_i = effects of sanitizers, and e_{ij} = random error associated with each observation.

RESULTS

Egg weight loss (%) differed significantly among the treatments ($P < 0.05$), ranging from 8.59 (ethanolic extract of propolis) to 13.40% (grain alcohol) (Table 2). The averages of egg weight loss were similar ($P > 0.05$) for the eggs treated with grain alcohol (13.40%), clove essential oil (12.96%), and paraformaldehyde (13.05%).

Fertility (%) differed significantly among the eggs (Table 3). The mean fertility rate in this study was 91.45 ± 3.99%.

The hatchability of the fertile eggs and the hatchability of the set eggs differed significantly among the treatments ($P < 0.05$; Table 3). The highest values were recorded in the paraformaldehyde (94.44%; 90.76%) and clove essential oil groups (92.37%; 80.26%), followed by the grain alcohol (85.00%; 81.58%) and ethanolic extract of propolis groups (51.39%; 44.74%).

Analysis of the unhatched eggs showed that late mortality (%) was higher than early mortality (%) ($P < 0.05$; Table 3) for the eggs treated with grain alcohol, clove essential oil, and ethanolic extract of propolis. The highest late embryo mortality rate was observed in the propolis group (36.63%) and the lowest in the paraformaldehyde group (2.78%). Early embryo mortality rates in the grain alcohol, clove essential oil, ethanolic extract of propolis, and paraformaldehyde treatments were 2.86, 3.03, 11.98, and 2.78%, respectively.

The means of the initial chick weights (average 41.07 ± 8.17 g) did not differ among treatments ($P > 0.05$). However, a difference in chick yield (Table 3) was detected among the treatments. The propolis group presented a higher yield value (69.25%, $P < 0.05$) than those of the clove essential oil (67.90%), paraformaldehyde (67.80%), and grain alcohol groups (67.50%).

Eggshell thickness was not significantly different among the treatments ($P > 0.05$; coefficient of variation = 5.92%). The mean eggshell thickness was 0.37 ± 0.029 mm.

DISCUSSION

The initial egg weight was similar among treatments (Table 2), reflecting the measures taken to homogenize the treatments for egg weight at the beginning of the experiment; therefore, variations in egg weight during the incubation period were due only to the effects of each treatment.

The eggs sprayed with ethanolic extract of propolis lost less weight than those treated with grain alcohol, clove essential oil, and paraformaldehyde (Table 2). Corroborating these results, Aygun et al. (2012)

Table 2. Egg weight before setting and during transfer and the percentage of egg weight loss in eggs treated with different sanitizers.[1]

Treatment	Egg weight before setting (g)	Egg weight during transfer (g)	Egg weight loss (%)
Grain alcohol	60.09 ± 4.88	52.05 ± 4.90^{b}	13.40 ± 2.87^{a}
Clove essential oil	60.20 ± 4.78	52.11 ± 4.57^{a,b}	12.96 ± 3.33^{a}
Ethanolic extract of propolis	59.69 ± 5.17	54.14 ± 4.85^{a}	8.59 ± 3.34^{b}
Paraformaldehyde	59.93 ± 4.30	52.19 ± 4.30^{a,b}	13.05 ± 3.24^{a}
P value	0.9201	0.0257	<0.0001
Coefficient of variation (%)	7.99	8.87	26.47

Means with different superscript letters in columns differ significantly ($P < 0.05$).
[1]Results are expressed as means ± SD.

Table 3. Fertile rate, hatchability of set eggs, hatchability of fertile eggs, early and late embryonic mortality, chick weight, and chick yield according to different sanitizers.[1]

Treatment	Fert (%)	Hatch (%)	Hatch fert (%)	Early dead (%)	Late dead (%)	Chick weight (g)	Chick yield (%)
Grain alcohol	96.05 ± 5.04^{a}	81.58 ± 3.04^{a}	85.00 ± 2.20^{b}	2.86 ± 3.30^{b}	12.14 ± 4.72^{b}	40.71 ± 8.85	67.50 ± 1.92^{b}
Clove essential oil	86.84 ± 3.04^{b}	80.26 ± 5.04^{a}	92.37 ± 3.25^{a,b}	3.03 ± 3.50^{b}	4.60 ± 5.95^{b}	41.22 ± 7.83	67.90 ± 1.87^{b}
Ethanolic extract of propolis	86.84 ± 5.26^{b}	44.74 ± 6.79^{b}	51.39 ± 5.81^{c}	11.98 ± 4.30^{a}	36.63 ± 6.60^{a}	41.69 ± 8.78	69.25 ± 1.68^{a}
Paraformaldehyde	96.05 ± 2.63^{a}	90.76 ± 6.62^{a}	94.44 ± 4.54^{a}	2.78 ± 3.21^{b}	2.78 ± 3.21^{b}	40.65 ± 7.23	67.80 ± 1.85^{b}
P value	0.0072	<0.0001	<0.0001	0.0079	<0.0001	0.4796	<0.0001
Coefficient of variation (%)	4.55	7.50	5.17	69.81	37.62	8.03	1.84

