Evaluation of propolis extract as a disinfectant of Japanese quail (*Coturnix coturnix japonica*) hatching eggs

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ABSTRACT This study was carried out to evaluate the effectiveness of an alcoholic propolis extract (15%) as a disinfectant for Japanese quail (*Coturnix coturnix japonica*) hatching eggs. A total of 600 eggs were randomly divided into four experimental groups: 1) negative control (NC), without disinfection, 2) control (C), fumigated with formaldehyde gas, 3) (A), sprayed with 96% alcohol, and 4) (P), sprayed with 15% alcoholic propolis extract. The eggs were incubated artificially in a BIOS hatching apparatus under standard conditions. On the 14th day, the eggs were candled to determine the number of infertile eggs and dead embryos and samples were collected for microbial analysis. After 17.5 d, fertility, hatchability, embryonic mortality, and eggshell conductance were calculated. Fertile eggs sprayed with propolis were shown to have a lower eggshell conductance constant (egg weight loss) than eggs from groups C and A. Total microbial activity on the eggshells did not differ significantly between groups, but *Staphylococcus aureus*, *Micrococcus* spp., *Bordetella* spp., and *Chryseobacterium meningosepticum* isolates were significantly affected by the propolis treatment. There were no significant differences between treatments for total hatchability, embryonic mortality, and chick body weight on the 1st, 7th, and 14th days of life. The total chick survivability during the first 2 wk was significantly higher in group P than in the other groups. The results indicate that spraying hatching eggs with 15% propolis as a disinfectant can be recommended as a safe and natural sanitizer in place of formaldehyde, with no negative effect on quail chicks.

Key words: chick survivability, egg conductance, eggshell disinfection, microbiological traits

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INTRODUCTION

The competitiveness of modern intensive poultry production creates the need to achieve high efficiency and to optimize hatchability, as well as chick viability and growth performance (Shane and Faust, 1996; Vilela et al., 2012). All of these are fundamentally dependent on disinfection of hatching eggs (Shahein and Sedeek, 2014). Some hatcheries use disinfectants for hatching eggs that are safe for human health and the environment. For this purpose, UV rays, ozone, hydrogen peroxide, colloidal silver, ethyl alcohol or plant derived substances have been used in previous experiments as an alternative to formaldehyde for disinfection of eggs, incubators, and livestock houses (Sander and Wilson, 1999; Gehan, 2009; Batkowska et al., 2017; Batkowska et al., 2018). Other authors (Scott and Swetnam, 1993; Debes and Basyony, 2011; Wells et al., 2011) have reported that formaldehyde fumigation should be excluded from decontamination programs for hatching eggs despite its efficacy as a disinfectant, due to exposure of farm workers or hatchery personnel to the toxic and potentially carcinogenic compound (Hayretda and Kolankaya, 2008).

Propolis is a natural and harmoniously balanced substance with strong antibiotic, anti-inflammatory, antibacterial, antifungal, and antiviral effects (Velikova et al., 2000). It is primarily composed of sticky and gummy resin (Valle, 2000) collected by worker honeybees from young shoots and buds of certain trees and shrubs (Schmidt, 1997). Bees use propolis to protect the colony and larvae from pathogenic microorganisms, by covering the inside of the hive during the construction of honeycombs (Ghisalberti, 1979) and using it to fill crevices and to seal and varnish honeycombs. Its biological properties improve immune response, depending on the concentration and the dose. Dias et al. (2012) reported that propolis can be used to

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increase the specific immune response after vaccination (Freitas et al., 2011) and has potential antibiotic effects against *Salmonella*, *Staphylococcus aureus*, *Proteus vulgaris*, and *Escherichia coli* (Powers, 1964).

The aim of this study was to assess the influence of an alcoholic extract of propolis in practical application as alternative to a traditional disinfectant (formaldehyde) for quail eggs (*Coturnix coturnix japonica*), applied by spraying, on some hatchability parameters, microbial activity, and chick quality.

