Original Article

Rosemary Essential Oil as a Natural Disinfectant in Japanese Quail Eggs (*Coturnix Coturnix Japonica*): Effect on the Bacterial Load and Hatchability Characteristics

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Abstract

Background: Maintaining hygiene in hatcheries is crucial for improving hatchability and ensuring healthy chick performance while reducing the risk of food contamination. Bacterial and fungal contamination, particularly on eggshells, is a major concern in the poultry industry. Egg disinfection before incubation has garnered global interest, especially with the rise in demand for natural alternatives to conventional disinfectants, which are often toxic and can lead to bacterial resistance. **Aims and Objectives:** This study evaluated the potential of rosemary essential oil (REO) as a natural disinfectant for eggshells and its impact on hatching success. **Materials and Methods:** A total of 216 Japanese quail eggs were divided into five groups: A negative control (nondisinfected), two positive controls treated with alcohol or SANIVIR (Quaternary Ammonium) (10%), and two groups disinfected with REO at the concentrations of 10 µl/ml and 100 µl/ml. Eggshells were swabbed to measure bacterial, coliform, fungal, and mold counts using serial dilutions and agar cultures. Hatching rate, chick weight, and mortality were also recorded. **Results:** The findings revealed that REO effectively reduced bacterial loads, with the 10 µl/ml group achieving the lowest contamination levels and significantly higher hatchability. In addition, chicks from this group had a greater average body weight. However, the 100 µl/ml group experienced higher embryonic mortality, likely due to its stronger concentration. **Conclusion:** In conclusion, REO at 10 µl/ml demonstrates promise as a safe and natural eggshell disinfectant. Its use not only improved hatchability but also positively influenced chick performance, making it a valuable alternative in commercial poultry operations.

Keywords: *Eggshell bacterial load, hatch parameters, natural disinfectant, rosemary essential oil*

Introduction

Poultry, as domesticated birds farmed for meat, eggs, or feathers, constitutes a diversified livestock, playing a major role in global food security by providing an affordable animal protein supply.[1] In response to the rising global demand for poultry products, intensive farming systems characterized by mass production have been increasingly implemented.[2] However, this intensification has raised concerns, particularly regarding microbial contamination in hatcheries, which compromises embryo development and increases mortality.[3,4] Moreover, eggs, known vectors of human and avian pathogens, require strict hygiene measures, as eggshell contamination is closely linked to internal infections, and reduced hatchability.[2,5]

In this context, maintaining sanitary conditions in poultry environments and industrial hatcheries remains a critical challenge for improving animal welfare and reducing the risk of pathogen transmission to both poultry and humans.[6,7] Indeed, among preventive strategies, egg disinfection before incubation has proven essential for limiting microbial load. This practice improves hatchability and chick viability and enhances the product quality while reducing economic losses.[8] Due to inadequate handling and storage conditions, eggs are highly vulnerable to bacterial

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contamination postlaying, mainly due to inadequate handling and storage conditions.[5,9] In addition, incubation conditions favorable to embryonic development such as high humidity (55%) and warm temperatures (37.7°C) – also promote bacterial proliferation.[10,11] As a result, bacteria can penetrate the eggshell, increasing embryo mortality and compromising chick quality.[3,8] Thus, to limit these risks, preincubation disinfection has become a critical step to ensure successful hatching and healthy chick development.[3,12]

From this perspective, a wide range of antimicrobial agents has been extensively used in intensive poultry systems.[11] Formaldehyde, in particular, has been the most widely used fumigant disinfectant.[6,13] However, the near-exclusive use of formaldehyde in hatcheries has raised concerns due to its embryo toxicity and role in the emergence of bacterial resistance, leading to increased embryo mortality. Its application is now restricted by regulatory protection agencies due to its known embryo toxic and carcinogenic effects, which also raise ethical concerns in the experimental research.[6,14-16] These limitations have highlighted the need for the poultry industry to explore safer and effective sanitizer alternatives.[17]