Means with different superscript letters in columns differ significantly ($P < 0.05$).
Abbreviations: Fert, fertility; Hatch, hatchability of set eggs; Hatch fert, hatchability of fertile eggs.
[1]Results are expressed as means ± SD.

evaluated the incubation of Japanese quail eggs and observed low weight losses in eggs sprayed with 5% (9.73%), 10% (9.28%), and 15% (9.21%) propolis solution. These authors observed that the propolis occluded the shell's pores, reducing the amount of moisture lost from the eggs during incubation. Akpinar et al. (2015) investigated the effect of propolis extract on the egg storage time of table quail (*Coturnix japonica*) eggs. Eggs were coated with various concentrations of propolis extract (0, 5, 10, and 15%) and treated with 70% ethyl alcohol. The authors reported that the highest egg weight loss values were obtained in the control group and samples coated with ethyl alcohol. Batkowska et al. (2018) observed that quail eggs sprayed with alcoholic extract of propolis (15%) presented significantly lower weight loss than nondisinfected eggs (negative control), eggs disinfected with formaldehyde (positive control), and eggs disinfected with 96% alcohol (group A).

Egg weight loss during incubation is due to the diffusion of water through the eggshell (Tona et al., 2001). Studies report that the ideal weight loss percentage to achieve good hatching results is between 10 and 15% (Rosa and de Avila, 2000; Schmidt et al., 2002; Molenaar et al., 2010). In this experiment, as all eggs were incubated under similar temperature and humidity conditions, the low weight loss of the eggs sprayed with ethanolic extract of propolis occurred because this sanitizer creates a coating on the eggshell, minimizing water loss through the eggshell pores.

Baylan et al. (2018) investigated the effects of garlic extract (*Allium sativum*) as an alternative to formaldehyde for the disinfection of hatching eggs. They used 2 different garlic extract percentages (2.5 and 5.0%, namely, garlic-1 and garlic-2), formaldehyde fumigation (positive control), and eggs not submitted to disinfection (negative control). They stated that there was a significant difference in the fertility rates of eggs that underwent control (93.24%), formaldehyde (89.62%), garlic-1 (87.78%), and garlic-2 (86.08%) treatments. It is known that egg fertility is determined before egg sanitization (Hrnčár et al., 2012). Thus, the difference in the fertility percentage in both studies can be explained by several factors related to the management and environmental conditions of the shed of breed hens, including incorrect control of bird body weight, heat stress, photoperiod, nutrition of breed hens, diseases, optimal number of sexually active males, behavioral changes of males, and physical impairment upon copulation (McGary et al., 2002; Rodenas et al., 2005; King'ori, 2011).

The differences observed between clove essential oil and paraformaldehyde for hatchability of set eggs can be explained by the significant difference in fertility of these treatments. Comparing the hatchability of fertile eggs (%) sprayed with grain alcohol, clove essential oil, and ethanolic extract of propolis with that of eggs treated with paraformaldehyde, we found that the clove essential oil treatment was as efficient as the paraformaldehyde treatment (Table 3). Similarly, Copur et al. (2010) evaluated the effect of oregano essential oil at 2 concentrations (0.55 and 0.75 $\mu L/cm^3$) and 2 exposure times (3 and 6 h) for hatching egg disinfection and observed that the hatchability of these eggs (90.00%) was higher than that of eggs disinfected with formaldehyde (89.91%); however, the differences were not statistically significant. Yildirim et al. (2003) observed that the hatchability percentage of the eggs in the formaldehyde fumigation group (50.53 ± 5.7%) was lower than that in the group sprayed with 0.2 mL of oregano essential oil (73.00 ± 3.7%). Other studies used essential oils for sanitizing hatching eggs and observed no negative effect on egg hatchability (Ulucay and Yildirim, 2010; Debes and Basyony, 2011; Zeweil et al., 2015).