MATERIAL AND METHODS

A total of 600 Japanese quail (*Coturnix coturnix japonica*) hatching eggs were divided in 4 groups before incubation, 150 eggs per treatment, 4 replication groups in each. Before being placed in the incubator, 1 group of eggs was not disinfected as a negative control (NC), the second group was disinfected by fumigation with formalin and $KMnO_4$ (C), the third group was disinfected by spraying with 96% alcohol (A), and the fourth group was disinfected by spraying with an alcoholic (96%) propolis extract (P) (APIS Apiculture Cooperative in Lublin, Poland) at 15% concentration. The concentration of propolis was chosen according to Aygun et al. (2012).

The eggs were hatched artificially using a BIOS hatching apparatus. Standard incubation conditions were maintained: 37.6 to 38.0°C with 50 to 65% relative humidity in the setting compartment and 37.0 to 37.5°C with 75 to 80% relative humidity in the hatching compartment. The eggs were turned 8 times a day during the incubation period. On the 14th day of incubation, the eggs were candled to determine the number of infertile eggs and dead embryos, after which they were transferred from the setter to the hatching compartment. In addition, samples for microbial analysis were collected on day 14 of the experiment. Three eggs per group were placed in sterile boxes containing 50 ml of sterile buffer salt solution (PBS) with 3 drops of TWEN 80. The containers with eggs were left on the stirrer for 1 h. Samples were serially diluted in PBS and plated on sterile medium to determine the total numbers of bacteria, coliform bacteria, haemolytic bacteria, *Salmonella* spp., *Staphylococcus* spp., yeasts, and moulds (Gentry and Quarles, 1972; Jones et al., 2002; PN-EN ISO 4833; PN-EN ISO 21528-1). After incubation, the colonies were counted and presented as CFU/ml of liquid from the egg. Bacterial colonies were identified by microscopic examination, Gram staining, and API biochemical tests (bioMérieuxPolska). Moulds were identified using special keys (Watanabe, 2002). The media used in the analysis are shown in Table 1.

After 17.5 d of incubation, healthy, deformed, and dead chicks were counted. Fertility, hatchability, periodical embryonic mortality, and the eggshell conductance constant (Christensen et al., 2001) were also calculated during the incubation. The chicks were reared for 14 d. Survivability and body weight were recorded at 7 and 14 d of rearing.

The data were analyzed with the SPSS 20.0PL statistical package (IBM, 2011). The normality of the data was verified by the Kolmogorov–Smirnov test. One-way ANOVA with Duncan's post-hoc test was carried out. The number of bacterial colony-forming units was verified by a nonparametric chi-square test.

RESULTS AND DISCUSSION

The use of different disinfection methods, i.e., formalin+$KMnO_4$ (C), alcohol (A), and propolis (P), did not affect the total counts of bacteria and fungi on the eggshell surface (Figure 1) as compared with the NC. The total bacterial count ranged from 1.15 to 1.68 log10 CFU/ml liquid from egg for groups A and NC, respectively, and the total number of fungi ranged from 0.00 to 0.37 log10 CFU/ml liquid from egg. At the same time, a significant decrease ($P \leq 0.05$), depending on the treatment, was noted in such strains of bacteria (% of total isolates) as *Micrococcus* spp., *Bordetella* spp., *Chryseobacterium meningosepticum*, and *Staphylococcus aureus*, as well as non-identified bacteria (Table 2).

No *Escherichia coli* or *Salmonella* spp. bacteria were found, which was indicative of a high level hygiene at the hatchery. The sensitivity of certain strains of bacteria (*Staphylococcus aureus*, *Micrococcus* spp., *Bordetella* spp., *Chryseobacterium meningosepticum,* and unidentified groups) to the propolis extract could be attributed to its antibacterial activity. Some researchers have reported that propolis exhibits broad-spectrum bioactivity against bacteria, chiefly Gram-positive bacteria (Kujumgiev et al., 1999; Miorin et al., 2003; Uzel et al., 2005). Bankova et al. (1999) and Marcucci et al. (2001) found that the antibacterial activity of propolis is higher against Gram-positive bacteria, probably due to flavonoids, acids, and aromatic esters found in the resin, which could cause disintegration of the cell wall structure of these microorganisms. Alencar et al. (2007), Cardoso et al. (2009), and Rahman et al. (2010) obtained similar results, reporting that an ethanolic propolis extract displayed antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus intermedius* isolates. Our results were in disagreement with the findings of Longhini et al. (2007), who showed that propolis had antifungal activity; with Vilela et al. (2012), who stated that immersion of hatching eggs in 2400 μg

Table 1. Incubation medium used in microbial analysis.