Natural compounds have been shown to enhance animal welfare by reducing the risk of foodborne zoonotic infections and limiting zoonotic transmission. Further, they have been investigated as natural growth-promoting agents, offering a promising alternative to antibiotics in animal production.[18,19] Beyond these benefits, they have also proven to be effective and safer alternatives to conventional chemical disinfectants, which may negatively affect embryonic development.[13,20] In this context, plant-based agents, particularly essential oils (EOs), known for their broad-spectrum antimicrobial properties, have emerged as promising solutions.[11,20] Their effectiveness in sanitizing hatching eggs has been demonstrated in several studies.[21,22]

In poultry production, REO has attracted interest due to its potent antimicrobial activity, primarily linked to its high phenolic content.[12,23] However, its practical application is limited by poor water solubility.[24] In this respect, to address this issue, polyethylene glycol, a commonly used solubilizer, has demonstrated the ability to improve EOs solubility without inducing toxicity when administered in ovo in quail embryos.[25]

To the best of our knowledge, there is limited evidence supporting the effectiveness of REO, especially for egg disinfection. For instance, Oliveira *et al*.[12] observed that a rice flour coating enriched with 1% REO reduced the bacterial load on quail eggshells during storage. However, its direct application as a disinfectant for incubating eggs remains unexplored. Therefore, the present study aimed to investigate the potential of REO as a natural eggshell disinfectant for Japanese quail eggs (*Coturnix Coturnix Japonica*) by evaluating its effect on bacterial load and hatch parameters.

Material and Methods

Preparation of treatments

Rosemary essential oil extraction and characterization

The rosemary officinalis plant material, aerial part (stems and leaves) in the El-Euchre region, Bordj Bou Arréridj department, located in northeastern Algeria. The harvested plants were dried in the shade for 10 days before extraction. EO extraction was carried out by the hydrodistillation method using a Clevenger-type apparatus. Distillations were carried out by boiling the aerial parts of the plant for 3 h, and the EO was kept in airtight sealed vials and stored at 4°C until further use.

The chemical composition of the extracted rosemary EO (REO) was analyzed using Gas Chromatography–Mass Spectrometry. The major bioactive constituents identified were camphor (18.88%), camphene (5.17%), 1,8-cineole (7.85%), β-thujene (13.66%), α-thujene (4.87%), chrysanthenone (12.05%), and β-cubenene (7.97%), confirming the antimicrobial potential of the EO.

The extracted REO was first solubilized by using polyethylene glycol (PEG 6000) as an emulsifying agent, resulting in complete dissolution through the solid dispersion technique. The oil was then transferred into airtight sealed vials and stored at 4°C until further use.[25]

Conventional disinfectants

In this study, two conventional egg disinfectants (ethyl alcohol 70% and SANIVIR) were used as positive controls.

Ethyl alcohol 70%

Ethyl alcohol (C + A) was used as the first conventional treatment for sanitizing hatching eggs.

SANIVIR

SANIVIR (Quaternary ammonium containing dodecyl dimethyl ammonium chloride, manufactured by Bioplagen, Seville, Spain) was used as the second conventional sanitizer for hatching eggs (C + S). The product is diluted in water before use.

The sanitizing solutions were prepared a day before use and stored at 4°C (Aberbour *et al*.).[25]

Application solution and egg incubation

The present study was conducted at the University of Bejaia, Algeria. A total of 216 fertile, fresh Japanese quail eggs (*Coturnix Coturnix Japonica*) were used. The eggs were numbered, weighed individually, and then randomly divided into five groups. The following treatments were applied to the eggs:

(a) T1 (C−): Negative control group (nondisinfected eggs)
(b) T2 (C + A): 70% ethanol (positive control)
(c) T3 (C + S): 10% SANIVIR (positive control)
(d) Two treatment groups were disinfected with 10 µl/ml (EO/distilled water) and 100 µl/ml (EO/distilled water), respectively.

Each treatment group consisted of two replicates of 18 eggs, for a total of 36 eggs/group, totaling 216 eggs. After treatment, eggs were left to dry for 30 min. Eggs were placed in an incubator (Cimuka, CT 180 SH Model) under standard conditions: 55% humidity, a temperature of 37.7°C, with one rotation every 2 h.