The hatchability of the fertile eggs and the hatchability of the set eggs sprayed with the ethanolic extract of propolis were negatively affected (Table 3). This result is in accordance with those of Mousa-Balabel et al. (2016), who investigated the effect of 14% alcoholic extract of propolis, 70% ethanol, 0.5% TH4 (Sogeval, Laval, Mayenne, France), and 0.5% Virkon S (Lanxess, Cologne, Germany) on the hatchability of hatching eggs and observed that the propolis-based disinfectant yielded a lower value (80.00%). In contrast, Aygun et al. (2012), Vilela et al. (2012), and Batkowska et al. (2018) reported that propolis did not affect egg hatchability. However, hatchability is directly related to embryonic mortality. Thus, the low hatchability of the eggs treated with the ethanolic extract of propolis in this study is most likely due to the low water loss during egg incubation, a factor that resulted in superhydration of the embryos, deficient gas exchange, and, consequently, embryonic mortality (Ribeiro et al., 2008).

The final period of incubation is characterized by the embryo turning toward the air cell to ventilate its lungs, redirect the blood circulation, and retract the yolk sac to

eventually hatch (COBB Hatchery Management Guide, 2008). In this experiment, late mortality was mainly affected by propolis, as it occluded the eggshell pores and impaired the physiological mechanisms of the embryo related to gas exchange during the last days of incubation. Corroborating this result, Copur et al. (2010) observed that eggs disinfected with oregano essential oil presented higher late embryonic mortality percentages (4.58%) than early embryonic mortality percentages (3.10%). Aygun et al. (2012) showed that applying propolis as a disinfectant on eggs before hatching did not affect the early mortality of quail embryos.

According to Aviagen (2011), achieving the ideal chick yield (between 67 and 68%) requires adequate incubation time and parameters as well as better chick quality (Boleli et al., 2016). In the present study, eggs sprayed with grain alcohol, clove essential oil, and paraformaldehyde presented chick yields classified as "ideal" (Table 3). Conversely, eggs treated with ethanolic extract of propolis were classified as "high" yield; that is, chicks with a superior yield, when housed on a farm, will move more slowly, weigh more, be less vocal, and will not be ready to eat or drink water (Aviagen, 2011). In this sense, Mousa-Balabel et al. (2016) reported that eggs disinfected with 14% propolis led to heavier chicks (44.04 g) than eggs disinfected with 70% ethanol (39.99 g), 0.5% TH4 (42.08 g), and 0.5% Virkon S (39.83 g). Ulucay and Yildirim (2010) observed that the weight of quail chicks at hatching was not affected by the compounds thymol (7.1 ± 0.23 g), carvacrol (6.8 ± 0.14 g), and cinnamaldehyde (7.0 ± 0.15 g).

The eggs used in the present study were from laying hens of the same line and age and were exposed to similar incubation conditions. This homogenization was crucial to minimizing possible variations that could compromise the study results. Once such variations were eliminated, the results for the eggs treated with propolis were likely explained by the internal water retention, causing excessive moisture on the chicks at hatching. Therefore, using ethanolic extract of propolis to sanitize fertile eggs is not recommended.

The mean eggshell thickness obtained in this study was considered adequate for 40-week-old breeders (Zita et al., 2009) and indicates good eggshell quality. Eggs with thicker shells are less likely to be penetrated by bacteria, and the additional thickness enables the embryo to better use the nutrients contained in the egg, provides better protection against mechanical damage, and, consequently, provides better conditions for normal embryo development (Narushin and Romanov, 2002; Guntzel, 2015).

According to Narushin and Romanov (2002), the eggshell thickness will determine the gas exchange and weight loss during incubation. Melo et al. (2019) evaluated the effect of egg disinfection with formaldehyde and other alternative products (ozone gas, UV light, hydrogen peroxide, and peracetic acid) and observed no significant difference in eggshell thickness among the treatments (average 0.36 ± 0.003 mm), and none of the tested disinfectants affected this variable in a negative manner.

CONCLUSIONS

Clove essential oil, when sprayed on fertile eggs as a sanitizing agent, did not differ from paraformaldehyde in relation to hatchery performance parameters. However, more studies are needed to confirm its efficacy. Conversely, using 15% ethanolic extract of propolis to sanitize hatching eggs is not recommended.

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