Microorganisms	Applied medium
Aerobic mesophilic bacteria	Conditions agar
Total number of bacteria	Conditions agar
Hemolytic bacteria	Conditions agar + 5% sheep blood
Mold-yeast	Sabouraud agar with chloramphenicol
Coliform bacteria	MacConkey's
Staphylococcus Spp.	Baird Parker agar (supplemented with 5% egg yolk-tellurite)
Salmonella Spp.	Agar *Salmonella-Shigella*

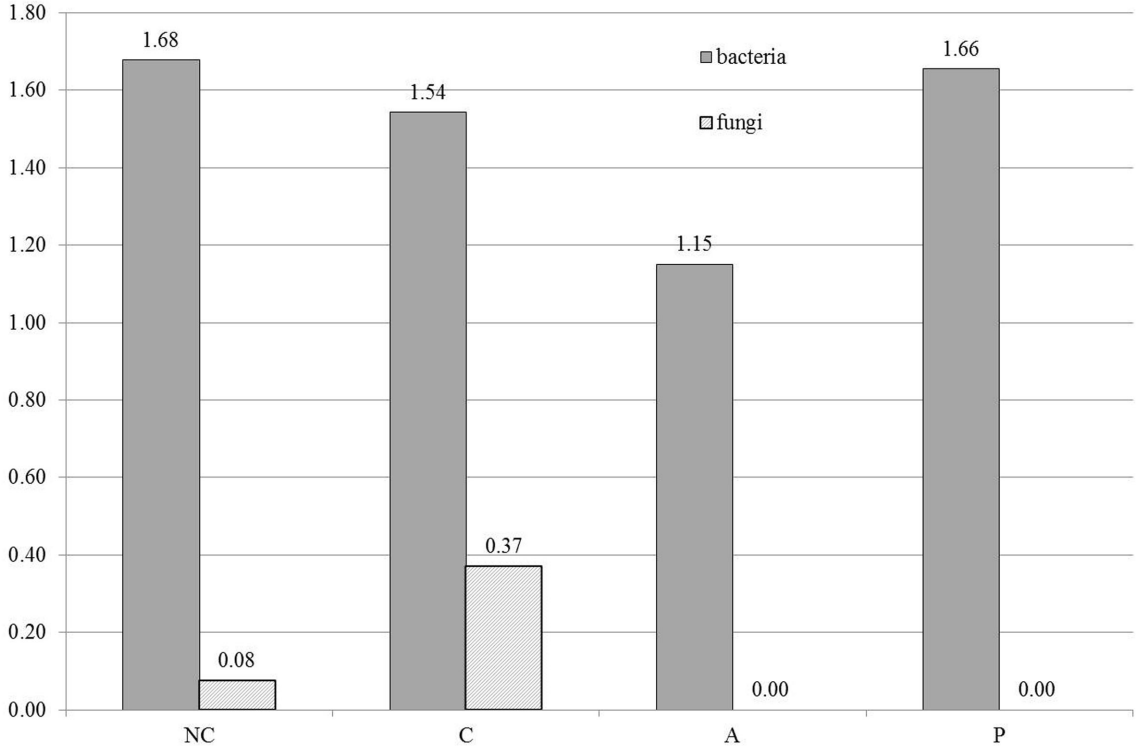

Figure 1. Total number of bacteria and fungi (log10 CFU/ml liquid from egg).

Table 2. More common bacteria isolated from different groups of eggs (% of total isolates).