Sanitizing procedures and microbiological analyses

Before incubation, eggs from each group, except for those of the negative control group (nontreated), were disinfected by manual surface application of the respective treatment solutions, following the methods described by Cadirci[6] and Ulucay and Yildirim.[13]

Disinfection was performed by thoroughly wiping (swabbing) the entire eggshell surface using sterile absorbent paper dampened with the corresponding disinfectant (EO or chemical agent). This method ensured uniform coverage while minimizing the risk of physical damage. We deliberately avoided immersion or spraying, as these techniques can alter eggshell permeability and increase the risk of microbial penetration when not precisely controlled. In contrast, wiping proved to be a safer and more controlled approach that preserves the natural barrier of the shell. It also aligns more closely with practical hatchery conditions, striking a balance between effective sanitation and protection of the developing embryo.

To preserve shell integrity and avoid pore dilation, all treatment solutions were applied at the room temperature (22°C–25°C). Following treatment, all eggs were left to air-dry under sterile conditions at the room temperature for 30 min before incubation.

Before incubation, the entire eggshell surface was swabbed, using sterile gloves, to recover the shell-associated microorganisms and determine total bacterial and coliform fungal counts in three eggs per treatment. After treatment, the eggshell's microbiological characteristics were examined according to quantitative analyses. The recovered microbial load was diluted (10^{-1}), (10^{-2}), and (10^{-3}) in physiological saline, and 0.1 ml of each dilution was inoculated onto sterile petri plates containing Plate Count Agar for the enumeration of the total bacteria, coliforms, and fungi. Plates were incubated at 37°C, for 24 h, and bacterial colonies were counted using conventional methods (CFU/ml). Results were expressed as log10 CFU/ml. Hatching rates and embryonic mortality rates were assessed for all groups.

$$\text{Colony Forming Unit (CFU / mL)} = (\text{Number of colonies} \times \text{dilution factors / volume of culture plate}) \quad (1)$$

Hatching parameters

In addition, hatching parameters were assessed for all groups. After hatching, all chicks were counted and weighed. The nonhatched eggs were opened, and the dead embryo stages were determined macroscopically to assess the time of death.

- Early Embryo Mortality (EEM): Death occurring between 0 and 6 days of incubation (non-visible embryo, partially developed embryo with distinct features)
- Medium Embryo Mortality (MEM): Death occurring between 7 and 14 days of incubation (partially developed embryo with distinct features)
- Late Embryo Mortality (LEM): Death occurring between the 14th and 17th days of incubation (fully formed embryo unable to hatch).

The hatching parameters were assessed using the following formula:

$$\text{Hatchability (\%)} = (\text{Number of hatched chicks / Total number of fertile incubated eggs}) \times 100 \quad (2)$$

$$\text{Relative hatching weight (\%)} = (\text{Absolute hatch chick weight / Initial egg weight before incubation}) \times 100 \quad (3)$$

$$\text{Total embryo mortality (\%)} = (\text{Number of unhatched fertile eggs / Number of incubated fertile eggs}) \times 100 \quad (4)$$

$$\text{Early embryo mortality (EEM \%)} = (\text{Number of dead embryos on days } 0-6 \text{ of incubation / Number of non-hatched fertile eggs}) \times 100 \quad (5)$$

$$\text{Medium embryo mortality (MEM \%)} = (\text{Number of dead embryos on days } 7-14 \text{ of incubation / Number of non-hatched fertile eggs}) \times 100 \quad (6)$$

$$\text{*Late embryo mortality (LEM \%)} = (\text{Number of dead embryos on days } 14-17 \text{ of incubation / Number of non-hatched fertile eggs}) \times 100 \quad (7)$$

Following the previously described microbiological procedure, swabs were also taken from dead embryos at different mortality stages (EEM and LEM) to assess the total bacterial load. The microbial load was then calculated using the following formula:

Embryo mortalities bacterial load (CFU/ml) = (Number of colonies × dilution factors/volume of culture plate) of dead embryo (EEM and LEM).

$$\text{Embryo mortalities bacterial load (CFU /ml)} = (\text{Number of colonies} \times \text{dilution factors / volume of culture plate}) \text{ of dead embryo (EEM and LEM)} \quad (8)$$

Statistical analysis

Regarding relative hatching weight and colony-forming units, statistical data were analyzed using a mixed model (Statview software, version 4.55). An analysis of variance (ANOVA) was employed to evaluate the obtained data. Values were considered statistically different when the $P < 0.05$. Microbial counts were transformed into log10 before statistical analyses.

Regarding hatching rate (%) and mortality rate (%), percentage data were analyzed using χ^2 test to determine

the statistical significance (χ^2 test, *P* value). Graphs were created using GraphPad Prism software, version 8.3.0.

Results

Effect of disinfection on eggshells and dead embryos bacterial load

The effect of eggshell sanitization on bacterial load is presented in Figure 1. Results indicate that REO-treated groups exhibited significantly lower mean bacterial counts compared with the nondisinfected eggs and the groups C + A and C + S treated with the conventional sanitizers ($P < 0.0001$). Treatments with 100 µl/egg REO recorded significantly the highest antibacterial activity than all other treatment groups, with only 3.636 ± 0.311 log10 CFU/ml, followed by the treatment with 10 µl/ml, which registered 4.507 ± 0.318 log10 CFU/ml.

Figure 2 illustrates the impact of eggshell disinfection on bacterial contamination recorded in embryonic mortalities occurring at early (EEM) and late (LEM) stages of incubation. Compared with the nondisinfected group (C−), egg sanitizing with 10 µl/ml of REO reduced bacterial load in early stage embryonic mortalities, with bacterial counts of 3.882 ± 0.15 log10 CFU/ml in the treated group and 4.171 ± 0.388 log10 CFU/ml in the control. However, this reduction was not statistically significant ($P = 0.526$). Similarly, in late-stage embryonic mortalities, bacterial loads were 3.190 ± 0.170 log10 CFU/ml in the treated group and 3.146 ± 0.458 log10 CFU/ml in the control, with no significant difference observed ($P = 0.932$).

Effect of eggshell disinfection on hatching parameter

The effects of REO eggshell sanitization on hatching parameters are presented in Figures 3-6. As shown in Figure 3, disinfection with 10 µl/ml of REO resulted in the highest hatching rate (81.82%), which was significantly higher ($P < 0.001$) than in all other groups. In contrast, treatment with 100 µl/ml showed a low hatching rate (34.6%). Intermediate hatching rates were observed in the alcohol (79.5%) and SANIVIR (69.3%) treatment groups.

Regarding total embryonic mortality [Figure 4], treatment with 10 µl/ml resulted in the lowest mortality rate (18.18%), which was significantly ($P < 0.001$) lower than in all other groups [Figure 3]. Conversely, treatment with 100 µl/ml REO exhibited significantly the highest mortality rate (65.4%) ($P < 0.001$). Among conventional sanitizers, alcohol treatment (C + A) recorded a lower embryonic mortality rate (20.5%) compared with SANIVIR (C + S) (30.7%). Interestingly, alcohol treatment resulted in a mortality rate similar to that of the negative control group (25.5%).

Sanitizing eggs with REO also influenced chick weight at hatch [Figure 5]. Treatment with 100 µl/ml REO resulted in the numerically highest relative weight of chicks at hatch (72.5%), followed by the treatment with 10 µl/ml REO, which registered 68.9%. However, no significant difference ($P = 0.932$) was recorded between all treatments. Among the conventional treatments, alcohol (C + A) resulted in a slightly higher relative weight than SANIVIR (C + S), with the values of 69.2 and 68.5%, respectively [Figure 4].

Concerning specific embryonic mortality [Figure 6], treatment with 100 µl/ml significantly reduced EEM ($P < 0.001$) compared with the 10 µl/ml group and the control [Figure 6a]. However, eggs sanitized with REO at 100 µl/ml exhibited a significantly ($P < 0.001$) higher LEM rate (95%) compared with the 10 µl/ml REO group and the control [Figure 6b]. Treatment with 10 µl/ml resulted in significantly lower MEM and LEM ($P < 0.001$) compared with the negative control group (nondisinfected) [Figure 6c].