Bacteria strain	Treatment				χ^2 (*P*-value)
	NC	C	A	P	
Bacillus spp.			6.3		0.227
Bordetella spp.	12.5				0.040
Chryseobacterium miningosepticum			12.5		0.040
Corynebacterium spp.		2.0	6.3		0.409
Corynobacterium propinguum	6.3				0.227
Kocuria kristinae	6.3				0.227
Micrococcus spp.			25.0		0.008
Staphylococcus aureus	12.5	10.0	31.3		0.047
Staphylococcus cohnii				57.1	0.076
Staphylococcus spp.	6.3	86.0	12.5		0.088
Staphylococcus xylosus	6.3		6.3		0.330
Streptococcus spp.	12.5	2.0		42.9	0.000
Salmonella spp.					
E. coli					
Non-indentified	37.5				0.000

Table 3. The eggshell conductance constant.

Trait		Treatment				SEM
		NC	C	A	P	
Day 15	Fertile eggs	$1.31^{a,c}$	$1.79^{b,c}$	2.20^{d}	1.04^{a}	0.126
	Infertile eggs	1.59	2.09	3.08	3.00	0.235
	Dead embryos	1.40^{a}	3.50^{b}	$2.80^{a,b}$	$1.52^{a,b}$	0.361
Day 17.5	Unhatched chicks	2.02	3.98	3.89	2.15	0.406
	Healthy chicks	6.60^{b}	6.61^{b}	7.26^{b}	5.36^{a}	0.153

[a–d]Differences between mean values for treatments are significant at $P \leq 0.05$.

and 240 μg of propolis had an antifungal effect; and with a recent study by Shahein and Sedeek (2014), who demonstrated that disinfection of hatching egg with 14% propolis affected the total bacterial, coliform, and *Staphylococcus* counts on the eggshell surface. Also, Aygun et al. (2012) reported that spraying Japanese quail eggs with 1%, 5%, and 15% propolis affected total antifungal and antibacterial activity.

The data obtained for the eggshell conductance constant during incubation at 15 and 17.5 d of embryonic development are summarized in Table 3. There were no significant differences in this trait among treatments for infertile eggs at 15 d of incubation or for unhatched chicks. However, a significant positive decrease in this trait for fertile eggs at 15 d of incubation (1.04) was noted for group P as compared with A and C, whereas in the case of dead embryos the P group did not differ significantly from the other groups. In the group disinfected with propolis, the loss of weight in hatched eggs was considerably smaller in comparison to both control groups and even to group A. This might be explained by minimization of water loss through occluded egg pores after spraying with propolis. The eggshell conductance constant refers to egg weight loss, and is therefore an important parameter for incubated eggs. An excessive rate of moisture loss is disadvantageous for active embryonic development and affects hatching success (Geng and Wang, 1990; Christensen et al., 2001). Bagley et al. (1988) and Yildirim et al. (2003) stated that the eggshell cuticle may be affected by the application of sanitizers that alter eggshell permeability and embryonic development, and noted that some of them diminished water vapor evaporation and the egg weight loss percentage. This result is in accordance with Aygun

Table 4. The hatching results depending on treatment.

Trait (%)	Treatment				SEM
	NC	C	A	P	
Eggs fertility	84.68	84.00	85.89	87.80	2.877
Hatchability of fertile eggs	79.63	77.88	77.27	76.00	1.983
Hatchability of set eggs	70.50	70.00	72.22	74.80	3.082
Total mortality of fertile eggs	20.37	21.24	27.27	24.00	2.097
Total mortality of set eggs	14.19	14.63	13.67	13.00	1.555

et al. (2012) and Shahein and Sedeek (2014), who concluded that spraying of alcoholic propolis on hatching eggs decreased the egg weight loss percentage.