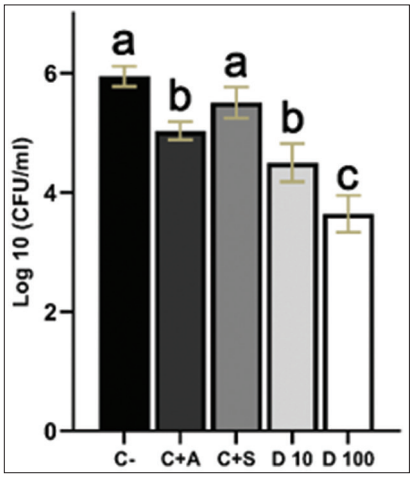

Figure 1: Effect of the antimicrobial activity of rosemary essential oil at 10 µl/ml (D10) and 100 µl/ml (D 100) on the bacterial load of hatching eggshell, compared with no treatment (C−), ethyl alcohol (C + A), and SANIVIR (C + S) Different letters (a, b, and c) indicate a statistically significant difference ($P < 0.05$). Values are represented as mean ± standard error

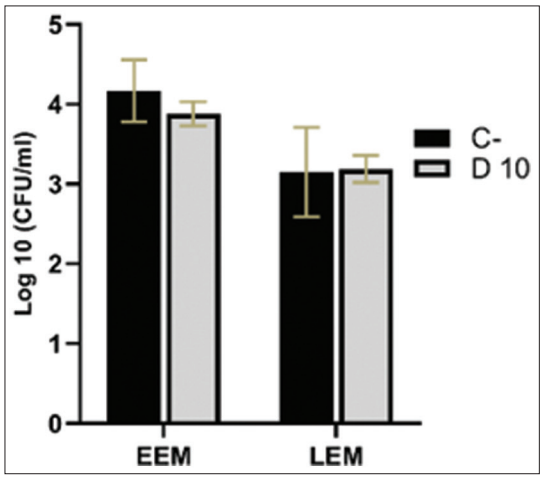

Figure 2: Effect of rosemary essential oil on the bacterial load recorded in early (EEM) and late (LEM) embryonic mortality. Values are represented as mean ± standard error. No statistically significant difference ($P > 0.05$) was recorded between the two treatments

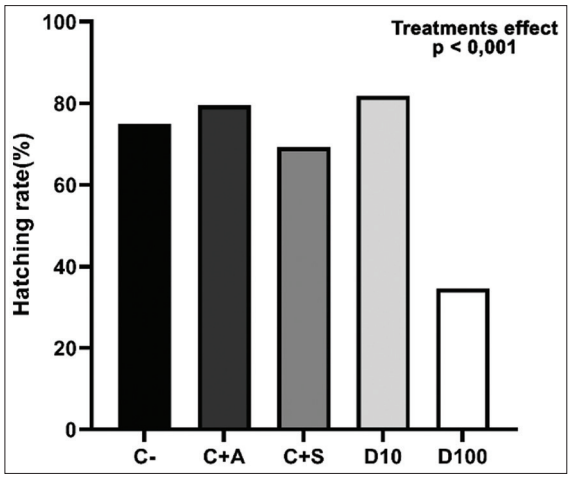

Figure 3: Effect eggshell disinfection with rosemary essential oil at 10 µl/ml (D10) and 100 µl/ml (D 100) on hatching rates, compared with no treatment (C−), ethyl alcohol (C + A), and SANIVIR (C + S). Values are represented as mean ± standard error (SE). A statistically significant difference (χ^2 test, P < 0.001) was observed among all treatments

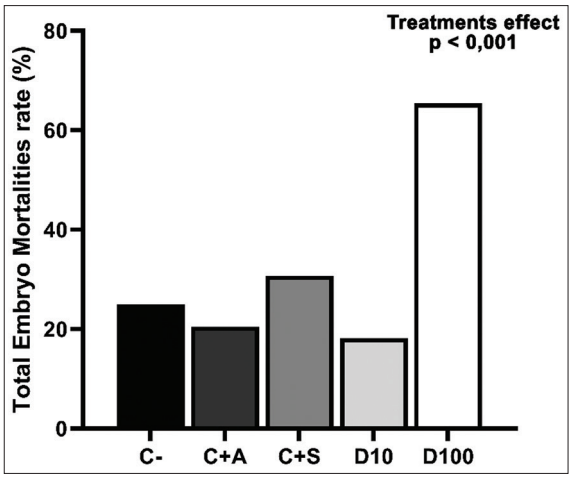

Figure 4: Effect of eggshell disinfection with rosemary essential oil at 10 µl/ml (D 10) and 100 µl/ml (D 100) on total embryonic mortality rates, compared with no treatment (C−), ethyl alcohol (C + A), and SANIVIR (C + S). Values are represented as mean ± standard error. A statistically significant difference (χ^2 test, *P* < 0.001) was observed among all treatments

Discussion

To improve productivity, the poultry industry focuses on optimizing incubation conditions and chick quality.[26] However, incubating contaminated eggs can reduce hatchability and chick performance.[11] In this context, the present study aimed to evaluate the effectiveness of REO as a natural eggshell disinfectant by assessing its antimicrobial potential and its impact on hatch parameters.

Our results demonstrated that REO, at both 10 and 100 µl/ml, significantly reduced the eggshell bacterial load ($P < 0.0001$) compared with the control groups (nondisinfected and alcohol-SANIVIR). Sanitizing eggs with 100 µl/ml REO exhibited the strongest antimicrobial effect against bacterial contamination. Moreover, this treatment was significantly more effective than the 10 µl/ml treatment. This efficacy is attributed to the chemical constituents of REO, particularly camphor, α-thujene, camphene, β-thujene, chrysanthenone, and β-cubene, which are well known for their broad-spectrum antimicrobial activity.[12] EOs exert their antimicrobial effects through multiple mechanisms. Their strong hydrophobic nature enables them to disrupt bacterial cell membranes, increasing membrane permeability, causing leakage of intracellular contents, and ultimately leading to cell death.[27,28]

Regarding the effectiveness of EOs in sanitizing hatching eggs, our findings are consistent with those of Copur *et al.*,[21] Shahein and Sedeek,[29] and Ulucay and Yildirim,[13] who demonstrated that EOs such as oregano, clove, thymol, and carvacrol effectively reduce microbial contamination on eggshells and can serve as alternatives to conventional chemical disinfectants. This is may be attributed to their broad-spectrum antimicrobial activity and lower embryo toxicity compared with some chemical disinfectants, when used at appropriate doses. Similarly, Oliveira *et al.*[12] reported that REO prestorage sanitization of quail eggs significantly decreased microbial loads due to its bioactive composition. Our findings reinforce the bactericidal potential of REO, particularly at higher doses, aligning with previous studies that have demonstrated the ability of EOs to disrupt key cellular functions in bacteria, ultimately leading to cell death.[30,31]

Furthermore, both conventional disinfectants - 70% alcohol and SANIVIR (a quaternary ammonium compound) – reduced microbial load compared with the negative control but were less effective than REO. This could be attributed to the limited residual activity and poor eggshell penetration of alcohol and quaternary ammonium compounds. In line with our results, Aygun *et al*.[32] reported that natural disinfectants such as propolis were more effective than 70% alcohol in reducing eggshell microbial contamination while also preserving hatchability. Similarly, Ulucay and Yildirim[13] found that disinfection with EOs before incubation led to a greater reduction in microbial load compared with quaternary ammonium-based treatments such as SANIVIR, further supporting the superior antimicrobial efficacy of EOs. This may be attributed to their broad-spectrum antimicrobial activity and lower embryo toxicity compared with some chemical disinfectants, particularly when used at appropriate concentrations. In addition, although distinguishing between bacteriostatic and bactericidal effects can be challenging, our results suggest that REO primarily exhibits a bactericidal effect, as evidenced by the significant reduction in bacterial contamination. This aligns with previous studies indicating that, at high concentrations, EOs disrupt key bacterial processes such as respiration and protein synthesis, ultimately causing cell death.[30,31]

Interestingly, this study also examined the bacterial load of dead embryos, a less explored area, by highlighting

the beneficial and potentially antimicrobial effects of REO, particularly in EEM [Figure 2]. Eggs sanitized with 10 µl/ml REO showed lower early embryo contamination than the untreated control group, although this difference was not statistically significant, suggesting a possible sustained antimicrobial action throughout incubation. Regarding LEM, no significant differences (P = 0.932) were observed between the REO-treated and control group (nondisinfected). This contamination observed in embryos may be attributed to the porous nature of the eggshell, which allows the bacteria to penetrate and reach the inner content of the egg, potentially compromising embryo development. Although the reduction in early embryonic contamination with 10 µl/ml REO was not statistically significant, we suggest that REO may help limit bacterial invasion during the initial stages of the development. The persistence of its bactericidal action throughout the incubation period was evidenced by the lower microbial load recorded in embryos up to day 19, when mortality was detected, indicating a prolonged protective effect of REO against internal contamination.