The results shown in Table 4 confirmed that there were no significant differences between experimental treatments (NC, C, A, and S) for any of the hatchability parameters, i.e., egg fertility and hatchability and total mortality of fertile and set eggs (%). Figure 2 shows the periodical embryonic mortality (%) of fertile and set eggs (1 to 14 d). There were significant differences between A and P and between NS and C, but there were no significant differences in the final periodical embryonic mortality of fertile and set eggs (15 to 17.5 d) between any of the experimental treatments. This result demonstrates that treating hatching eggs with propolis as a disinfectant had no negative impact on hatching results as compared with traditional sanitation treatment (formalin + $KMnO_4$) or alcohol. Propolis can be used as an alternative disinfectant for hatching eggs instead of fumigation by formaldehyde, which is considered an irritant to the eyes and nose, has a lingering noxious odor, and exerts a carcinogenic effect. Furthermore, venting of its vapors is difficult (Whistler and Sheldon, 1989; Debes and Basyony, 2011) and it may decrease hatchability (Fasenko et al., 2009; Lowman and Parkhurst, 2014). Thus, effective alternative disinfectants are needed to replace formaldehyde. Propolis seems to be an effective, safe, nontoxic, and natural hatching egg sanitizer that can improve or at least maintain chick hatchability. Our results are in line with Aygun et al. (2012) and Vilela et al. (2012), who used propolis for egg disinfection by either spraying or immersion and found that it had no adverse effect on egg fertility, hatchability of fertile and set eggs, or embryonic mortality. Shahein and Sedeek (2014) have reported that spraying hatching eggs with 14% propolis led to higher hatchability and significantly reduced embryonic mortality.

The body weight of newly hatched chicks and at 7 and 14 d of rearing did not differ significantly between the experimental groups (Figure 3). These results are in contrast with those reported by Peebles et al. (1987), who showed that chick weight was reduced by increased egg weight loss during incubation from 0 to 18 d. Davis and Ackerman (1987) also reported that a deficiency or excess of water incorporated into new tissues influences future chick weight. Moreover, an increase in the chick weight loss percentage might be linked to the eggshell conductance constant (indicator of egg weight loss). These results are in agreement with Aygun et al. (2012), who demonstrated that egg disinfection by spraying with propolis had no effect on body weight at 1 and 10 days or on weight gain. However, Shahein and Sedeek (2014) recently achieved high chick body weight from fertile eggs sprayed with an alcoholic propolis extract.

Table 5 shows the survivability of the chicks during their first week of life. There were significant

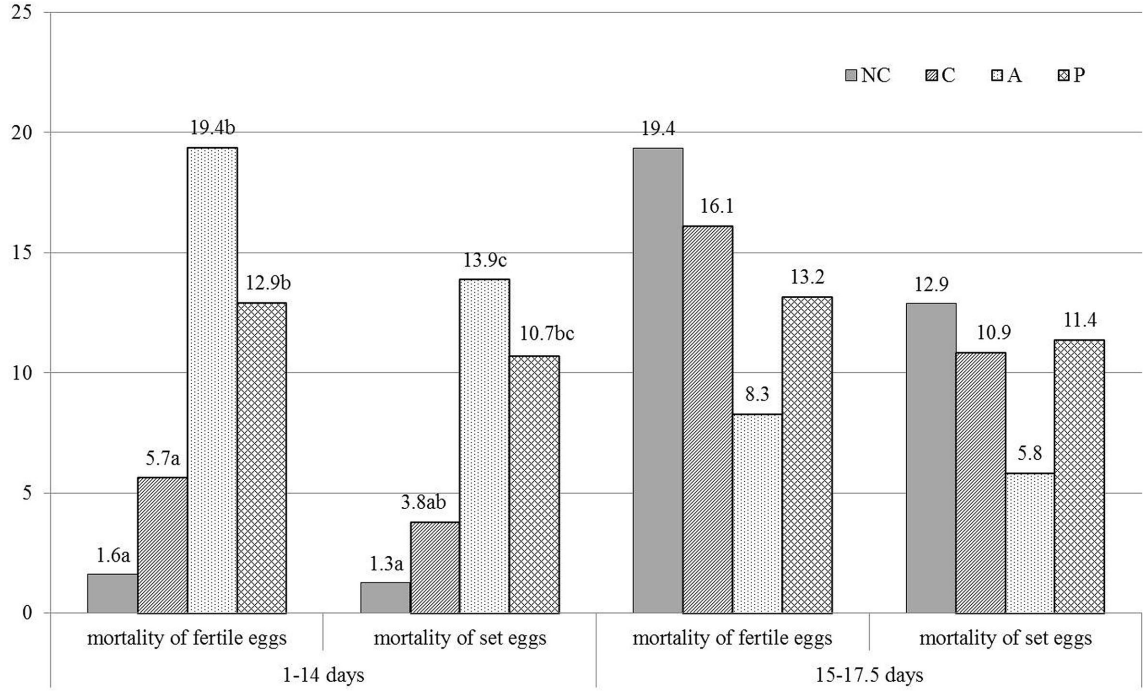

Figure 2. Mean values (±SE) of periodical embryonic mortality (%). a, b—differences between mean values for treatments are significant at $P \leq 0.05$.