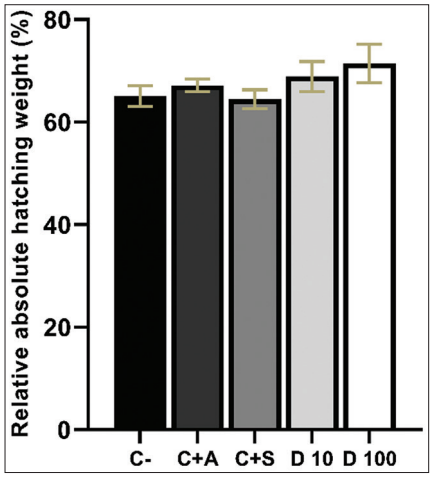

Figure 5: Effect of eggshell disinfection with rosemary essential oil at 10 µl/ml (D 10) and 100 µl/ml (D 100) on relative hatch chick weight rates, compared with no treatment (C−), ethyl alcohol (C + A), and SANIVIR (C + S). Values are represented as mean ± standard error. No statistically significant difference (χ^2 test, $P > 0.05$) was recorded between all treatments

On the other hand, hatchability was significantly improved ($P < 0.001$) in the 10 µl/ml REO group compared with both the control and the 100 µl/ml REO [Figure 3]. This outcome may be attributed to effective bacterial control at 10 µl/ml without inducing toxicity, unlike the higher concentration that adversely affected embryo development. This supports previous studies showing that EO-based disinfection improves hatchability and embryo safety compared with conventional chemical agents.[3,11,21] In contrast, the 100 µl/ml dose, despite achieving lower bacterial loads, led to the highest embryonic mortality highlighting that microbial contamination was not the primary cause but rather the toxic concentration of REO. This may be linked to the permeability of the eggshell, allowing absorption of excessive bioactive compounds. These results are in line with those demonstrated in our previous research,[25] in which high doses of rosemary oil injected in in ovo at 100 µl/ml (3 µl/egg) proved to be toxic for quail embryo development. In addition, regarding embryo exposure to high levels of EOs, Damasco and Lemonica[33] reported that high concentrations of REO impaired embryo implantation in mice, likely due to terpenoids like camphor and 1,8-cineole, which can cross biological membranes and disrupt early developmental processes, including implantation and early organogenesis.

On the other hand, the results of the current study revealed that sanitizing eggs with REO increased the chicks' hatch weight. In this respect, we hypothesize that the powerful antioxidant and antimicrobial properties of REO could enhance embryonic development by limiting oxidative stress and microbial challenges during incubation. Improved antioxidant status is known to support tissue development and metabolic efficiency, which may result in better growth performance. The current results are similar to those reported by Oliveira *et al*.,[11] who reported that disinfecting eggs with clove EO improved hatching weight. Although our study focused on disinfection, residual REO compounds may have influenced posthatch intestinal development. In this perspective, Youssef *et al*.[34] reported that dietary supplementation with a blend of EOs significantly increased villus height and the villus-to-crypt ratio in the small

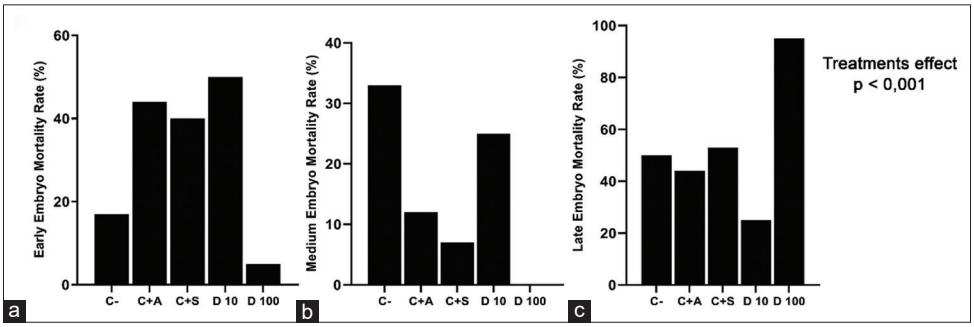

Figure 6: Effect of eggshell disinfection with rosemary essential oil at 10 µl/mL (D 10) and 100 µl/ml (D 100) on specific embryo mortality rates (early (a), medium (b) and late (c)) compared with no treatment (C−), ethyl alcohol (C + A), and SANIVIR (C + S). Values are represented as mean ± standard error. A statistically significant difference (χ^2 test, $P < 0.001$) was observed among all treatments