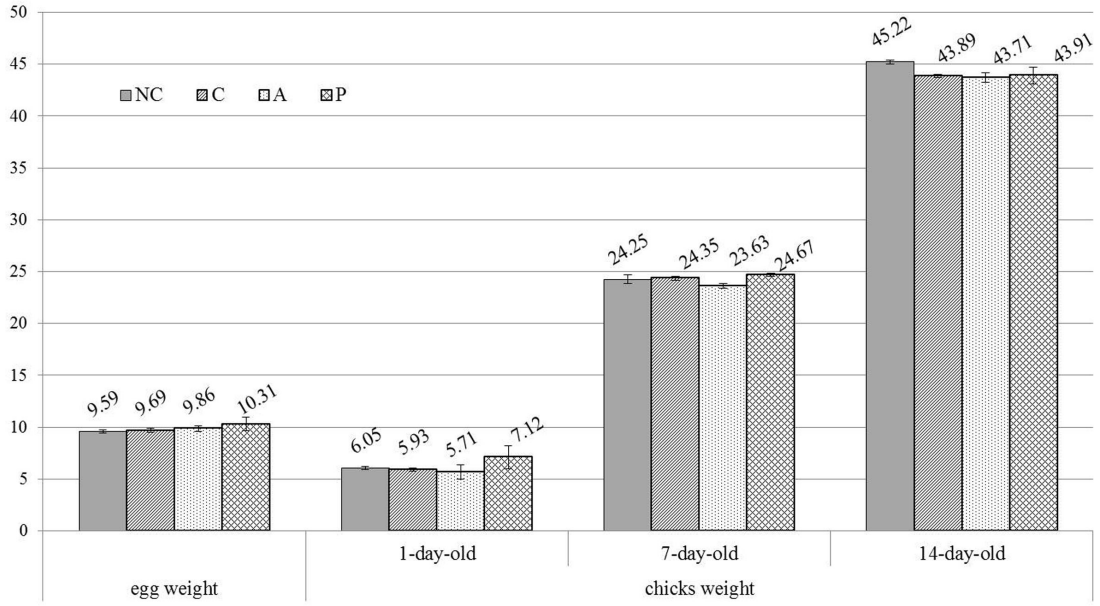

Figure 3. Mean values (±SE) of egg weight and body weight of quail chicks at days 1, 7, and 14 of rearing depending on the treatment.

Table 5. The chicks survivability depending on treatment (% of hatched chick).

	Treatment				
Time (days of rearing)	NC	C	A	P	χ^2 (P-value)
1 to 7	70.00	67.14	85.29	88.89	0.056
8 to 14	100	100	100	99.00	0.398
1 to 14	69.80	76.14	86.29	87.66	0.052

differences depending on the treatment, with the highest value noted for group P (88.89%). Although chick survivability did not differ significantly among treatment groups in week 2, for the entire rearing period (1 to 14 d) there were differences ($P \leq 0.05$) ranging from 69.80 to 87.66%, depending on the treatment, with the highest value noted for group P. Poultry eggs are vulnerable to microbial infections affecting the health and performance of hatched chicks. Therefore, good egg sanitation procedures may reduce mortality and ensure the biological survivability of hatched chicks.

CONCLUSIONS

The alcoholic propolis extract used as a disinfectant by spraying hatching eggs did not affect total bacterial or fungal counts, but it did affect certain strains of bacteria, without harming embryonic development but also without improving hatchability rates. It is regarded as a potential tool to increase chick survivability during rearing. The alcoholic propolis extract can be recommended as a natural and safe alternative to formaldehyde fumigation for disinfection of hatching eggs.

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