intestine of broiler chickens, which was associated with improved growth performance. Further research is needed to confirm this mechanism in the context of egg disinfection; such an effect could also contribute to the observed increase in hatch weight. Interestingly, despite the higher embryonic mortality observed at 100 µl/ml, the surviving chicks showed greater body weight, likely due to a selection effect favoring more resilient embryos with higher metabolic efficiency. This supports our previous findings,[25] in which in ovo injection of REO at 3 µl/egg (100 µl/ml) increased chick weight despite reducing hatchability.

The final stage of incubation is critical for embryo survival. Moreover, this study highlights how REO, depending on its concentration, can exert both beneficial and adverse effects on embryonic development. At 10 µl/ml, REO provided sustained antimicrobial activity throughout incubation, significantly reducing LEM (LEM; $P < 0.001$) without inducing toxicity. In contrast, 100 µl/ml was more effective in reducing early (EEM) and MEM, but led to increased ($P < 0.001$) LEM, likely due to prolonged exposure and deeper penetration interfering with late-stage metabolism. These findings emphasize the importance of concentration in balancing antimicrobial efficacy and toxicity. These findings suggest that REO at a higher concentration (100 µl/ml) controls early bacterial infections effectively, but its potential toxicity negatively impacts embryo survival in the later stages, while the lower concentration (10 µl/ml) offers a more protective and balanced effect, ultimately improving hatching. Similar results were reported by Copur *et al*.,[21] who found that oregano EO increased LEM, with no significant impact on EEM or MEM.

Conclusion

The results of this study revealed the usefulness of sanitizing incubating eggs with REO at 10 µl/ml for egg shell bacterial load, hatchability, and absolute relative hatching weight. Strong toxic effects were observed at 100 µl/ml, but it showed a better effect on eggshell bacterial load compared with the control groups. The results demonstrated that pure REO at 10 µl/ml can be an interesting and suitable alternative as a safe and natural disinfectant for incubated eggshells and as a growth promoter in poultry farming. Future studies should focus on characterizing the microbiome and aerobic bacterial load of hatching eggs before disinfection and on investigating how REO disinfection procedures impact eggshell microbiomes during the subsequent disinfection step.

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Author contributions

Conceptualization, L.T., A.A., M.I.O., J.L.H., and N.M.; methodology, L.T., A.A., K.D., A.B., M.B., M.I.O., J.L.H., and N.M.; software, S.A., L.T., A.A., M.I.O., J.L.H., and N.M.; validation, L.T., A.A., M.I.O., J.L.H., and N.M.; formal analysis, S.A. and N.M., investigation, L.T., A.A., K.D., M.I.O., J.L.H., and N.M.; resources, L.T., M.I.O., J.L.H., and N.M.; data curation, L.T., A.A., S.A., and N.M.; writing – original draft preparation, L.T., A.A.; writing – review and editing, L.T., K.D., M.I.O., J.L.H., and N.M.; visualization, L.T., M.I.O., J.L.H., and N.M.; supervision, L.T., K.D., M.I.O., J.L.H., and N.M.; project administration, J.L.H. and N.M.; funding acquisition, L.T., M.I.O., J.L.H., and N.M.

All authors have read and agreed to the published version of the manuscript.

A.A. and L.T. contributed equally to this work.

Data availability statement

All the data generated or analyzed during this study are reported in this published article. The data sets used and/or analyzed in this study are available from the corresponding author upon request.

Ethical approval

All the study procedures and guidelines for experimental animals were approved by the Association Algérienne des Sciences en Expérimentation Animale (58 AASEA: N°45/DGLPAG/DVA/SDA/14).

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Conflicts of interest

There are no conflicts of interest.

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